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Contents

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Neuronal Organization and Synaptic Mechanisms of Supraspinal Motor Control in Vertebrates*

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Contents

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I. Introduction

Motor coordination is one of the most essential functions of the central nervous system. In vertebrates it is executed mainly by the brain centers. By way of descending synaptic projections these centers govern the spinal motoneurons and the movements of the animal as a whole "as a single machine" (SHERRINGTON, 1906). In contrast, reflex movements may be accomplished at segmental levels. The progressive evolution of the supraspinal motor control may be considered as the main content of the process of encephalization. SHERRINGTON (1906, 1940), SPERRY (1969), and BERNSTEIN (1966) pointed out that central motor mechanisms are subject to considerable changes in the course of phylogenetic development and may be considered as the key factor in the evolution of the brain. The comparison of neuronal and synaptic organization of supraspinal control in different vertebrates may give considerable insight into the nature of the neuronal circuitry subserving the central regulation of motor activity and the trends of its evolution.

The influence of brainstem structures on reflex movements was first discovered by SECHENOV (1863). FRITSCH and HITZIG (1870) demonstrated the electrical excitability of the cortical motor area; the corticospinal system has been most extensively investigated, largely because of its evident and paramount importance in mammals, particularly primates and man. The direct connections between corticopyramidal cells and alpha-motoneurons found in primates are thought to provide an important mechanism for the fine control or skilled voluntary movements, especially of the distal muscles of the extremities. The phylogenetically older motor outflow—the brainstem-spinal or extrapyramidal system—was assumed to be concerned with the execution of the rather crude reflex movements

of posture and locomotion governed only through diffuse multisynaptic projections. This concept, however, required critical reassessment.

Modern electrophysiological and morphological approaches have provided important details of the complex "electroanatomical" picture of supraspinal organization and function. Since morphological studies have been unable to substantiate any synaptic connection between descending axons and identified spinal neurons, the electrophysiological approach remains especially important. Over the last 15 years the increase of efforts in this area has been dramatic. It has become necessary to review current concepts and research on neuronal organization and synaptic mechanisms of supraspinal motor control. This review is limited to the relatively few vertebrate species where the most detailed cellular studies of supraspinal effects are available.

II. Brainstem-Spinal Monosynaptic Control of Alpha-Motoneurons

The brainstem developed very early in the course of evolution and is rather uniform in structure and organization (TABER, 1961), although there are, of course, some variations among species. Descending axons originating from the brainstem nuclei make up the final output, descending through the spinal cord as the reticulospinal, vestibulospinal and rubrospinal tracts. It was generally believed that, in contrast to the cortico-motoneuronal system of primates, influences coming to the spinal motoneurons from the brainstem are exclusively polysynaptic and nonspecific (BRODAL, 1957; MAGOUN, 1950; ROSSI and ZANCHETTI, 1957). This concept is in accord with recent morphological investigations which could not detect terminations of brainstem-spinal pathways in Rexed's lamina IX of the ventral gray matter, where cell bodies of alpha-motoneurons are located (NYBERG-HANSEN and BRODAL, 1964; NYBERG-HANSEN and MASCITTI, 1964; NYBERG-HANSEN, 1969; KUYPERS, 1964).

One of the earliest lines of evidence suggesting the possibility of direct brainstem-motoneuronal linkage was obtained by LLOYD (1941); he placed stimulating electrodes in the floor of the fourth ventricle of the cat and expected to excite neurons of the bulbospinal (both reticulo- and vestibulospinal) system. LLOYD'S ventral root recordings allowed him to suggest that this complex descending system has feeble excitatory endings on spinal motoneurons. However, the first convincing evidence that neurons located in the brainstem can monosynaptically activate the spinal alpha-motoneurons was obtained only with the aid of intracellular recording. As was first reported in 1965 at the First Nobel Symposium (cf. SHAPOVALOV, 1966), local stimulation of the lateral vestibular, pontomedullary reticular, and red nuclei in the cat elicits short-latency monosynaptic EPSPs in lumbar motoneurons. Further detailed observations have confirmed this conclusion (GRILLNER et al., 1968, 1970; HONGO et al., 1969; HONGO, 1966; LUND and POMPEIANO, 1965, 1968; POMPEIANO, 1966; SHAPOVALOV et al., 1966, 1967; SHAPOVALOV and SHAPOVALOVA, 1966; WILSON and YOSHIDA, 1969, 1970).

The work on the cat has been followed by several other studies in various mammalian (SHAPOVALOV and GUREVITCH, 1970; SHAPOVALOV et al., 1971a, b, c) and inframammalian species (AUERBACH and BENNET, 1969; BATUEVA, 1972; YASARGIL and DIAMOND, 1968; SHAPOVALOV and SHIRJAEV, 1973). There is now general agreement on the existence of monosynaptic articulations between neurons of brainstem nuclei and spinal motoneurons, but specific projections may differ among different species.

A. Reticulo-Motoneuronal Effects, Comparative Aspects, and Functional Organization

In cyclostomes, some of the most primitive living vertebrates, supraspinal motor control is exerted mainly by reticulospinal neurons known as Müller's cells. The few giant Müller's neurons are located in the dorsomedial region of medulla and midbrain and only two such neurons can be seen on the cross-section of the medulla (Fig. 1.1) (BATUEVA and SHAPOVALOV, 1968).

Reticulospinal fibers are in close anatomical relationship to the cell bodies and dendrites of segmental motoneurons at all levels of the spinal cord of the lamprey. This is clearly demonstrated by intracellular or intra-axonal staining of the motoneurons with procion yellow dye (TEROVAINEN and ROVAINEN, 1971; SHAPOVALOV, 1972); the thick, fast-conducting reticulospinal axons may be seen to be less than one micron distant from the dendrites of segmental motoneurons. Actual synapses of the thickest reticulospinal fibers with motoneurons have been demonstrated by intracellular recording from antidromically identified cells and by stimulation of the medulla (Fig. 1). Moreover, the synaptic transmission from Müller's axons to segmental motoneurons in apparently accomplished both via electrical and chemical synapses as evidenced by different segmental delay of the monosynaptic EPSPs, their diphasic character, ability to follow repetitive stimulation and different sensitivity to injected currents (BATUEVA, SHAPOVALOV, 1974).

In teleosts the reticulospinal system is formed by large neurons located in the medial region of the brainstem (RESTINAUX, 1958). Such specialized and peculiar brainstem-spinal neurons as Mauthner cells also belong to the reticulospinal system. It was established that they cause monosynaptic excitation of the spinal motoneurons. Another pair of giant descending axons of unknown origin project down the cord from the brainstem and establish monosynaptic articulations with the motoneurons innervating the thoracic fins (AUERBACH and BENNET, 1969); here, the excitatory transmission is accomplished via electrical synapses.

In the frog the large reticulospinal neurons undergoing chromatolysis after spinal lesions are located in the medial reticular formation of the medulla (SHAPOVALOV, 1972b). Reticulospinal fibers conducting at a rate of 20 to 30 m/sec descend in the ventrolateral columns and evoke monosynaptic EPSPs in the motoneurons of the cervical and lumbar cord (SHAPOVALOV and SHIRJAEV, 1973) (Fig. 1.2). The mean amplitude of reticulo-motoneuronal EPSPs is about 2 mV, and in some cells EPSPs appear in all-or-none fashion, suggesting that a restricted number of reticulospinal axons are involved in the production of monosynaptic responses.

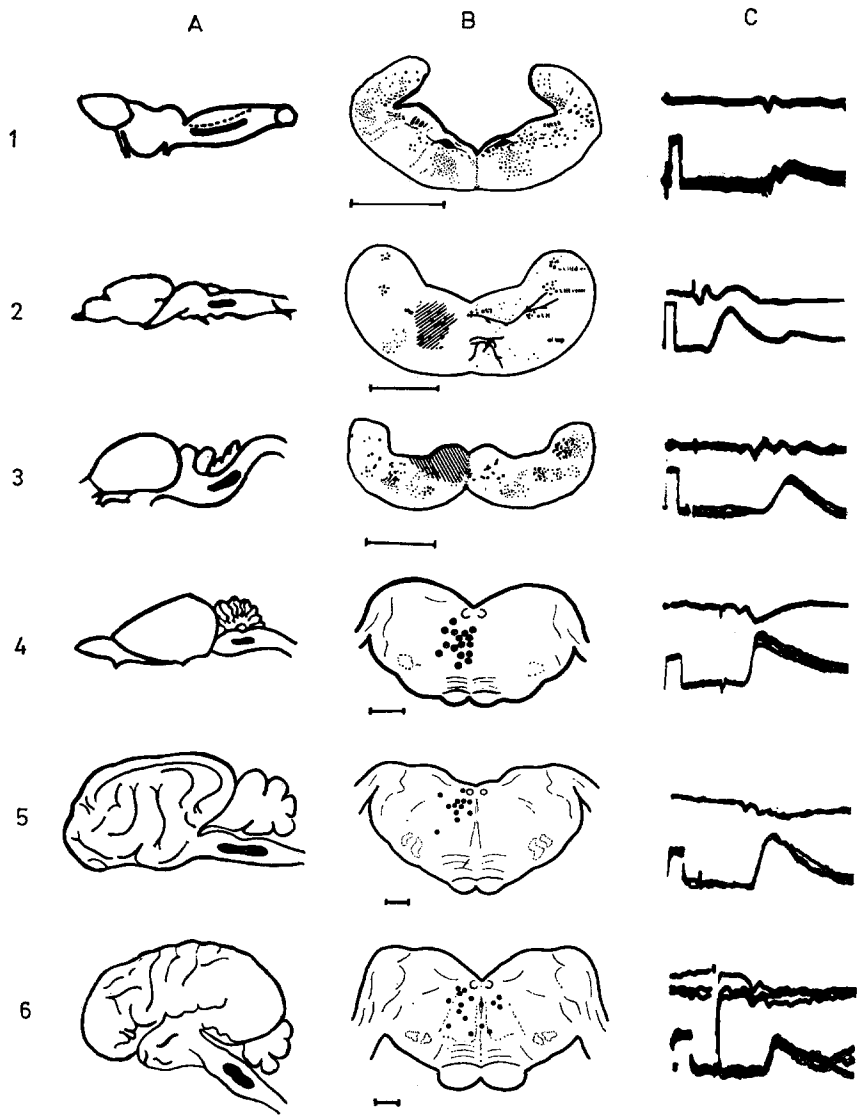


Fig. 1A—C. Reticulo-motoneuronal actions in lamprey (1), frog (2), turtle (3), rat (4), cat (5) and rhesus monkey (6). (A) Diagrams of the lateral aspect of the brain. Black area shows the medial reticular formation. (B) Transverse plane of the brainstem at medullar level. Hatched area or dots show the loci of stimulation. Scale line: 1 mm. (C) Monosynaptic EPSPs evoked by single shock from the medial reticular formation. The upper traces are records of the cord dorsum potentials, the lower traces are intracellular records. Calibration pulses 2 mV, 1 msec. (Modified after SHAPOVALOV, 1972a, c)

In reptilia the reticulospinal tract originates from the medial reticular formation of the caudal brainstem (ROBINSON, 1969). Experiments with turtles have shown that stimulation of this region elicits a descending volley at 30 to 40 m/sec and monosynaptic EPSP (Fig. 1.3). Monosynaptic EPSPs were recorded from motoneurons innervating both flexors and extensors of the hindlimb.

In mammals the reticulospinal fibers originate from the medial reticular formation of the lower pons and medulla (BRODAL, 1957; NYBERG-HANSEN, 1965; PETRAS, 1969) and descend throughout the cord to lumbosacral levels. In the rat reticulospinal fibers conduct at 60 to 80 m/sec (FOX, 1970; SHAPOVALOV and GUREVITCH, 1970). The reticulospinal volley evokes monosynaptic EPSPs in the lumbar motoneurons innervating proximal and distal, flexor and extensor muscles of the hindlimb (SHAPOVALOV and GUREVITCH, 1970; KIBAKINA and SHAPOVALOV, 1975) (Fig. 1.4).

In the cat the conduction velocity of the fastest reticulospinal axons was found to be about 120 to 150 m/sec (ECCLES et al., 1974; ORLOVSKY, 1970a; SHAPOVALOV et al., 1967; WOLSTENCROFT, 1964). The descending volley mediated by fast-conducting reticulospinal fibers monosynaptically excites lumbar the (GRILLNER et al., 1971; LUND and POMPEIANO, 1966, 1968; SHAPOVALOV et al., 1967; WILSON and YOSHIDA, 1969) and cervical motoneurons (WILSON and YOSHIDA, 1969, 1970). Monosynaptic effects are evoked by stimulation of the n. reticularis giganto-cellularis (RGC), n. reticularis pontis caudalis (RPC), and medial longitudinal fasciculus (MLF). These effects are preserved after chronic destruction of the Deiter's nucleus and the red nucleus (SHAPOVALOV et al., 1967). Amplitudes of reticulo-motoneuronal EPSPs usually do not exceed 2 to 3 mV (Fig. 1.5). In the cat lumbar cord monosynaptic reticulospinal effects preferentially select motoneurons innervating the flexor muscles of the hindlimb (GRILLNER et al., 1971). However, motoneurons of extensor muscles can also receive monosynaptic reticular input (WILSON and YOSHIDA, 1969). The amplitude of monosynaptic EPSPs in motoneurons innervating proximal muscles is larger (SHAPOVALOV, 1972a).

In the rhesus monkey stimulation of the medial reticular nuclei of the lower pons and medulla elicits a descending volley mediated at 80 to 90 m/sec, and an EPSP is recorded from hindlimb motoneurons with monosynaptic segmental delay (SHAPOVALOV et al., 1971c) (Fig. 1.6). The maximal amplitude of reticulo-motoneuronal EPSPs was in the range 0.15 to 2.1 (mean 0.64 ± 0.03) mV. In contrast to effects in subprimates, the reticulospinal monosynaptic input in the rhesus monkey was found primarily in motoneurons of the proximal muscles of the hindlimb, and more rarely in DP motoneurons (SHAPOVALOV, 1973a).

B. Vestibulo-Motoneuronal Effects, Comparative Aspects, and Functional Organization

Vestibulospinal fibers originating from the vestibular nuclei in the medulla can be identified in cyclostomes. A number of medium-sized axons located in the ventral columns of the lamprey spinal cord were traced from the posterior vestibular nucleus (STEFANELLY and CARAVITA, 1970). However, this vestibulospinal pathway, in contrast to the Deiterospinal projection of higher vertebrates, is contralateral.

Unlike other vestibular nuclei, the lateral vestibular nucleus of Deiters is derived from the reticular formation (KAPPERS et al., 1960). The homolateral vestibulospinal pathway was traced in reptilia (ROBINSON, 1969) but the pro-

perties of the relevant fibers were not investigated. It is not known what connections, if any, exist between vestibulospinal axons and spinal motoneurons in inframammalian species. Stimulation of vestibular nuclei in the frog elicits EPSPs in the lumbar motoneurons after a longer latency than for reticulo-motoneuronal effects (SHIRJAEV, 1972).

Within the mammalian world development of the Deiterospinal tract is not uniform. In lower mammals such as rodents (rat) it is of a modest extent (SCHOEN, 1964). In carnivores (cat) and in lower primates (rhesus monkey) the Deiterospinal tract consists of thick fibers which have been traced as far as lumbosacral levels (KUYPERS, 1964; NYBERG-HANSEN and MASCITTI, 1964; SCHOEN, 1964). On the other hand, this tract is scarcely visible in chimpanzee, gibbon, and man (SCHOEN, 1964).

Stimulation of Deiters' nucleus and recordings from lumbar motoneurons in the rat failed to disclose definite monosynaptic actions, even in extensor motoneurons of the hindlimb (KIBAKINA and SHAPOVALOV, 1975). The conduction velocity of the vestibulospinal axons ranged from 23 to 67 m/sec with a peak velocity of 39 m/sec (KORN et al., 1973) and thus is somewhat slower than that of the fastest reticulospinal axons in the same species (KIBAKINA and SHAPOVALOV, 1975).

In the cat the conduction velocity of the fastest vestibulospinal fibers is about 100 to 140 m/sec (ITO et al., 1964). These values are very similar to those reported for the fastest reticulospinal axons. The descending volley in the fast vestibulospinal path excites the motoneurons of the lumbar (SHAPOVALOV, 1966; SHAPOVALOV et al., 1966; GRILLNER et al., 1970; LUND and POMPEIANO, 1968) and cervical cord (WILSON and YOSHIDA, 1969) at a monosynaptic latency.

In the lumbar cord monosynaptic connections select predominantly the motoneurons innervating the gastrocnemius (GS) and quadriceps (Q) muscles, as indicated by both the mean size of the EPSP and its frequency of occurrence (GRILLNER et al., 1970) (Fig. 2). A illustrates the monosynaptic EPSP evoked in a GS cell of the cat. GRILLNER et al. (1970) also found that motoneurons of other extensors (m. anterior biceps-semimembranosus ABSm, plantaris Pl, flexor digitorum longus FDL and tibialis Tib) may also receive a monosynaptic input from Deiters' nucleus. Thus, Deitero-motoneuronal EPSPs appear to be common in knee and ankle extensors, and rather rare in hip and toe extensors. WILSON and YOSHIDA (1969) recorded monosynaptic EPSPs evoked from Deiters' nucleus exclusively from GS cells, but they found little evidence of a monosynaptic vestibulo-motoneuronal input to hip extensors. The differences between the results of WILSON and YOSHIDA and those of GRILLNER and coworkers may be due, at least in part, to the fact that these authors also stimulated structures other than Deiters' nucleus.

The amplitude of vestibulo-motoneuronal EPSPs recorded from lumbar motoneurons varied from 0.2 to 2.5 mV (mean less than 1 mV) (SHAPOVALOV et al., 1966; SHAPOVALOV and SAFIANTS, 1968; GRILLNER et al., 1970). GRILLNER et al. (1970, 1971) found a considerable variation among different cats in terms of both amplitude and frequency of occurrence of vestibulo-motoneuronal EPSP. In some animals virtually no monosynaptic connection could be revealed from Deiters' nucleus.

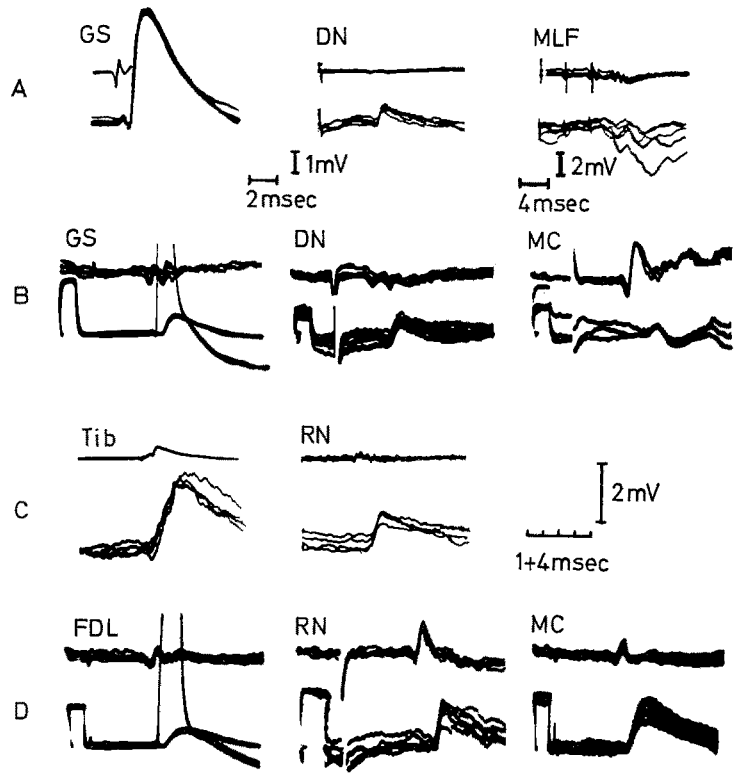


Fig. 2A—D. Vestibulo-motoneuronal (A, B) and rubro-motoneuronal (C, D) EPSPs in the cat (A, C) and rhesus monkey (B, D). (A) a GS motoneuron of the cat (GRILLNER et al, 1968) (B) a GS motoneuron of the monkey (SHAPOVALOV et al., 1971) Calibration pulse 1 msec, 5 mV (GS) and 2 mV. (C) a Tib motoneuron of the cat (HONGO et al., 1969a) (D) a FDL motoneuron of the monkey (SHAPOVALOV et al., 1971) Calibration pulse 1 msec, 5 mV (FDL), 1 mV (RN) and 2 mV (MC)

Whereas monosynaptic Deitero-motoneuronal excitatory connections are found in motoneurons of some extensors of the hindlimb, WILSON and YOSHIDA (1969) could not detect such an input in any forelimb motoneurons. On the other hand, the motoneurons of neck muscles acting on the head receive a powerful monosynaptic input from Deiters' nucleus. This pathway seems to be the predominant one, linking the vestibulospinal cells with these motoneurons. The presence of direct connections between vestibulospinal axons and neck motoneurons apparently reflects the special role of Deiters' nucleus in the regulation of neck reflexes. Thus it appears that the Deitero-motoneuronal projection in the cat is highly specialized and, in contrast to the reticulomotoneuronal input, selects only particular groups of alpha-motoneurons reflecting the close functional relation between the labyrinth and some particular muscle groups.

In the monkey the vestibulospinal effects were investigated only in the lumbar cord. The monosynaptic EPSPs mediated by fast-conducting volleys in the vestibulospinal tract were recorded mainly from GS motoneurons (Fig. 2B).

C. Rubro-Motoneuronal Effects, Comparative Aspects, and Functional Organization

The rubrospinal pathway connecting the midbrain with the spinal motor centers first appears in reptilia. ROBINSON (1969) clearly demonstrated the existence of the crossed rubrospinal tract in the lizard. Stimulation of the red nucleus in the turtle failed to disclose any synaptic action in lumbar motoneurons. This suggests that oligosynaptic rubro-motoneuronal projection has not yet developed in reptilia (cf. SHAPOVALOV, 1972b).

In the rat the maximum conduction velocity of the rubrospinal pathway is about 40 to 55 m/sec (GUREVITCH and BELOZEROVA, 1971) and thus does not reach the maximal rates of conduction in reticulospinal axons of the same species. Stimulation of the contralateral red nucleus in the rat rarely evoked monosynaptic effects in lumbar motoneurons.

In the cat rubrospinal axons running in the lateral columns terminate at all levels of the spinal cord in Rexed's laminae V–VII (NYBERG-HANSEN and BRODAL, 1964). The rubrospinal axons were found to conduct at velocities up to 120 to 150 m/sec, although the majority were between 40 and 90 m/sec (TSUKAHARA et al., 1967; FANARDJAN and SARKISJAN, 1969; PADEL et al., 1972). Thus, the conduction velocity of the fastest rubrospinal and reticulospinal fibers is quite similar.

Monosynaptic rubro-motoneuronal EPSPs evoked by fast rubrospinal volleys were found in only a few hindlimb motoneurons of the cat (SHAPOVALOV, 1966; HONGO et al., 1969). An example of monosynaptic EPSP elicited by a rubrospinal volley in the toe extensor motoneuron is shown in Fig. 2C. Taking into account that rubro-motoneuronal EPSPs can rarely be recorded from cat motoneurons, HONGO et al. (1969) considered them to be aberrant. However, MOTORINA (1974) using the experimental morphological methods of GOLGI-DEYNEKA, found degenerating rubrospinal boutons on the dendrites of lumbar motoneurons, mainly in the dorsolateral motor nuclei. It may be suggested that the rubro-motoneuronal input in the cat is highly specialized and can be found only in a few particular groups of alpha-motoneurons.

In the rhesus monkey the rubrospinal tract originating from the large rubral neurons terminates in Rexed's laminae V–VII at all levels of the spinal cord (KUYPERS et al., 1962; MILLER and STROMINGER, 1973). The conduction velocity of the rubrospinal pathway was found to be 70 to 80 m/sec (SHAPOVALOV et al., 1971a) and that of the fastest rubrospinal axons activated antidromically rose to 90 to 110 m/sec (TAMAROVA, 1975). Thus, there is practically no difference between the conduction velocity of the reticulo-spinal and rubro-spinal pathways in the same species.

Intracellular recordings from lumbar alpha-motoneurons revealed that the monkey possesses a considerable monosynaptic connection with hindlimb motoneurons innervating the distal muscles (EDL, TA, FDL, Ll), but monosynaptic EPSPs were absent in motoneurons of proximal muscle groups (ABSm, Q, PBST, H) and GS (SHAPOVALOV et al., 1971a). An example of rubro-motoneuronal EPSPs in a FDL motoneuron is shown in Fig. 2D. It should be noted that the motoneurons receiving a monosynaptic input from the red nucleus were located

in the dorsolateral region of lamina IX, as evidenced by intracellular injection of procion yellow dye (SHAPOVALOV, 1972a).

Although both toe flexor and extensors receive a rubral monosynaptic excitatory input, the amplitude of the monosynaptic EPSPs recorded from the flexor motoneurons was a rule larger than that from the extensors. The mean size of the maximal rubro-motoneuronal EPSPs in DP, EDL and TA motoneurons was found to be 0.84 mV, whereas the mean size of the EPSPs in FDL motoneurons was only 0.54 mV. The difference is statistically significant ($p > 0.01$).

Thus, the direct rubro-motoneuronal monosynaptic input, like the vestibulo-motoneuronal input, is more specialized in terms of activation of particular alpha-motoneurons than the direct reticulo-motoneuronal path. However, it appears inappropriate to specify monosynaptic excitatory connections of the rubrospinal tract in terms of extensor and flexor motoneurons, since the tract selects motoneurons of both functional groups, but rather in terms of motoneurons of distal muscles.

The available evidence demonstrates that there has been a considerable change in rubrospinal projection in the course of evolution. It is generally believed (SCHOEN, 1964) to be very poorly developed in subhuman primates and in man. It may be suggested that rubrospinal synaptic projections reach their peak in carnivores and lower primates and then begin to regress. It is remarkable that the size of the rubrospinal tract generally parallels that of the vestibulospinal tract (SCHOEN, 1964).

D. Properties of Brainstem-Motoneuronal Inputs

The descending monosynaptic actions evoked from different brainstem nuclei in spinal motoneurons of various vertebrates have many properties in common.

1. Monosynaptic input is mostly excitatory. Only in cervical and thoracic motoneurons of the cat were monosynaptic IPSPs reported to be evoked from the lower brainstem (WILSON et al., 1970). In the lumbar cord monosynaptic effects were exclusively excitatory.

2. Monosynaptic actions are mediated only by fast-conducting fibers running in the corresponding pathway. Such organization may be due to the specific functional task of the direct communication system. As RUSHTON (1951) pointed out, when there is a limited amount of space into which nerve fibers can safely be fitted, the choice lies between a few fast-conducting or many slowly conducting fibers. Considerations of economy alone would suggest that large fibers should supply mechanisms involving rapid reaction with little analysis of the input at segmental level. This might be an important factor, since it is evident that monosynaptic commands supply motoneurons with information that is processed at brain level and is not modified at spinal level.

In cyclostomes 12 pairs of thick reticulospinal axons occupy about 10% of the cross-sectional area of the spinal cord. In this case, as in invertebrates, high conduction speed was achieved by developing giant axons. Myelination, which appeared at later stages of evolution, greatly increases axonal conduction rates without greatly increasing diameter, and this permits an increase in the number

Table 1. Comparison of the latencies of monosynaptic EPSPs evoked in hindlimb motoneurons by reticulospinal and afferents volleys and of conduction velocities of the fastest reticulospinal and primary afferent fibers in different tetrapods

Animal		Frog	Turtle	Rat	Cat	Monkey
Latency of	reticulospinal	2.5–3.5	5–7	1.5–2.0	3–4	3–4
EPSP msec	afferent	5–6	5–7	2–3	2–4	3–6
Conduction	reticulospinal	25–30	30–40	60–80	120–150	70–80
velocity m/sec	afferent	35–40	15–20	60–70	90–120	60–70

of fast-conducting fibers. Therefore the fast-conducting pathway in evolutionarily more advanced animals may occupy a relatively smaller part of the projection system although it consists of a greater number of descending axons. It is remarkable that the evolutionary maturation of the Deitero-motoneuronal and rubro-motoneuronal projection is accompanied by an increase in the conduction speed of the relevant fibers in relation to the conduction speed of the more ancient reticulo-motoneuronal system.

It should be noted in this connection that the absolute values of the conduction velocity of the brainstem-motoneuronal axons may vary considerably in different vertebrates. In some species (lamprey, turtle, rat, cat, monkey) these descending fibers conduct at faster rates than any other fibers of the central and peripheral nervous system. In other species (frog) they may conduct more slowly than the fastest primary afferents. The data summarized in Table 1 show that the conduction speed of the fastest reticulospinal and the fastest primaries is often different. However, the latency of the reticulo-motoneuronal EPSPs is shorter than or at least equal to that of the monosynaptic EPSPs evoked from peripheral nerves. In other words, descending volleys from the brainstem can always reach the target motoneurons innervating the muscles of the extremities before or at the same instant as impulses from muscle spindles. This feature may be sufficient for the integration of descending and afferent information in the motoneuron. It may be concluded, therefore, that conduction time is an especially important factor for brainstem-motoneuronal input. It may be surmised, further, that once the central mechanism for coactivation of direct descending and afferent actions on alpha-motoneurons has developed, it does not need any further modification because of changes in the actual length of each corresponding pathway in relation to the size of the body or extremity. This conclusion is consistent with the ontogenetic maturation of the direct bulbo-motoneuronal system as evidenced by the constancy of conduction time of monosynaptic actions recorded in young cats (LENKOV and SHAPOVALOV, 1969).

3. Monosynaptic EPSPs evoked by single reticulospinal, vestibulospinal, or rubrospinal volleys are subthreshold for initiation of the spike. The small amplitude of monosynaptic brainstem-motoneuronal actions may be due to a restricted number of descending neurons innervating spinal motoneurons. This interpretation fits in with the relation between stimulus strength and amplitude of the resulting EPSP. The monosynaptic actions are usually saturated at small increase of stimulating current. Once the maximum size has been attained, the

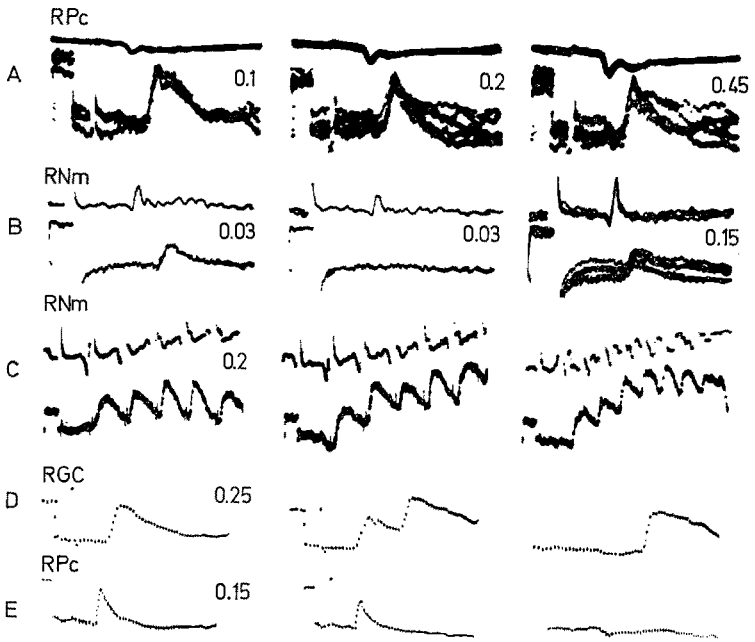


Fig. 3A—E. Properties of monosynaptic EPSPs evoked from different brainstem structures in pyramidotomized monkeys. (A) Reticulo-motoneuronal EPSP elicited by different current intensities. (B) Single traces of rubro-motoneuronal EPSP evoked by shocks decreased to the threshold value (0,03 mA) and occurring in an all-or-none manner. Final record: superimposed tracts of EPSP evoked by shocks incremented to 0.15 mA. (C) Effects of repetitive rubral stimulation at different frequencies. (D) Averaged record of reticulo-motoneuronal EPSP evoked by single (first trace), paired shocks (middle trace), and the result of subtracting the single response from the double by the computer (third trace). (E) Averaged records of reticulo-motoneuronal EPSP. Left, no polarizing current; center, a hyperpolarizing current at intensity 1×10^{-8} A is passed; right, difference between the other two records. The strength of the stimulating current (mA) is noted beside the records. Calibration pulses 1 mV, 1 msec. The upper traces are records of the cord dorsum potentials, the lower traces are intracellular records. (SHAPOVALOV, 1973a)

stimulus intensity can be very much increased without inducing any further change in the height of the EPSP (Fig. 3A). In many cases it is possible to reduce the intensity of the stimulating shocks to threshold level so that the resulting EPSP occurs in all-or-none manner; the increase of stimulus intensity does not alter the height of the recorded EPSP but restores the regularity of its generation (Fig. 3B). These properties and the fast rate of monosynaptic actions imply that they depend on an initial near-synchronous synaptic impact.

Since stimulation of direct brainstem-motoneuronal projections can activate only a small part of the synaptic scale of alpha-motoneurons, the mechanism by which the firing of the motoneuron is brought about by monosynaptic action suggests the coactivation of other depolarizing inputs converging onto the same motoneuron. It was shown that some monosynaptic EPSPs of the rhythmic series step up the depolarization to cross the firing level and lead to a propagated response when reinforced by background subthreshold depolarization (SHAPOVALOV, 1972a). This means that each outgoing discharge is temporally related

to a certain ingoing descending volley and that the discharge patterns may be closely related to the patterns of descending monosynaptic input. This provides a control mechanism in keeping with the small amplitude of the descending monosynaptic actions. Therefore, monosynaptic effects exerted by a relatively small number of brainstem neurons can be quite effective and need far less energy in order to reach the same effect as ordinary sustained depolarization. As GRANIT (1970) has pointed out, a nonspecific effect of the polysynaptic pathway may be all that is needed to bring out a specific monosynaptic effect by providing a kind of necessary screen of general depolarization for the commands mediated by direct specific inputs.

4. Monosynaptic actions evoked from different brain levels can follow at a high frequency of stimulation, up to 500–800/sec (SHAPOVALOV et al., 1966, 1967, 1971; GRILLNER et al., 1971). They do not sum appreciably until the stimulus interval falls below 3–5 m/sec (Fig. 3C) and reveal, as a rule, only moderate frequency potentiation (Fig. 3D). Powerful frequency potentiation of monosynaptic reticular EPSPs, reported by WILSON and YOSHIDA (1969) in cat motoneurons, is demonstrated by EPSPs of unknown segmental delay and of longer latency than is characteristic for a strictly monosynaptic linkage; this potentiation may be due to the involvement of disynaptic actions.

The ability to follow the high frequency stimulation without block or reinforcement may reflect the high functional stability of descending monosynaptic commands.

5. Monosynaptic brainstem-motoneuronal EPSPs reveal a relatively low sensitivity to trans-membrane polarization. SHAPOVALOV et al. (1966, 1967, 1970, 1971c) investigated the effects of intracellularly injected de- and hyperpolarizing currents on the monosynaptic vestibulo- and reticulo-motoneuronal EPSPs in the cat. They did not find any substantial change in the amplitude of these EPSPs, although marked effects were encountered on the descending polysynaptic actions recorded from the same cells (Fig. 3E). This finding may indicate a dendritic location of the direct monosynaptic input, and such an interpretation agrees with the anatomical evidence (NYBERG-HANSEN, 1969). From the functional point of view, this implies that the amplitude of the direct synaptic actions exerted by long descending axons is relatively independent of other inputs converging onto the same motoneuron. Such an arrangement may be very appropriate for the high stability of the brainstem-motoneuronal commands, as is also evidenced by other properties of this input.

6. Investigation of interaction of monosynaptic brainstem-motoneuronal and group 1a EPSPs showed that they sum linearly (KURCHAVYI and SHAPOVALOV, 1971). This suggests the spatial segregation of direct synapses formed by long descending and group 1a fibers on the motoneuronal membrane. This conclusion is in accord with experiments conducted with chromatolyzed motoneurons in the cat (SHAPOVALOV and GRANTYN, 1968). It was shown that reticulo-motoneuronal and group 1a EPSPs of equal size can frequently be quite different in terms of initiation of partial response in the same alpha-motoneuron.

The suggestion that the two inputs are spatially segregated is consistent with the anatomical data about the direction of descending and afferent projections toward the different dendrites of the motoneuron (SPRAGUE and HA, 1964).

E. Conclusion

Available evidence implies that a direct cerebro-motoneuronal linkage exists in all vertebrates above Acrania. The most ancient direct input is represented by the reticulo-motoneuronal system. Fast-conducting reticulospinal axons establish monosynaptic articulations with spinal motoneurons of all spinal levels in all vertebrates, and nearly all the main features found in mammals can already be recorded in lower vertebrates. The differences between species are generally much smaller than their similarities. In the course of evolution the efficient reticulo-motoneuronal projection evidently developed at the beginning and changed relatively little thereafter. One may thus conclude that it plays an important role in supraspinal motor control, otherwise it would not have been preserved throughout the evolutionary scale.

More elaborate supraspinal coordination apparently required the development of an additional, more specialized descending system, the vestibulo-spinal and rubrospinal system, which can be considered as a derivative of the reticulospinal system. Development of these projections in the course of vertebrate phylogeny is accompanied by the increase of conductance speed relative to the reticulospinal path and the appearance of direct synaptic articulations with particular alpha-motoneurons.

It was shown in the cat that individual hindlimb motoneurons receive monosynaptic input from only one supraspinal source: either the reticulospinal tract or Deiters' nucleus, depending on the species of motoneurons (GRILLNER et al., 1971). On the other hand, cervical motoneurons in the cat regularly receive monosynaptic input from both supraspinal sources (WILSON and YOSHIDA, 1969). In the monkey, motoneurons innervating distal muscles of the hindlimb in addition to the rubral input tend to receive monosynaptic excitation from the motor cortex (SHAPOVALOV et al., 1971). Thus, the same motoneurons may receive only one or several monosynaptic excitatory inputs from different descending pathways. This implies that supraspinal projections may be functionally coupled in a manner different from the simple reciprocal relation proposed by GRILLNER et al. (1971). This conclusions is also in accord with the evidence that a given direct pathway may innervate both extensor and flexor motoneurons (SHAPOVALOV and GUREVITCH, 1970; SHAPOVALOV et al., 1971a, b; SHAPOVALOV, 1973a; WILSON and YOSHIDA, 1969).

III. Brainstem-Spinal Disynaptic Control of Alpha-Motoneurons

A. Comparative Aspects

Fast-conducting fibers of brainstem-spinal pathways also exert disynaptic influences on spinal motoneurons in addition to or instead of monosynaptic actions. Even in lower vertebrates with their relatively poor interneuronal apparatus of the spinal gray matter (NIEUWENHUYS, 1964) disynaptic control may be quite prominent. In the lamprey the large nerve cells located at the lateral edge of the

spinal gray matter and projecting caudally into the ventrolateral column receive direct excitatory synapses from thick reticulospinal axons of the bulbar reticular cell groups (ROVAINEN et al., 1973). It may be supposed, therefore, that these propriospinal descending neurons mediate reticular influences to segmental motoneurons. In fact, the substantial part of post-synaptic effects recorded from the motoneurons has a segmental delay suggesting a disynaptic linkage (BATUEVA and SHAPOVALOV, 1974).

In the amphibian cord, descending monosynaptic EPSPs can be elicited in motoneurons of lower lumbar segments by stimulation of lateral (FADIGA and BROOKHART, 1960) and ventral (SHAPOVALOV and SHIRJAEV, 1973; SHIRJAEV, 1973) columns. Since these monosynaptic actions persist after chronic spinal section, they may be tentatively regarded as propriospinal (SHAPOVALOV and SHIRJAEV, 1973). Neurons of the propriospinal descending system are the most probable relay station for the disynaptic reticular influences, since reticulospinal axons terminate mainly in the region where cell bodies of the propriospinal neurons are located within the spinal gray matter (ABBIE and ADEY, 1950). The participation of segmental interneurons is, of course, also very likely.

Disynaptic excitatory and inhibitory synaptic actions mediated by fast-conducting bulbospinal pathways are frequent in lumbar motoneurons of the turtle (BATUEVA, 1972).

Disynaptic EPSPs and IPSPs were recorded in different mammalian species from motoneurons of the lumbar cord when electrical stimuli were applied to reticular formations (SHAPOVALOV et al., 1967, 1971c; SHAPOVALOV, 1969; GRILLNER et al., 1971; SHAPOVALOV and GUREVITCH, 1970) vestibular nuclei (GRILLNER et al., 1970; SHAPOVALOV and SAFJANTS, 1968; WILSON and YOSHIDA, 1969, 1970) and red nuclei (HONGO et al., 1969; GUREVITCH and BELOZEROVA, 1971; SHAPOVALOV, 1966; SHAPOVALOV et al., 1971a).

B. Properties of Disynaptic EPSPs and IPSPs

In alpha-motoneurons of the cat lumbar cord directed reticulospinal or vestibulospinal volleys frequently evoke short-latency EPSPs and IPSPs with a segmental delay of 1–2 msec; this suggests a disynaptic linkage. Usually a later positive volley of the cord dorsum potential precedes the beginning of disynaptic response for 0.2–0.5 msec (Fig. 4); this may indicate cellular activity deep in the ventral horn. On the basis of its latency, time course, temporal facilitation, post-tetanic potentiation and sensitivity to drugs, it is assumed that the second positive volley of the cord dorsum potential reflects the activation of propriospinal elements included in the disynaptic pathway between brainstem-spinal neurons and alpha-motoneurons (SHAPOVALOV and SAFJANTS, 1968; SHAPOVALOV, 1969). It is noteworthy that the relayed component of the cord dorsum potential changes in parallel with the disynaptic actions.

The properties of the disynaptic actions evoked in alpha-motoneurons of different mamalian specimens are similar in many respects.

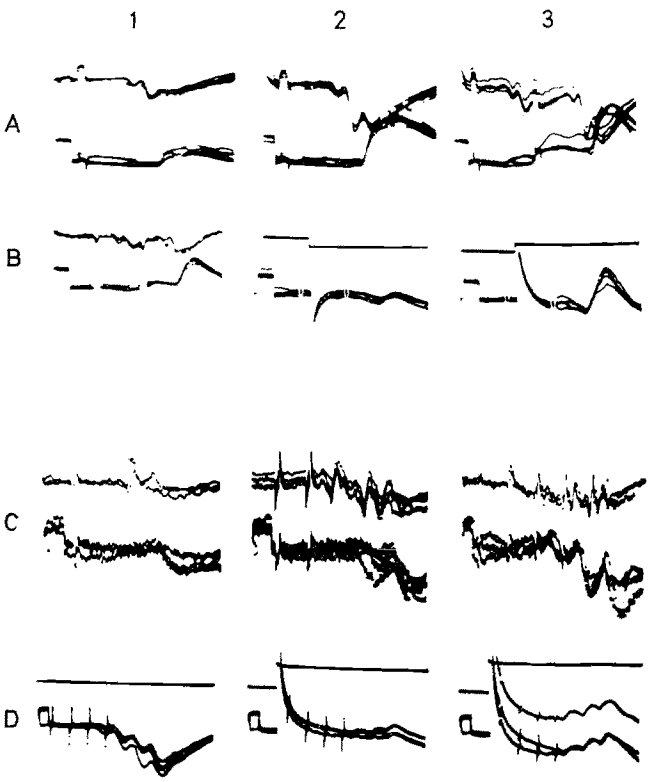


Fig. 4A—D. Disynaptic brainstem-spinal actions. (A) Reticulospinal EPSPs in lumbar motoneuron of the cat. 1 Control records; 2 Test response after conditioning tetanus at 600-sec for 40 sec; 3 responses to double shocks. Calibration pulses 5 mV, 1 msec. (SHAPOVALOV, 1969). (B) Effects of polarizing currents on disynaptic reticulospinal EPSPs in the rat. 1 no polarizing current; 2 a pulse of depolarizing current at intensity 13.2×10^{-9} A is passed; 3 a pulse of hyperpolarizing current at intensity 11×10^{-9} A is passed. Calibration pulses 2 mV, 1 msec. (SHAPOVALOV and GUREVITCH, 1970). (C) Rubrospinal EPSPs in GS motoneuron of the monkey evoked by single and repetitive shocks (D) Effects of polarizing currents of disynaptic rubrospinal EPSPs. 1 no polarizing current; 2 a pulse of hyperpolarizing current at intensity 1.5×10^{-8} A is passed; 3 a pulse of hyperpolarizing current at intensity 2×10^{-8} A is passed. Calibration pulses 0.5 mV, 1 msec. (SHAPOVALOV et al., 1971 a)

1. In contrast to monosynaptic EPSPs, disynaptic excitatory and inhibitory responses show a progressive increase in amplitude when descending volleys are repeated or after a succession of stimuli (Fig. 4A). The augmentation of successive PSPs depends very much upon the frequency of stimulation increasing with the frequency of the applied stimuli. This temporal facilitation or frequency potentiation is often quite marked even at rates of 50–100/sec and reaches its peak at frequencies of about 200–400/sec. The special property of frequency potentiation of disynaptic effects produced by repetitive activation of the brainstem-spinal excitatory pathways may be considered significant in the initiation of spike discharges in target motoneurons (SHAPOVALOV et al., 1966, 1967). The potentiation of disynaptic IPSPs may lead to a great enhancement of the inhibitory effects produced by the relevant pathways.

The frequency potentiation of disynaptic actions may be due to the fact that synapses between fast descending fibers originating from supraspinal structures and propriospinal neurons, or between axons of propriospinal neurons and alpha-motoneurons, have special properties distinct from those of direct brainstem-motoneuronal synapses. Another possible interpretation is that frequency potentiation is due to the existence of a large subliminal fringe in the relay between axons of supraspinal tracts and propriospinal neurons. The fact that frequency potentiation of disynaptic EPSPs depends on their amplitude (SHAPOVALOV and SAFJANTS, 1968; SHAPOVALOV, 1969) favors the latter supposition.

Because of frequency potentiation, disynaptic responses that cannot be disclosed by a single shock can easily be evoked by paired shocks or by a short train of stimulating impulses.

2. In contrast to the results obtained during the study of monosynaptic brainstem-motoneuronal actions, polarizing currents passed through an intracellular microelectrode readily influence disynaptic EPSPs (Fig. 4B) and identify the accompanying change in membrane impedance (SHAPOVALOV et al., 1966, 1967, 1971). These results suggest that the loci of corresponding synapses are relatively near the recording site, presumably in the cell body. On the other hand, if some IPSPs take part in the recorded post-synaptic depolarization, which is quite probable as the disynaptic EPSPs and IPSPs have similar latencies, the effect of the injected current on disynaptic EPSPs will be much amplified.

The records shown in Fig. 4D further indicate that the disynaptic IPSPs can be easily reversed to depolarizing potential following the passage of hyperpolarizing current through the impaled motoneuron. The inversion of disynaptic inhibition induced from reticulospinal, vestibulospinal and rubrospinal pathways (SHAPOVALOV et al., 1966, 1967, 1971a) shows that an increase in conductance is associated with the generation of the corresponding IPSP. Moreover, these results indicate that the loci of IPSP synapses are relatively near the recording site, presumably in the cell body.

3. Disynaptic actions are much more sensitive than monosynaptic EPSPs to the blocking action of anesthetic drugs (SHAPOVALOV and SAFJANTS, 1968). However, rhythmic stimulation can readily restore the partially blocked disynaptic transmission because of the property of frequency potentiation.

C. Functional Organization

Disynaptic actions are evidently less specific in terms of selective activation of particular motoneurons than direct brainstem-motoneuronal EPSPs. Disynaptic reticulospinal EPSPs can be found in motoneurons of proximal and distal muscle groups, of flexors and extensors (GRILLNER et al., 1970, 1971). In the cat many flexor and extensor motoneurons also receive disynaptic EPSPs from Deiter's nucleus (GRILLNER et al., 1970). For instance, many DP and FDL cells received disynaptic EPSPs from the vestibulospinal tract and monosynaptic and disynaptic input from the reticulospinal pathway. This implies that propriospinal neurons included in the disynaptic pathway to the spinal motoneurons receive convergent excitation from both these supraspinal sources. In fact, the heterosynaptic facili-

tation of disynaptic EPSPs evoked from reticulospinal and vestibulospinal tracts supports this possibility (SHAPOVALOV and SAFJANTS, 1968). Disynaptic IPSPs evoked from Deiters' nucleus are usually recorded from motoneurons receiving monosynaptic excitatory input from the reticulospinal pathway. The input provides reciprocal organization of vestibulospinal and reticulospinal effects (GRILLNER et al., 1971).

Disynaptic EPSPs evoked in feline alpha-motoneurons from the rubrospinal tract were found in cells innervating both flexor and extensor muscles; this shows that there is not a simple reciprocal relationship between effects to the motor nuclei of antagonists (HONGO et al., 1969). In the monkey disynaptic IPSPs from the red nucleus occur preferentially in motoneurons of the proximal muscles of the hindlimb and in the GS cells (SHAPOVALOV et al., 1971a).

D. Control of Propriospinal Neurons and Last-Order Interneurons of Reflex Pathways

Propriospinal neurons relay information that can be transmitted caudally for long distances along the spinal cord and contribute significantly to suprasegmental control of the motor systems. In the cat the cell bodies of propriospinal neurons innervating lumbosacral motoneurons are located mainly at the level of L_2–L_4 (LLOYD, 1941a; SZENTAGOTHAI, 1964). Axons of propriospinal neurons descend in the ventrolateral columns and make synaptic articulations with motoneurons of lower lumbar segments (MCLAUGHLIN, 1971). Stimulation of the ventrolateral funiculi evokes monosynaptic EPSPs in ipsilateral motoneurons (WILLIS et al., 1967) and these effects are preserved after chronic section of the spinal cord at thoracic or cervical levels (REPINA et al., 1969; KOJANOV, 1974). Microstimulation of different points of the ventral quadrants in cats with chronic cord section evokes monosynaptic EPSPs and less often monosynaptic IPSPs in the hindlimb motoneurons. The records in Fig. 5C and D show monosynaptic EPSPs evoked from two differents points of spinal gray matter at L_3 in PP and PI motoneurons in cats with complete cord section made 2 to 3 weeks before recording. Paired shocks reveal moderate frequency potentiation.

Recording from antidromically identified propriospinal neurons located in the same region from which monosynaptic actions were elicited in hindlimb motoneurons, has shown that many of them received monosynaptic excitatory input from a fastconducting reticulospinal path (Fig. 5A). The reticulo-propriospinal input is so powerful that it evokes action potentials in many propriospinal cells. The latency of these discharges is in the range of 3 to 4 msec. The antidromic spikes evoked by shocks applied to the ventrolateral columns at L_7 appear after a latency of 0.5 to 0.8 msec. These values taken together with the synaptic delay (0.3 to 0.8 msec) in the relay between propriospinal axons and alpha-motoneurons give a total value of 4.2 to 5.5 msec, which agrees very well with the latencies of brainstem-spinal disynaptic EPSPs and IPSPs recorded from hindlimb motoneurons (SHAPOVALOV and SAFJANTS, 1968).

It should be noted that in some propriospinal cells discharges evoked by brainstem stimulation appeared after a longer delay (2 to 6 msec) following a

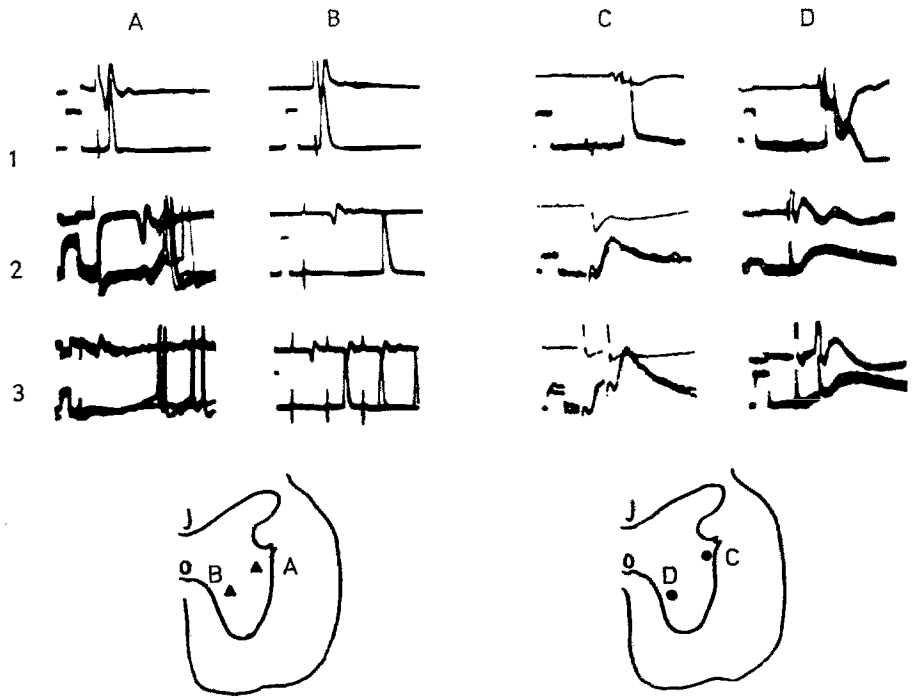


Fig. 5A–D. Intracellular records from propriospinal interneurons at L_3 (A, B) and motoneurons at L_7 (C, D) of the cat. (A) 1 Antidromic response evoked by ventral column stimulation at L_7; 2 response evoked by reticular stimulation; 3 response evoked by stimulation of Q nerve. Calibration pulses 1 msec, 25 mV (1) and 5 mV (2, 3). (B) 1 Antidromic response evoked by ventral column stimulation; 2, 3 responses evoked by reticular stimulation. Calibration pulses 1 mseci, 25 mV. (C) 1 Antidromic response evoked from PP nerve; 2, 3 responses evoked from L_3. Calibration pulses 1 msec, 5 mV (I) and 1 mV (2, 3). (D) 1 Antidromic response from Pl nerve; 2, 3 responses evoked from L_3. Calibration pulses 1 msec, 5 mV (1) and 1 mV (2, 3). On the diagrams: triangles—recording points, circles—stimulating points

synchronous fast-conducting descending volley (Fig. 5B). Since the latencies of these later discharges were stable and the responses could follow rhythmic stimulation, they may be considered as result of monosynaptic excitation produced by brainstem-spinal fibers with slower conduction velocity. If this suggestion is correct, the propriospinal cells may receive monosynaptic input from a much broader spectrum of descending fibers than alpha-motoneurons.

Propriospinal neurons projecting down to lower lumbar segments usually received synaptic actions from primary afferents (Fig. 5A).

KOSTYUK and PILYAVSKY (1969), VASILENKO et al. (1972) and KOSTYUKOV (1973) claim that cat spinal interneurons receiving monosynaptic input from the rubrospinal and reticulospinal pathways are not influenced by primary afferents. These authors consider such spinal neurons as a "private" disynaptic pathway from the supraspinal centers to the alpha-motoneurons. In contrast, HONGO et al. (1972b) regularly recorded monosynaptic excitation from the rubrospinal tract simultaneously with various kinds of synaptic actions from primary afferents in

interneurons of the same species. HONGO et al. presented convincing evidence that disynaptic excitation of spinal motoneurons by rubrospinal impulses is well explained by monosynaptic connections between rubrospinal fibers and last-order interneurons of reflex pathways. This conclusion agrees with the data of BALDISSERA et al. (1971), who examined the effects of a conditioning volley in peripheral nerves on the disynaptic PSPs evoked in alpha-motoneurons from the rubrospinal tract.

Monosynaptic vestibulospinal excitation of last-order interneurons mediating the crossed extensor actions was described by BRUGGENCATE et al. (1969).

E. Conclusion

Available evidence indicates that long descending pathways of supraspinal origin are supplemented by a short descending path intrinsic to the cord. This phylogenetically old system receives a powerful monosynaptic excitation from the brainstem. It has not been established whether the fast reticulo-, vestibulo- and rubrospinal fibers having monosynaptic connections with the alpha-motoneurons are the same as those that influence them disynaptically, although this is highly probable. On the other hand, propriospinal interneurons may also be activated by supraspinal fibers with slower conduction velocities.

The inclusion of even a single additional relay in the path between brainstem-spinal neurons and alpha-motoneurons greatly modifies the properties of this projection, as is evidenced by the marked frequency potentiation of disynaptic effects and by their lower specificity in terms of selective activation of particular alpha-motoneurons.

There is increasing evidence that spinal interneurons receiving monosynaptic input from brainstem-spinal pathways also receive synaptic effects from the periphery. Many of these interneurons may be the last-order interneurons (i.e. interneurons directly connected to motoneurons) of segmental reflex areas.

IV. Brainstem-Spinal Polysynaptic Control

A. Comparative Aspects

It seems clear on the basis of available evidence, both anatomical and physiological, that a good many of the descending motor effects from the brainstem-spinal pathways may be exerted on alpha-motoneurons by way of internuncials located within the spinal cord. Progressive differentiation of the interneuronal apparatus in the course of evolution (NIENWENHUYS, 1964) played a conspicuous role in the evolutionary development of supraspinal control.

Already in cyclostomes, the most primitive vertebrates, a substantial part of the synaptic actions evoked by descending impulses may have features characteristic of polysynaptic linkage (BATUEVA and SHAPOVALOV, 1974). Polysynaptic bulbospinal influences were recorded from amphibian motoneurons (SHAPOVALOV

and SHIRJAEV, 1973; SHIRJAEV, 1972) and in the turtle (BATUEVA, 1972). In mammals polysynaptic EPSPs and IPSPs were considered to be the main constituens of the brainstem-spinal motor outflow (LUNDBERG, 1969; JUNG and HASSLER, 1960).

The technique of identifying particular functional interneuronal systems, developed chiefly in LUNDBERG's laboratory, made it possible to evaluate the role of the different spinal elements in descending motor control.

As most of the electrophysiological experiments were performed on mammals, mainly on the cat, which is by far the most popular preparation for work in this area, consideration of the properties of polysynaptic effects may be based largely on this species.

B. Polysynaptic Excitatory and Inhibitory Actions

1. Polysynaptic EPSPs

Polysynaptic EPSPs evoked in feline alpha-motoneurons by reticulo-, vestibulo- and rubrospinal impulses are characterized by a long latency. This latency may be caused by interneuronal relays, but also by slower conduction rates of the relevant descending fibers. Usually a single stimulating pulse cannot evoke a response but with increase in the number of stimuli the amount of post-synaptic depolarization rapidly mounts (Fig. 6A). Increasing the frequency of stimulation without changing the number of shocks also very strongly affects the intensity and the slope of the depolarizing response (Fig. 6B). The polysynaptic EPSPs

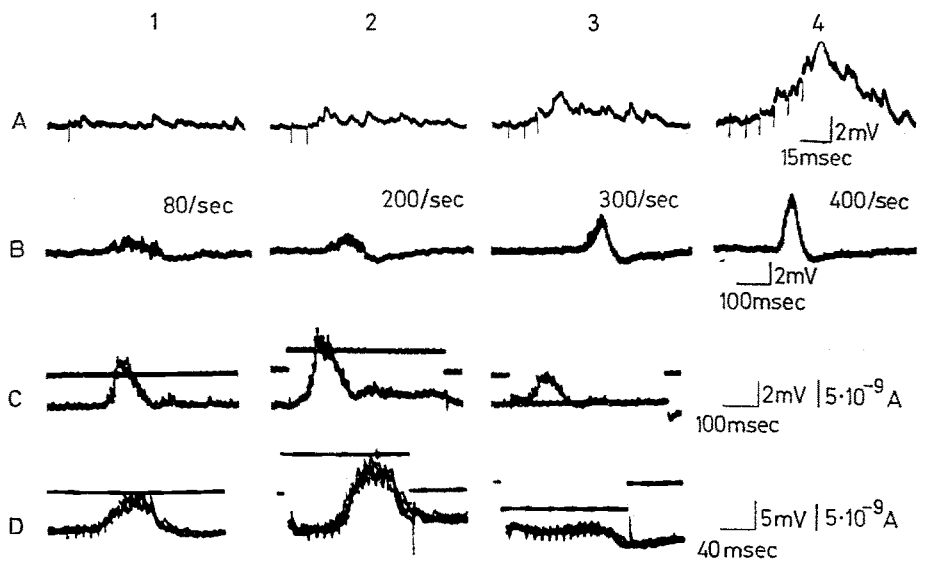


Fig. 6A—D. Polysynaptic EPSPs evoked in 4 different cat motoneurons (A—D) by rhythmic rubral (A—C) and vestibular (D) stimulation. (A) The number of stimuli is increased (1–4) (B) The frequency of stimulation is increased (1–4) (C) Effects of polarizing currents (D) Effects of polarizing currents (SHAPOVALOV, 1966; SHAPOVALOV et al., 1966)

induced by any brainstem-spinal pathway can easily be augmented by artificial membrane hyperpolarization, and depressed or inverted by injection of depolarizing current (Fig. 6C, D). This suggests that the the equilibrium potential is not far from the resting potential, although the effects may be due to contamination by inhibitory actions. The above suggestion agrees with the finding that polysynaptic EPSPs are not very specific and can be recorded from motoneurons of antagonists (HONGO et al., 1969; LUNDBERG, 1966).

2. Polysynaptic IPSPs in Alpha-Motoneurons

The polysynaptic inhibitory actions may be evoked from all brainstem nuclei from which the main descending tracts originate. Descending impulses mediated by a ventral reticulospinal pathway evoke polysynaptic IPSPs in both flexor and extensor motoneurons (JANKOWSKA et al., 1968).

Reticulospinal IPSPs recorded from lumbar motoneurons reverse during injection of hyperpolarizing current or chloride ions (JANKOWSKA et al., 1968; SHAPOVALOV et al., 1967). By contrast, LLINAS and TERZUOLO (1965) and KOSTYUK (1968) found that the reticular IPSPs in flexor motoneurons, unlike the postsynaptic inhibition evoked by stimulation of primary afferents, could hardly be reversed by injection of chloride ions; they also found little or no change in conductance. These findings led them to postulate that the reticular inhibition in flexor motoneurons is exerted via the synapses located on the dendrites. It is not clear why the findings of LLINAS and TERZUOLO (1965) and of KOSTYUK (1968) differ from those of JANKOWSKA et al. (1968) and of SHAPOVALOV et al. (1967), since there is convincing evidence that the descending inhibition is transmitted via interneurons mediating mediating reflex inhibition and located at segmental level (LUNDBERG, 1969).

Stimulation of Deiters' nucleus and red nucleus also evokes polysynaptic IPSPs in both flexor and extensor motoneurons (GRILLNER et al., 1970; HONGO et al., 1969; SASAKI et al., 1960) although Deiterospinal inhibition is found more frequently in flexor motoneurons and rubrospinal inhibition in extensor motoneurons. IPSPs mediated by Deiterospinal and rubrospinal fibers are reversed by injection of hyperpolarizing current and thus reveal a definite change in conductance (SHAPOVALOV, 1966; SHAPOVALOV et al., 1966).

C. Brainstem-Spinal Influences on Different Spinal Elements

1. Segmental Interneurons

Recording from cat segmental interneurons as well as the combination of afferent stimulation with stimulation of brainstem structures have shown that interneurons transmitting descending influences on alpha-motoneurons are also included in various reflex pathways (LUNDBERG, 1967). These interneurons are located mainly in the areas of spinal gray matter in which descending fibers terminate.

The reticulospinal system and its dorsal component in particular can influence first-order spinal interneurons by giving post-synaptic inhibition (ENG-

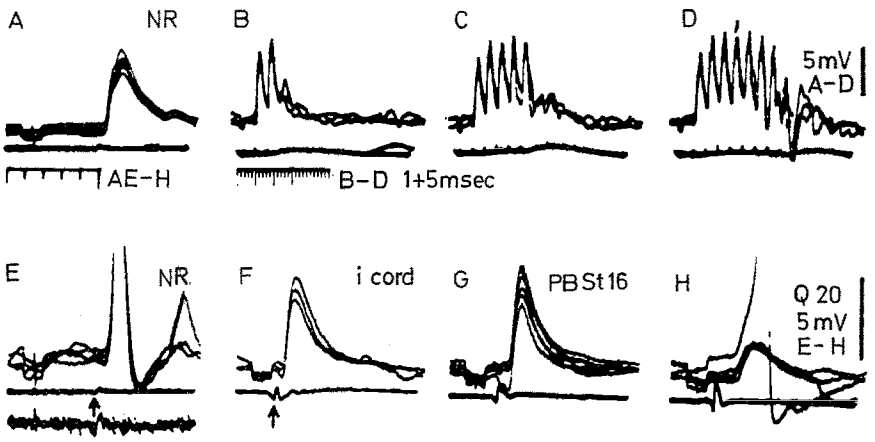


Fig. 7A—H. Monosynaptic excitation from the red nucleus in a Group 1 excited interneuron. (A—E) Potentials evoked from the red nucleus by single and repetitive shocks. (E—F) Response evoked by stimulation of lateral funiculus. (G, H) Group 1 EPSPs (HONGO, et al., 1972b)

BERG et al., 1968). Impulses mediated by fibers running in the ventral reticulospinal pathway frequently evoke EPSPs in segmental interneurons. The dominating effect of Deiterospinal and rubrospinal volleys on interneurons is facilitatory (LUNDBERG, 1967; HONGO et al., 1972; BRUGGENCATE and LUNDBERG, 1974). Fig. 7 shows monosynaptic rubro-interneuronal EPSPs recorded from cells also receiving monosynaptic excitatory input from primary afferents.

HAASE et al. (1971) have shown in the cat that impulses from different brainstem nuclei evoke excitation and inhibition of Renshaw cells.

2. Gamma-Motoneurons

Intracellular recording from feline gamma-motoneurons has demonstrated that they receive monosynaptic excitatory input from fast-conducting fibers of the reticulospinal and vestibulospinal pathways (GRILLNER et al., 1969). The descending monosynaptic EPSPs are larger in gamma- than in alpha-motoneurons. These large values do not necessarily imply a difference in the synaptic organization of descending effects to alpha- and gamma-cells but may be explained by the smaller cell size and thus higher input resistance of gamma-motoneurons (GRILLNER et al., 1969).

It was postulated (GRILLNER, 1969) that gamma- and alpha-motoneurons are co-controlled as illustrated in Fig. 8. This suggests an arrangement for alpha-gamma linkage of a reciprocal nature, so that fast Deiterospinal axons monosynaptically excite static gammas of extensor muscles, whereas fast reticulospinal axons excite static gammas of flexor muscles. As the latencies of monosynaptic EPSPs in alpha- and gamma-cells coincide, both descending projections activate alpha- and gamma-motoneurons of the same muscle in parallel; such organization may be important for efficient supraspinal control.

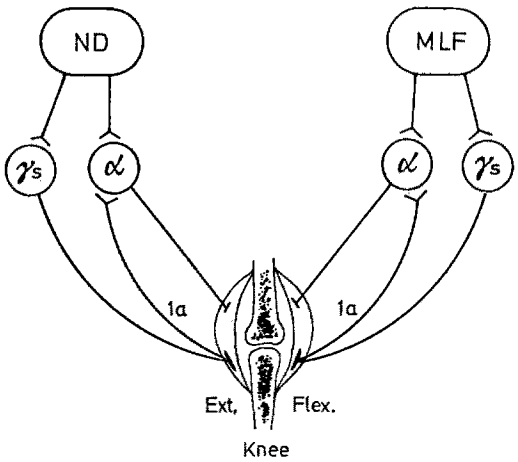


Fig. 8. Schematic representation of descending monosynaptic effects via fast-conducting fibers from Deiter's nucleus and medial reticular formation (MLF) via to alpha- and static gamma-motoneurons to flexor and extensors acting at the knee joint (GRILLNER, 1969)

It should be noted, however, that amphibia lack the gammasystem. Therefore, in these species fast-conducting brainstem-spinal fibers directly innervating spinal motoneurons do not co-control the muscle spindles. In newborn kittens alpha-motoneurons receive monosynaptic input from the brainstem (LENKOV and SHAPOVALOV, 1969) but alpha-gamma linkage is not yet developed (SCOGLUND, 1966). On the other hand, the persistant correlation between conduction time in the thickiest afferents and fastest descending fibers (see Table 1) suggests that information about muscle contraction must reach the alpha-motoneurons after the central commands.

3. Primary Afferents

In the cat depolarization of primary afferent terminals can be evoked from many brainstem structures including the medullary reticular formation (CARPENTER et al., 1966), the vestibular nuclei (BARNES and POMPEIANO, 1970; CARPENTER et al., 1966; COOK et al., 1969) and the red nucleus (HONGO et al., 1972a). It is generally assumed that the depolarization effects presynaptic inhibition of transmission from the primary afferents (ECCLES, 1964).

Excitability measurements from the intraspinal terminals have revealed primary afferent depolarization in 1a, 1b and cutaneous afferents due to stimulation of the medial longitudinal fasciculus and the medial reticular formation of the medulla (CARPENTER et al., 1966). Descending depolarization of 1a afferents has been found on stimulation of the medial vestibular nucleus (BARNES and POMPEIANO, 1970).

The rubrospinal volleys cause primary afferent depolarization in large cutaneous and 1b afferents (HONGO et al., 1972a). It was suggested by these authors that descending effects on primary afferents might be related to motor

regulation rather than to sensory transmission. The depression of transmission from the afferents via their presynaptic inhibition can act jointly with the descending postsynaptic inhibition of first-order interneurons (ENGBERG et al., 1968).

4. Neurons of Ascending Systems

Brainstem-spinal pathways also influence neurons mediating information about movement to the different brain levels. HOLMQVIST et al. (1960) have shown that spinal neurons projecting rostrally on the contralateral ventral funiculi receive monosynaptic input from the bulbo-spinal tracts. Analysis of these descending projections has shown (GRILLNER et al., 1968) that the main monosynaptic route to the spino-reticular neurons is the vestibulo-spinal tract. Monosynaptic effects are also produced by reticulospinal pathways. Disynaptic effects from these bulbospinal fibers are also common.

Neurons of the ventral spino-cerebellar tract receive monosynaptic excitatory and inhibitory input from Deiters' nucleus and the pontomedullary reticular formation (BALDISSERA and WEIGHT, 1969), and from the red nucleus (BALDISSERA

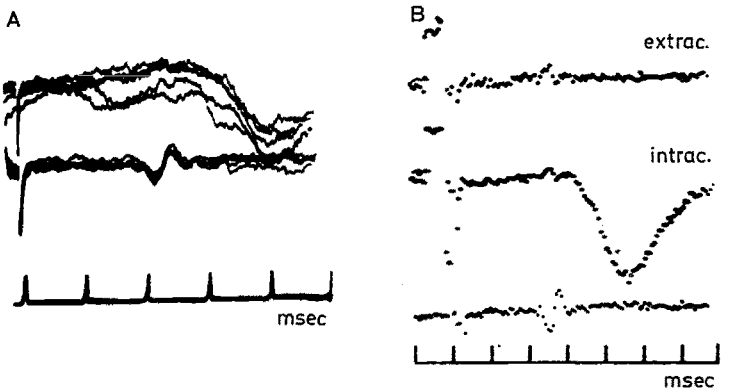


Fig. 9A and B. IPSP evoked in a spinal border cell by MLF stimulation. (A) intracellular potential (upper trace) and descending volley (lower trace. (B) averaged response; upper trace gives the extracellular field potential. Calibration in averaged records 0.5 mV (BALDISSERA and WEIGHT, 1969)

and BRUGGENCATE, 1969) and also powerful polysynaptic influences as well. Fig. 9 illustrates the monosynaptic IPSPs evoked in a spinal border cell by long inhibitory axons descending from the brain stem to the lumbar enlargement. If one takes into account the monosynaptic connections of fast-conducting brainstem-spinal fibers with alpha-motoneurons, the coactivation of motoneurons and neurons of ascending pathways may provide a mechanism for effective internal feedback.

D. Conclusion

Recent evidence indicates that in mammals brainstem-spinal pathways control all neuronal elements of the spinal cord participating in motor control. Moreover,

most of the functionally identified neuronal systems receive direct monosynaptic inputs from the brainstem.

Control of segmental interneurons is concerned with descending polysynaptic influences on alpha-motoneurons and modification of transmission in spinal reflex pathways. Synaptic effects on gamma-motoneurons, primary afferents, and neurons of ascending pathways provide an efficient control of the mechanisms subserving the motor regulation at every stage of information processing at the spinal level.

V. Participation of Brainstem-Spinal Projections in Cerebral Control of Motor Systems

A. Cerebello-Spinal Effects

In evolutionary terms the cerebellum is a very old structure. It already appears in cyclostomes and is basically similar from fish to man, being primarily concerned with the regulation of motor coordination (DOW and MORUZZI, 1958; ECCLES, 1969). The cerebellum has been presumed to exert its influence on spinal neurons indirectly through various brainstem-spinal and also corticospinal neurons.

1. Cerebello-Reticulospinal Effects

One of the major projections from the fastigial nucleus of the cerebellum is to the medial reticular formation of the medulla (JANSEN and BRODAL, 1954; WALBERG et al., 1962). Fastigial stimulation in the cat powerfully exites fast-conducting reticulospinal neurons at a monosynaptic latency (ITO et al., 1970; ECCLES et al., 1974) and this pathway provides a high degree of urgency in the transmission of the cerebellar commands to spinal motoneurons.

The activity of reticulospinal neurons giving fast-conducting axons to the lumbar cord was recorded during locomotion in mesencephalic and thalamic cats before and after cerebellectomy (ORLOVSKY, 1970a, b). While in animals with intact cerebellum the modulation of activity of reticulospinal neurons with locomotor rhythm was observed in more than half of all recorded units, after cerebellectomy only 8% of reticulospinal neurons displayed modulation of their rhythmic firing in phase with locomotory movements.

2. Cerebello-Vestibulospinal Effects

Connections between the cerebellum and Deiters' nucleus are so powerful and so specific that the latter may be regarded as a displaced cerebellar nucleus (GRANIT, 1970). The efferent projections from the cerebellar vermis to the vestibular nuclei are made by long corticofugal fibers originating from Purkinje cells (WALBERG and JANSEN, 1961; VOOGD, 1964) and from fastigio-vestibular neurons

(WALBERG et al., 1962). Projection from Purkinje cells is inhibitory (ITO and YOSHIDA, 1964; ITO et al., 1970). Projection from the fastigial nucleus is excitatory (ITO et al., 1970).

In an attempt to estimate the role of the cerebellum in the regulation of vestibulospinal neurons during locomotion, ORLOVSKY (1972b) recorded firing of Deiters' neurons in decerebellate cats and in animals with an intact cerebellum. His experiments have shown that the periodic alternations of the activity of fast vestibulospinal neurons projecting to the lumbar cord in relation to the locomotor cycle were observed only in cats with intact cerebellum and disappeared after the cerebellum was removed. Apparently a substantial part of the cerebello-spinal control is exerted by cells projecting to Deiters' nucleus.

3. Cerebello-Rubrospinal Effects

The motor effects from the intermediate cortex of the cerebellum and the interpositus nucleus are transmitted to the spinal cord through the red nucleus. Anatomical investigations in cats and monkeys have shown that the cells in the interpositus nucleus project through the brachium conjunctions to the contralateral red nucleus where fibers of the brachium conjunctions end at all levels from caudal to rostral (CARPENTER and STEVENS, 1957; FLUMERFELT et al., 1971). Phylogenetially the brachium conjunctivum and the red nucleus develop at the same time (MASSION, 1967).

In cats TOYAMA et al. (1970) demonstrated monosynaptic excitation of red nucleus neurons by stimulation of the interposite nucleus. Interposito-rubral connections are so potent that a single electrical shock applied to the red nucleus through a microelectrode fires the rubrospinal neurons not only directly, but also trans-synaptically due to activation of interposito-rubral efferents (BALDISSERA et al., 1972; SHAPOVALOV et al., 1972).

In the monkey stimulation of the interpositus nucleus produces short-latency EPSPs in ipsilateral motoneurons controlling distal muscles of the hindlimb, and polysynaptic actions in motoneurons of the proximal muscle groups (SHAPOVALOV et al., 1972). A striking analogy has been found between the patterns of cerebellar and rubral synaptic influences on different motoneuronal pools (Fig. 10).

Comparison of the effects exerted on the same motoneuron from the interpositus nucleus and red nucleus has shown that synaptic actions evoked in lumbar motoneurons by stimulation of the former are transmitted via the rubrospinal pathway.

Extracellular recording from single identified rubrospinal neurons projecting to the lumbar cord demonstrated modulation of activity in fast rubrospinal cells according to locomotor rhythm only when the cerebellum was intact (ORLOVSKY, 1972c).

4. Cerebro-Spinal Synaptic Actions and Effects of Strychnine

As the brainstem neurons projecting down to the spinal cord represent the intermediate stations between the cerebellum and the spinal neurons, similar synaptic

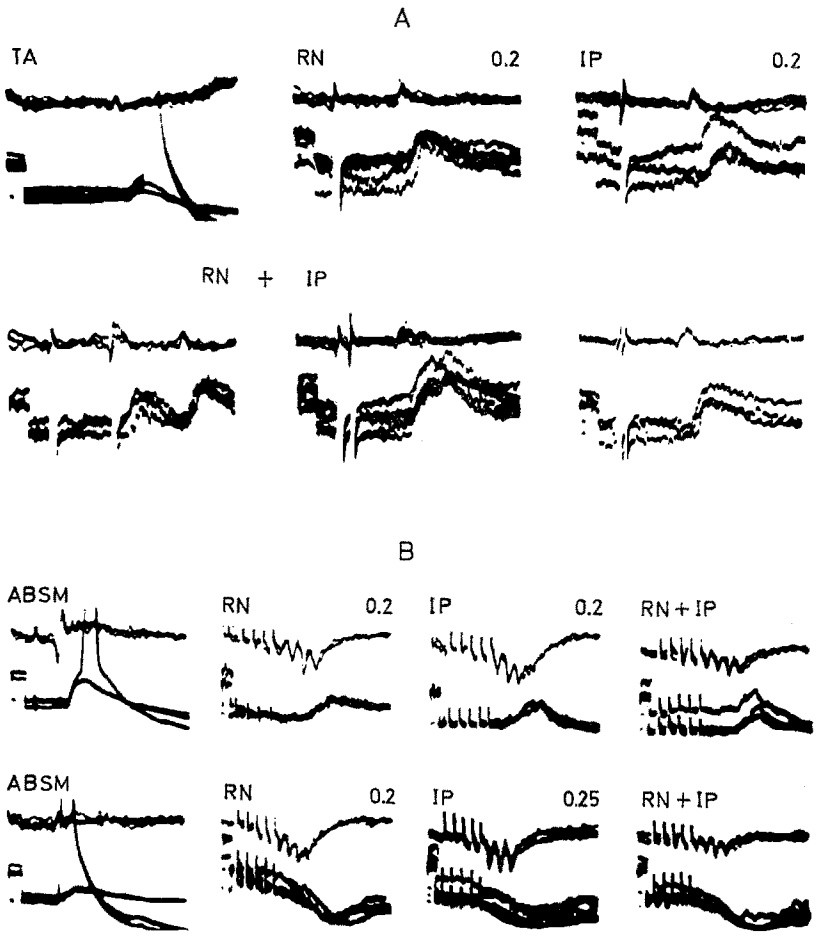


Fig. 10A and B. Cerebello-spinal and rubrospinal effects in hindlimb motoneurons of the monkey. (A) Responses elicited in a TA motoneuron by stimulation of red and interpositus nuclei, first separately (first row) and then together at different intervals (second row). Calibration pulses 1 msec and 1 mV for all records except TA (5 mV) (B) Effects evoked in two different ABSM motoneurons by repetitive stimulation of red nucleus, interpositus nucleus, and both simultaneously. Calibration pulses 1 msec and 1 mV for all records except ABSM (5 mV) (SHAPOVALOV et al., 1972)

actions are evoked in alpha-motoneurons of the cat and the rhesus monkey by stimulation of different cerebellar and brainstem nuclei (SASAKI and TANAKA, 1963; SHAPOVALOV et al., 1972).

It was stressed in several old reports that, contrary to segmental inhibition, inhibitory effects obtained from the cerebellum and the brainstem still remain after strychnization (BREMER, 1953; GERNANDT and TERZUOLO, 1955; TERZUOLO and GERNANDT, 1956). These authors demonstrated in the cat that strychnine tetanus can be effectively suppressed by repetitive stimulation of the anterior lobe of the cerebellum, vestibular and reticular nuclei. It is well-known that strychnine

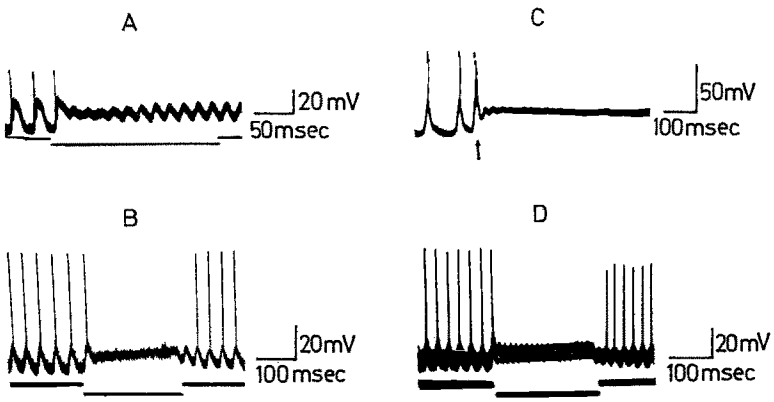


Fig. 11 A—D. Abolition of strychnine tetanus during sustained depolarization produced in cat motoneurons by repetitive stimulation of different supraspinal structures. (A) Stimulation of anterior lobe of the cerebellum. (SHAPOVALOV and ARUCHANJAN, 1963). (B) Stimulation of the red nucleus. (SHAPOVALOV and SHAPOVALOVA, 1966). (C) Stimulation of the pontine reticular formation (KAWAI and SASAKI, 1964). (D) Stimulation of Deiter's nucleus

blocks segmental post-synaptic inhibition; thus might be assumed that in the descending projections there should be some other post-synaptic inhibitory mechanism which is hardly depressed by strychnine (ECCLES, 1957). However, recording of IPSPs evoked from the cerebellum (CURTIS, 1959; SHAPOVALOV and ARUCHANYAN, 1963), reticular nuclei (SHAPOVALOV and ARUCHANYAN, 1965), and red nucleus (SHAPOVALOV, 1966) demonstrated the supressive action of strychnine on descending IPSPs.

Recording from alpha-motoneurons under heavy strychnization which evoked strychnine tetanus has further shown that rhythmic stimulation of the anterior lobe of the cerebellum (SHAPOVALOV and ARUCHANYAN, 1963), pontomedullary reticular formation (KAWAI and SASAKI, 1964), vestibular and red nuclei (SHAPOVALOV, 1966) usually stopped the seizures by development of sustained depolarization and subsequent inactivation of the spike-generating mechanism (Fig. 11). The shunting effect of strychnine depolarization (FUORTES and NELSON, 1963) may also be of importance for suppression of the spikes. The available evidence suggests that the sustained depolarization is due to the excitation of segmentary interneurons on which descending and afferent impulses converge, and to the partial elimination of post-synaptic inhibition (SHAPOVALOV, 1966).

B. Hypothalamo-Spinal Effects

According to CROSBY and WOODBURNE (1940), the hypothalamus is well developed even in cyclostomes and is basically similar throughout the series of vertebrates. GRANTYN et al. (1973) demonstrated in the cat that lateral hypothalamic stimulation evokes oligosynaptic EPSPs in reticulospinal neurons. ORLOVSKY (1973) found that most reticulospinal cells projecting to the lumbar cord received mono-

synaptic excitatory input from the posterior hypothalamus. However, no such input could be determined in the vestibulospinal neurons.

Electrical stimulation of lateral and posterior hypothalamus in the cat evokes EPSPs and IPSPs in lumbar motoneurons (SHAPOVALOV and ARUTYUNYAN, 1964). The shortest latencies of EPSPs evoked in hindlimb motoneurons by hypothalamic stimulations were only 6 to 7.5 msec, suggesting a disynaptic linkage with the first relay at the hypothalamo-reticulospinal junction.

C. Cortico-Extrapyramidal Effects

In contrast to the cerebellum and the hypothalamus, the motor cortex represents the most recent evolutionary development of the vertebrate nervous system, and is present exclusively in mammals. Anatomic studies have been made in various mammalian species: rat (GOLDBY and KACKER, 1963; VALVERDE, 1966; ZIMMERMAN et al., 1964), cat (ROSSI and BRODAL, 1956), and monkey (KUYPERS, 1960). These studies revealed that in its passage through the brainstem the corticospinal tract projects collateral branches to the reticular formation of the pontine and medullary tegmentum, which give off reticulospinal fibers and branches to the red nucleus. There are also special cortico-reticular and cortico-

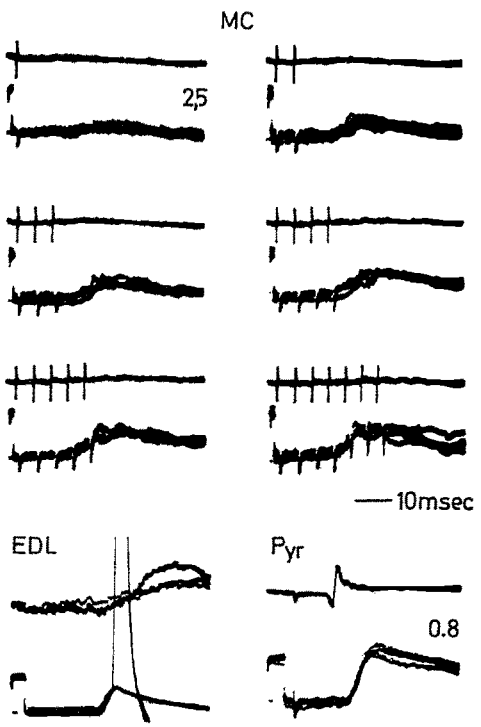


Fig. 12. Cortico-extrapyramidal effects in a pyramidotomized monkey. Effects evoked by single and repetitive stimulation of motor cortex (mc) at intensity 2.5 mA and single shock applied to bulbar pyramid (Pyr) below the lesion. Calibration pulses 1 msec, 2 mV for MC, 5 mV for EDL (TAMAROVA et al., 1972)

rubral projections. It was suggested by NOBACK and SHRIVER (1966) that the original corticospinal fibers were probably cortico-reticular fibers, whose branches extended in the course of evolution into the spinal cord. Monosynaptic and polysynaptic excitatory and polysynaptic inhibitory actions from the motor cortex on reticulospinal neurons have been demonstrated by MAGNI and WILLIS (1964) in the cat, and both monosynaptic and polysynaptic excitation of rubrospinal cells in the red nucleus by stimulation of the sensomotor cortex have been reported in the same species (TSUKAHARA and KOSAKA, 1964).

Electrical stimulation of the sensomotor cortex in pyramidotomized cats revealed that cortico-extrapyramidal volleys evoke PSPs in lumbar motoneurons, facilitation of spinal reflexes at an interneuronal level and depolarization of the presynaptic terminals of group 1 b and cutaneous fibers (HONGO and JANKOWSKA, 1967). The latencies and amplitudes of cortico-pyramidal and cortico-extrapyramidal synaptic actions in spinal motoneurons are very similar.

In rhesus monkeys with pyramidal section there is a dramatic decrease in cortically induced synaptic effects, especially in motoneurons of distal muscles of extremities (SHAPOVALOV, 1972b; SHAPOVALOV, 1973b). Cortical stimulation is effective only with repetitive stimulation, and the latency of PSP is much longer than when the pyramids are left intact (Fig. 12). The patterns of synaptic activation of motoneurons tend to be diffuse, i.e. not time-locked with the applied shocks. Thus, even in primates with their highly developed pyramidal tract, this tract is not the sole pathway that is important for execution of cortically induced motor commands.

D. Conclusion

The brainstem nuclei projecting down to the spinal cord are shown to be governed from different cerebral structures. While some of brainstem-spinal projections are more specific in terms of processing and transmitting commands prefentially from one or several particular centers (vestibulospinal and rubrospinal systems), others (reticulospinal) receive convergent inputs from all central structures involved in cerebral control of movements. The excitation and inhibition of spinal neurons exerted from the cerebellum, hypothalamus and cortico-extrapyramidal systems is undoubtedly secondary to the various patterns of activation of the reticulo-vestibulo- and rubrospinal pathways projecting onto particular interneuronal and motoneuronal groups.

VI. Corticospinal Polysynaptic Control of Mammalian Alpha-Motoneurons

A. Comparative Aspect

The outstanding feature of the organization of supraspinal control in mammals, a feature not shared by other vertebrates, is the existence of the corticospinal tract connecting the neocortex with the spinal cord. This phylogenetically new

cerebral motor outflow becomes increasingly prominent throughout from primitive mammals to primates, and in primates it has established direct contacts with alpha-motoneurons. However, in subprimate species the corticopyramidal control of motoneurons is exclusively polysynaptic, and polysynaptic influences evidently maintain a functional role in primates. Electrophysiological analysis of corticospinal synaptic actions is performed mostly on rodents (rats), carnivores (cats), and primates (monkeys). Although these usual laboratory animals do not appear to fit the criteria for a direct phylogenetic line, they allow comparison of the properties of corticospinal control in species with a different degree of encephalization.

1. Rodents

Anatomical investigations have revealed that in rodents the pyramidal tract descends in the dorsal columns of the white matter and terminals of the corticospinal fibers are distributed only in the dorsal regions of the dorsal horn (BROWN, 1971). Estimates of the conduction velocities of the fastest pyramidal fibers range from 5 to 15 m/sec (MCCOMAS and WILSON, 1966) and thus are 5–10 times smaller than the maximum conduction velocities of reticulospinal fibers in the same species.

Microstimulation deep in the rat sensorimotor cortex evoked synaptic actions in lumbar alpha-motoneurons, which were mostly excitatory in motoneurons of flexors and extensors, distal and proximal muscles of the hindlimb (SHAPOVALOV, 1972b, 1973b; BOGATYREVA and SHAPOVALOV, 1973).

Practically no effects could be elicited by a single shock. At least a pair of shocks was necessary to produce the minimal detectable EPSP. This requirement together with the long latency of response and its relatively slow rise and complex time course strongly suggest a polysynaptic linkage.

When the distribution of effective spots within the depth of the cortex was examined, the most effective depth of stimulation was found to be about 200 and 500–800 μm below the surface of the cortex. This means that the histological distribution of the pyramidal cells is in good correlation with the location of the stimulating electrode (Fig. 13A). Systematic mapping of the sites of stimulation revealed that the points with low threshold for evoking EPSPs were concentrated in the same region from which movements of the hindlimb were produced by the trains of stimuli.

A pyramidal section abolished the synaptic effects induced by intracortical microstimulation even when much stronger stimuli (up to 500–600 μA) were used (Fig. 13B); however, synaptic action evoked by surface cortical stimulation persisted in pyramidotomized rats, suggesting the involvement of brainstem-spinal pathways. Transection of the brainstem sparing the bulbar pyramids did not affect the responses to intracortical stimulation (Fig. 13C). These results strongly suggest that the pyramidal tract mediates polysynaptic effects to the hindlimb motoneurons. Moreover, acute sections of the spinal cord have shown that the relevant fibers are located in the dorsal funiculi of the spinal cord.

It should be noted that JANZEN et al. (1972), using single electrical stimuli of unknown strength applied to cerebral cortex of rats, recorded short-latency

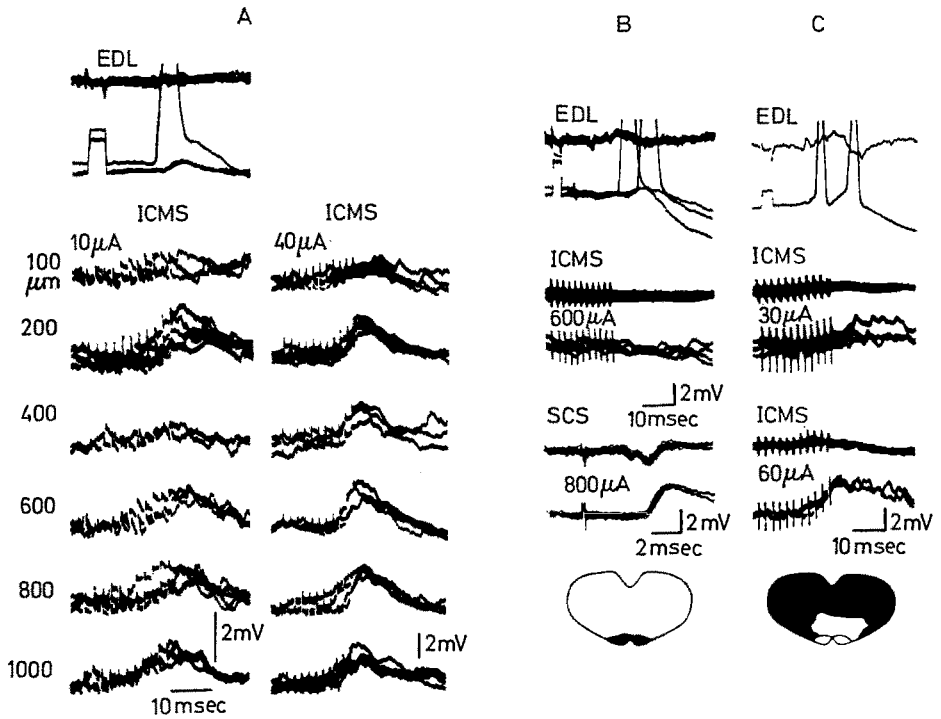


Fig. 13A—C. Effects of intracortical microstimulation (ICMS) on three different EDL motoneurons of the rat. (A) Effects of ICMS at different depths (indicated beside records) from the surface. (B) Effects of ICMS and surface cortical stimulation (SCS) in rats subjected to pyramidotomy. (C) Effects of ICMS in rats subjected to brainstem section. (BOGATYREVA and SHAPOVALOV, 1973)

EPSPs in hindlimb motoneurons. The short latency (2–3 msec) of these responses and rapid conduction velocity of the descending volley (60 m/sec) suggests that these effects are mediated by the reticulospinal pathway. It was shown (Fig. 13B) that similar results may be obtained in pyramidotomized rats.

BANNISTER and PORTER (1967) recorded in the rat short-latency EPSPs evoked by single shocks applied to medullary pyramids. These results may also be explained by involvement of the reticulospinal pathway.

2. Carnivores

In the cat rhythmic stimulation of the sensorimotor cortex evokes excitatory and inhibitory PSPs in motoneurons of the lumbar and cervical cord (CORAZZA et al., 1963), EPSPs are more marked in the motoneurons of flexors (LUNDBERG and VOORHOEVE, 1962; KATO et al., 1964; KOSTYUK, 1967; VASILENKO and VUCHO, 1966). In accord with earlier findings of LLOYD (1941), corticopyramidal impulses may activate alpha-motoneurons only via the internuncials. However, the latency of pyramidal effects may be very short (KATO et al., 1964), possibly due to the fact that thickest pyramidal tract fibers in the cat have conduction

velocity of about 70 to 80 m-sec (LLOYD, 1941; HERN et al., 1962). This velocity is only about half that of the fastest reticulospinal fibers in the same species.

Terminals of the pyramidal tract fibers in the spinal gray matter in the cat are located mainly in the lateral parts of the base of the dorsal horn (NYBERG-HANSEN and BRODAL, 1963; PETRAS, 1969). Thus, it may be noticed that the sites of termination of corticospinal fibers in the cat are shifted considerably in the ventral direction as compared with rodents. This ventral shift is even more prominent in other carnivores such as dogs and racoons (BUXTON and GOODMAN, 1967) and suggests simpler connections between pyramidal axons and alpha-motoneurons.

3. Primates

In the monkey the motor cortex and the pyramidal tract are evidently much more developed than in the cat. The corticospinal pathway contains thick fibers which conduct at rates similar to those of the fastest brainstem-spinal axons (SHAPOVALOV et al., 1971c; SHAPOVALOV, 1972b). Anatomic studies show that the bulk of pyramidal tract terminals are distributed among the interneurons (KUYPERS, 1960; LIU and CHAMBERS, 1964). Although descending volleys from the primary cortex have been shown to initiate monosynaptic EPSPs in lumbar motoneurons in monkeys, the majority of motoneurons are excited after a delay which would allow ample time for interneuronal relay (PRESTON and WHITLOCK, 1961).

The properties of corticospinal polysynaptic EPSPs and IPSPs are very similar to those of polysynaptic brainstem-spinal actions. They are frequency-dependent and very sensitive to the injected currents (SHAPOVALOV and KURCHAVYI, 1970).

B. Pyramidal Effects on Different Spinal Elements Participating in Motor Control

1. Segmental Interneurons

It was found in the cat that pyramidal volleys influence interneurons of spinal reflex pathways (LUNDBERG et al., 1962; LUNDBERG, 1967). In many cases the linkage was monosynaptic (Fig. 14). In contrast, ZADOROZHNY et al. (1970) claim that pyramidal impulses monosynpatically excite only interneurons of a "private" channel largely independent of segmental reflex activity. The results of ZADOROZHNY et al. may be due to the fact that they did not stimulate all afferent pathways converging upon the cells under study. FETZ (1968) showed that almost all lumbar interneurons activated from the pyramidal tract were influenced by natural and electrical stimulation of the periphery. ASANUMA et al. (1971) examined in the cat the characteristics of cervical interneurons activated by intracortical microstimulation and found that essentially all of those interneurons received input from the primary afferents. LLOYD (1968) found convergent impingement upon spinal interneurons of activity engendered by pyramidal and primary afferent impulses.

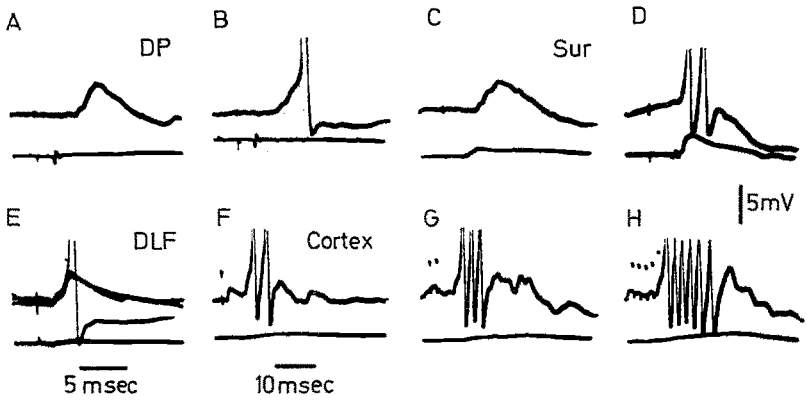


Fig. 14. Intracellular recordings (upper traces) from a dorsal horn interneuron (the upper part of L_7) activated from cortex and flexor reflex afferents in the cat. The responses F—H were evoked on stimulation of the contralateral sensorimotor cortex with an increasing number of stimuli. The latency (6 msec) of cortically induced EPSPs suggests a monosynaptic linkage (LUNDBERG et al., 1962)

2. Gamma-Motoneurons

In the cat gamma-motoneurons receive short-latency excitation from the sensorimotor cortex (MORTIMER and AKERT, 1961).

A monosynaptic pryamidal path to the gamma-motoneurons has been found in the baboon (CLOUGH et al., 1971). There is evidence that gamma-cells are coactivated with alpha-cells upon electrical stimulation of the motor cortex in this species, although the alpha and gamma effects can be elicited independently (KOEZE, 1968; PHILLIPS, 1969).

3. Primary Afferents

Transmission of information in afferent pathways can be modulated by the corticospinal system by mechanisms operating on the presynaptic elements (LUNDBERG, 1967, 1969). Investigations with cats have revealed that depolarization of presynaptic terminals of primary afferents may be induced by stimulation of the sensorimotor cortex (CARPENTER et al., 1962). It was found with intracellular recording preterminally from axons that corticospinal impulses evoked these effects in cutaneous and 1b afferents, but not in 1a afferents.

4. Neurons of Ascending Pathways

Experiments carried out on cats have shown marked effects from the cortex on ascending pathways influenced by the flexor reflex afferents (LUNDBERG et al., 1963). The neurons of the ventral and dorsal spinocerebellar tracts are inhibited from the cortex, whereas cells of ventral spinobulbar tract are excited from the cortex (LUNDBERG et al., 1963; HONGO and OKADA, 1967). Thus, corticospinal projections can control the pathways carrying information to the brain centers.

C. Conclusion

Rapid alterations characterize the development of the mammalian corticospinal system. From rodents to primates this development discloses important modifications of its conducting projections and synaptic organization. However, the polysynaptic projections are present in mammals at different levels of encephalization. The corticospinal system also controls the main neuronal systems of the spinal cord participating in regulation of motor activity.

VII. Corticospinal Monosynaptic Control of Alpha-Motoneurons

A. Comparative Aspect

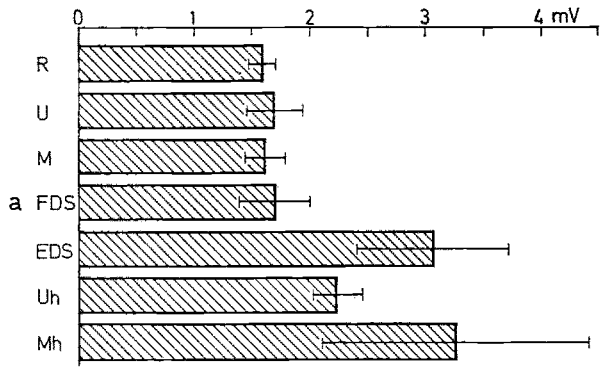
Direct cortico-motoneuronal linkage is present only in primates and it has been shown to increase in ascending the phylogenetic scale. Electrophysiological analysis of the cortico-motoneural system has been accomplished only in the rhesus monkey and the baboon. Although the baboon represents a somewhat higher level than the rhesus monkey in the primate order a direct comparison of results obtained from each species is rather difficult in evolutionary terms, since the monosynaptic actions in the baboon were studied mainly on the forelimb motoneurons, but mainly on the hindlimb cells in the rhesus monkey. However HOFF and HOFF (1934) found that there are more pyramidal fiber boutons applied to motoneurons in the baboon than in the macaque, and still more in the chimpanzee. KUYPERS (1964) and PETRAS (1969) demonstrated that the direct projection of the pyramidal tract fibers to the spinal motoneurons becomes increasingly prominent throughout the primate series from lower to subhuman primates. In the higher primates, such a gibbon and chimpanzee, the direct cortico-motoneuronal fibers make up about half of the pyramidal endings in the spinal cord. In man the figure may be even higher (cf. GRANIT and BURKE, 1973).

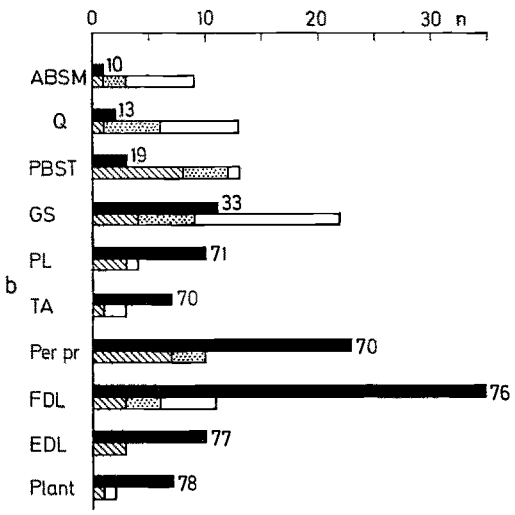
B. Properties of EPSPs and Functional Organization

The monosynaptic cortico-motoneuronal EPSPs are mediated by the fastest pyramidal axons (BERNHARD et al., 1953; PHILLIPS and PORTER, 1964; TAMAROVA et al., 1972). The latencies of monosynaptic cortico-motoneuronal and group 1a EPSPs are similar (LANDGREN et al., 1962; PHILLIPS and PORTER, 1964; TAMAROVA et al., 1972).

In the baboon, the largest numbers of monosynaptic cortico-pyramidal projections are found in the motoneurons controlling finger movements (Fig. 15A). Similarly, in the rhesus monkey the monosynaptic input from the motor cortex was found preferentially in motoneurons innervating the distal muscles of the hindlimb (Fig. 15B). The stimulus strength necessary to produce a monosynaptic EPSP was considerably smaller for motoneurons of distal muscles (Fig. 15C).

The largest cortico-motoneuronal EPSPs induced by single pyramidal volleys are still below the level required for generation of a spike. However, PHILLIPS





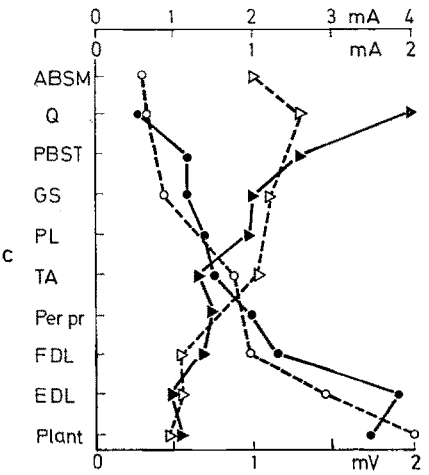


Fig. 15 A—C. Cortico-pyramidal effects on motoneurons innervating different muscles in the baboon (A) and in the rhesus monkey (B, C). (A) Mean amplitudes of maximal monosynaptic EPSPs evoked by corticospinal volleys in different forlimb motoneurons (CLOUGH et al., 1968). (B) Distribution of excitatory and inhibitory effects in hindlimb motoneurons. Black columns show monosynaptic EPSPs. Shaded columns show di- and polysynaptic EPSPs. Dotted columns show IPSPs, and white columns absence of response. Percentage of monosynaptic response for every motor nucleus is indicated beside each motoneuronal group (TAMAROVA et al., 1972). The ordinate shows the number of cells. (C) Average amplitudes of maximal monosynaptic EPSPs evoked from the motor cortex (solid circles) and bulbar pyramids (open circles) and threshold currents for monosynaptic EPSPs evoked from motor cortex (solid triangles) and bulbar pyramids (open triangles). Ordinate: right, amplitude (mV), left, current: 0–4 mA for the motor cortex, 0 to 2 mA for the bulbar pyramids (TAMAROVA et al., 1972)

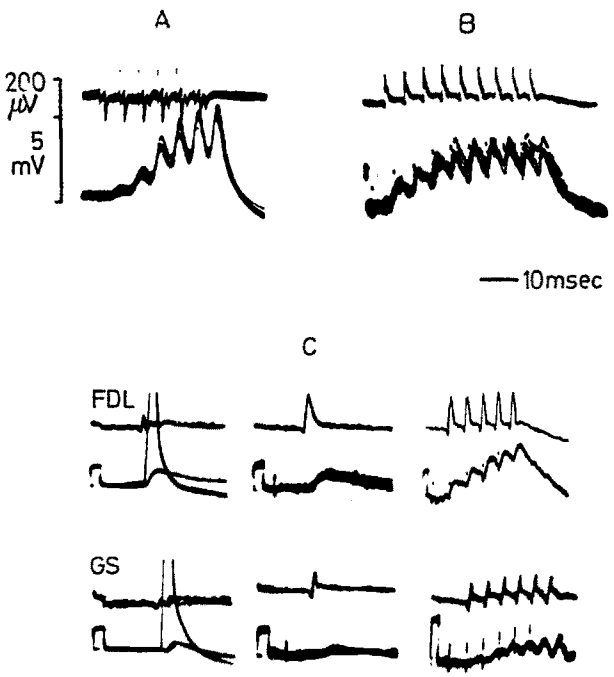


Fig. 16A—C. Frequency potentiation of monosynaptic EPSPs evoked from the motor cortex (A) and bulbar pyramids (B, C) in the baboon (A) and rhesus monkey (B, C). (A) Monosynaptic EPSPs in a motoneuron of the median nerve evoked from the motor cortex. *Upper trace*: corticospinal volleys recorded from the dorsolateral white matter; *lower trace*: intracellular records. Time scale given by shock artifacts at 5 msec intervals (PHILLIPS and PORTER, 1964). (B) Monosynaptic EPSPs in a FDL motoneuron evoked from bulbar pyramids. *Upper trace*: cord dorsum potentials; *lower trace*: intracellular records. Calibration pulse 5 mV (SHAPOVALOV, 1972b). (C) Monosynaptic EPSPs evoked in FDL and GS motoneurons. *Upper trace*: cord dorsum potentials; *lower trace*: intracellular records. Calibration pulse 1 msec, 5 mV (FDL, GS), 2 mV for all other records (TAMAROVA et al., 1972)

and PORTER (1964) have found that high-frequency stimulation leads to an enormous increase in successive EPSPs in cervical motoneurons (Fig. 16A). Similar results were reported by PORTER (1970) during recording from lumbar motoneurons in rhesus monkeys. The increasing size of the EPSPs produced by successive cortico-pyramidal volleys was attributed to an increased transmitting potency at the cortico-motoneuronal synapses. On the other hand, this effect may be partly accounted for by a recruitment of pyramidal neurons at the site of cortical stimulation because the amplitude and wave form of the descending volley may change in parallel (Fig. 16A). Direct stimulation of the bulbar pyramids reveals only moderate frequency potentiation (Fig. 16B). The powerful frequency potentiation might be also due to the involvement of concomitant disynaptic effects. The larger monosynaptic EPSPs in the motoneurons of distal muscles are less susceptible to frequency potentiation than the smaller EPSPs recorded from motoneurons of the proximal muscle groups (TAMAROVA et al., 1972) (Fig. 16C).

VIII. Comparison of Pyramidal and Nonpyramidal Synaptic Projections to Alpha-Motoneurons and Their Interaction

Supraspinal motor control is accomplished through coordination of different descending projections which collaborate closely during any reflex or voluntary movement. In mammals nonpyramidal influences are correlated with pyramidal impulses at all levels of the neuroaxis (JUNG and HASSLER, 1960). The intracerebral circuitry of different descending systems does not fall within the scope of the present survey. For the purpose of the present discussion it seems reasonable to call attention to the interaction of pyramidal and nonpyramidal commands within the spinal cord at motoneuronal and interneuronal levels.

A. Monosynaptic Inputs to Alpha-Motoneurons

Although all mammalian species possess direct brainstem-motoneuronal projections, only in primates do the same alpha-motoneurons receive direct monosynaptic input from both the brainstem and the motor cortex (SHAPOVALOV et al., 1971a; SHAPOVALOV, 1972a). It may be tentatively supposed that this convergence contributes to the integrative mechanisms of the motoneuron as the final common path (SHERRINGTON, 1906). Consideration of the interaction of monosynaptic actions produced from different sources that impinge directly on motoneurons also has a direct bearing on the problem of synaptic organization and on the mechanisms underlying synaptic transmission at particular synapses.

1. Convergence of Rubro-Motoneuronal and Cortico-Motoneuronal Inputs. Comparison of these Inputs

In the rhesus monkey the motoneurons innervating distal muscles of the hindlimb often receive monosynaptic inputs from both the motor cortex and the red nucleus (SHAPOVALOV, 1972a; SHAPOVALOV et al., 1971a, b). Although rubro-motoneuronal and cortico-motoneuronal EPSPs have several points of resemblance, which are also common to all direct cerebro-motoneuronal influences, i.e. they are evoked by fast-conducting fibers and are subthreshold for initiation of the spike, there are certain obvious differences between them.

a) Cortico-motoneuronal EPSPs have larger average and individual amplitudes. Moreover, in many motoneurons from which records were made, the amplitudes of rubro-motoneuronal EPSPs were practically unaffected by variations in stimulating current strength, in contrast to cortico-motoneuronal actions (Fig. 17A). This may be accounted for by the fact that rubro-motoneuronal EPSPs originate from a restricted number of presynaptic terminals, whereas the cortico-motoneuronal colonies contain more cells. Therefore the latter allow a finer subdivision and fractionation of descending commands. This conclusion is consistent with other differences between cortico- and rubro-motoneuronal EPSPs, and especially with their time course.

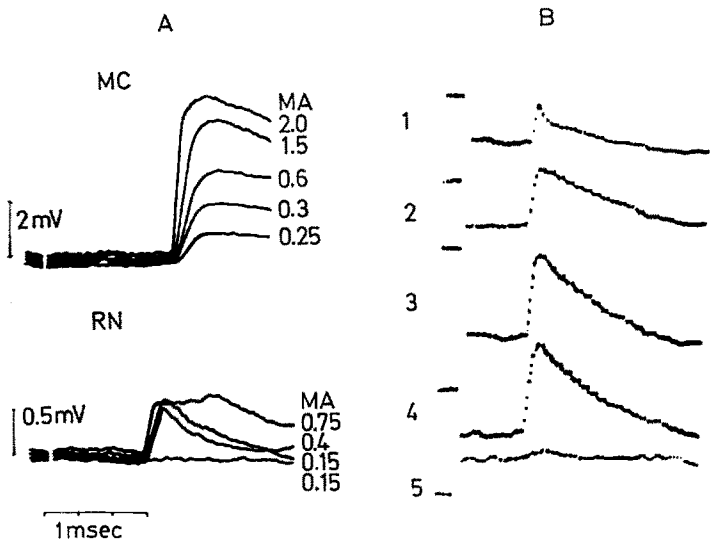


Fig. 17A and B. Monosynaptic cortico-motoneuronal and rubro-motoneuronal EPSPs in two different motoneurons (A and B) of the rhesus monkey. A Superimposed traces of monosynaptic EPSPs in FDL motoneurons evoked by stimulation of the motor cortex (MC) and the red nucleus (RN) at different intensities (SHAPOVALOV, 1972a). B Averaged records of monosynaptic EPSPs evoked by rubrospinal (1) and corticospinal (2) volleys, first separately and then together (4), their algebric sum (3) and the difference between the algebraic and motoneuronal sum (5). Calibration pulse 1 msec, 1 mV (doubled by the computer in 3). (SHAPOVALOV et al., 1971a).

b) The slope of the rubro-motoneuronal EPSP rising phase is steeper and time of decay faster than that of the cortico-motoneuronal EPSP.

This general finding was sustained whether the individual EPSPs in the same cell or the whole population were examined. Comparison of the mean temporal characteristics of both types of EPSPs shows that the difference between them is statistically significant (SHAPOVALOV et al., 1971a). The slower time course of the cortico-motoneuronal EPSPs may be attributed to the more asynchronous generation of unit synaptic actions or to the more distal location of the synaptic input.

c) The total latencies of cortico-motoneuronal EPSPs were larger than those of rubro-motoneuronal EPSPs, presumably owing to the somewhat slower conduction velocity of the relevant fibers and the longer distance between the stimulation point and the target motoneurons. Moreover, the segmental delay of cortico-motoneuronal EPSPs tended to be longer than that of rubro-motoneuronal EPSPs. The difference is statistically significant (SHAPOVALOV et al., 1971a). The exact reason for the observed difference remains uncertain, but it may be a result of better synchronization of the incoming rubrospinal volley.

2. Interaction

The records in Fig. 17B are tracings of the averaged monosynaptic EPSPs elicited in the same motoneuron by volleys in the rubrospinal and corticospinal tracts,

first separately and then simultaneously. Paired rubral and cortical stimulation demonstrated the absence of any occlusion. The near-perfect linearity of the observed summation suggests spatial segregation of the corresponding synaptic inputs on the motoneuronal membrane. This finding is in good agreement with the different time course of each type of EPSP.

The particular distribution of synapses across the somadendritic complex of the motoneuron might have some functional importance in the integration of inputs of different origin (TERZUOLO and LLINAS, 1966).

3. Spatial Organization and Mechanisms of Synaptic Transmission

The shape indices of an EPSP can be used to estimate the probable position on the neuron surface of the synaptic sites producing the synaptic current, and much of the analysis of the group 1a monosynaptic actions in cat motoneurons performed by RALL and co-workers (RALL, 1967; RALL et al., 1967) rests on the assumption that the synapses generating fast EPSPs are located nearer the cell soma than those responsible for the slower EPSPs. The results of summation suggesting the spatial segregation of cortico-motoneuronal and rubro-motoneuronal inputs are compatible with this interpretation.

On the other hand, the location of the synapses may be indicated by the degree of dependence of the amplitude of the EPSPs on the membrane potential, since current injection into the soma region would not uniformly depolarize all the somadendritic membrane but would mainly affect the synapses located in the vicinity of the current source, presumably the cell soma.

The influence of artificially induced alteration of membrane potential on the amplitude of the monosynaptic EPSP evoked in simian motoneurons from the motorcortex and red nucleus is shown in Fig. 18. It may be seen that, although the monosynaptic EPSP elicited by a pyramidal volley has a very much slower rise and decay than the rubro-motoneuronal EPSP, the latter is no more effectively influenced by depolarizing currents than is the slow EPSP produced by a corticospinal volley. Moreover, cortico-motoneuronal EPSPs were frequently more sensitive to depolarization currents than rubro-motoneuronal or group 1a EPSPs (SHAPOVALOV and KURCHAVYI, 1974) recorded from the same cell. The lack of more pronounced sensitivity to depolarizing currents in group 1a and rubro-motoneuronal EPSPs was in sharp contrast to their faster time course.

The intracellular records in Fig. 18 further indicate that injected depolarizing currents fail to reverse monosynaptic EPSPs. There is no clearcut equilibrium or reversal potential for monosynaptic EPSPs of different type, although reduction almost to zero was quite common. When the equilibrium potential was estimated by extrapolation, it was found that the values of the equilibrium potential different for different synaptic inputs converging on to the same cell (Fig. 19). It was usually observed that monosynaptic EPSPs evoked by pyramide impulses had an equilibrium potential at a more negative level than that measured for monosynaptic EPSPs produced by rubrospinal or group 1a volleys in the same cell, although the difference between mean equilibrium potentials measured in the whole population of cells was not statistically significant.

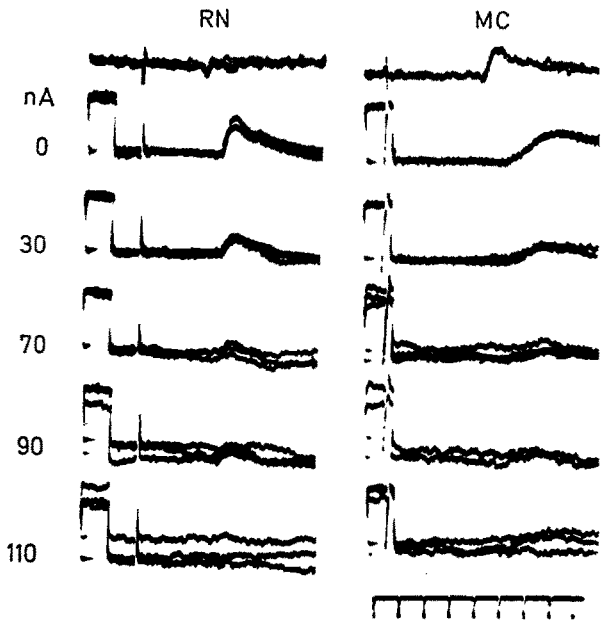


Fig. 18. Changes in monosynaptic rubro-motoneuronal (RN) and cortico-motoneuronal (MC) EPSPs during application of depolarizing currents across the same cell. Calibration pulse 2 mV. Time marker 1 msec. The number on each record indicates the amount of current injected (SHAPOVALOV and KURCHAVYI, 1974)

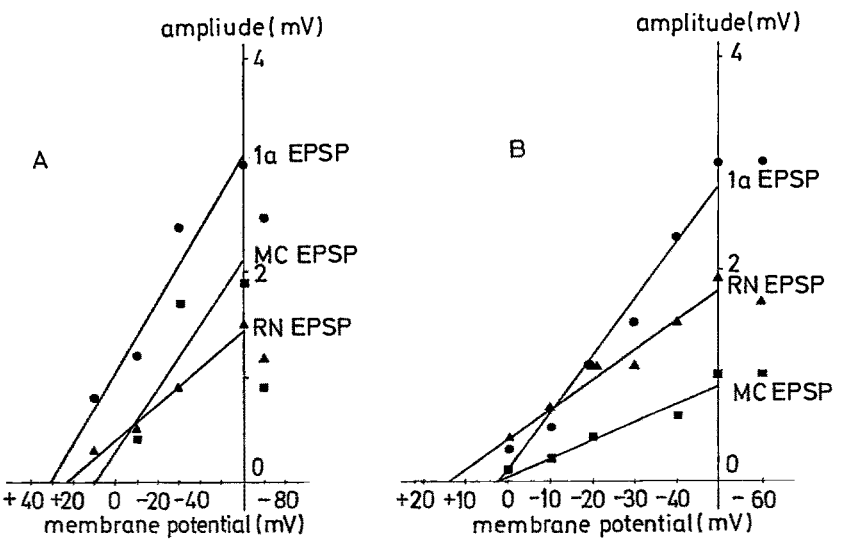


Fig. 19. Plot of the membrane potential (abscissa) versus the amplitudes of monosynaptic EPSs (ordinates) evoked by group 1a (filled circles), MC (squares) and RN (triangles) stimulation in two different motoneurons (A und B). The lines are computed curves giving the best fit with the observed data and are extrapolated to the X intercept, the theoretical equilibrium potential (SHAPOVALOV and KURCHAVYI, 1974)

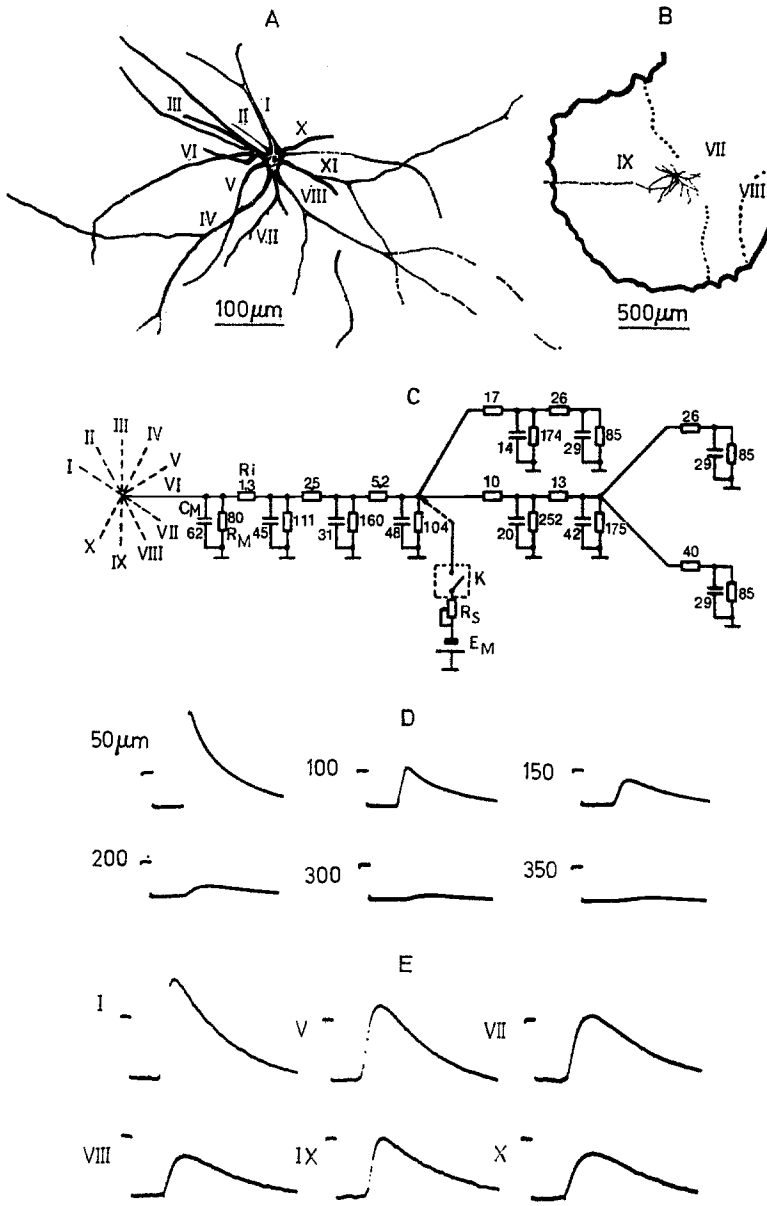


Fig. 20A—E. Structure, localization and analog model of the monkey motoneuron and the time course of the artificial EPSPs produced at different points of the model. (A) Reconstruction of the structure of the motoneuron stained with procion yellow dye. Roman numericals indicate the different dendrites. (B) Diagram showing the localization of the stained motoneuron on the transverse plane of the spinal gray matter. Roman numericals indicate Rexed's laminae. (C) Electrical model of the stained cell. Roman numerals indicate different dendrites. Arabic numerals indicate the resistance (MOM) and capacitance (PF). R_m membrane resistance; R_i successive resistance; R_s shunting resistance; C_m membrane capacitance; K electronic switch. (D) Amplitudes and time course of the artificial EPSPs produced on the same dendrite but at different distances from the soma. (E) Amplitudes and time course of the artificial EPSPs produced at the same distance from the soma but on different dendrites (indicated by roman numerals) (KURCHAVYI et al., 1973)

It should be stressed in this connection that determination of equilibrium potentials by extrapolation may be invalid because of possible errors introduced by the nonlinear properties of the membrane and the spatial distribution of the synapses. However, these uncertainties cannot introduce a large error when different monosynaptic inputs are compared in the same cell. Thus these findings can be tentatively explained by the possible involvement of differences in the specific synaptic mechanisms, such as transmitter nature and kinetics, or ionic mechanisms producing EPSPs. At present the nature of the excitatory transmitter substances subserving different inputs and the ionic fluxes involved in their action cannot be definitely stated. However, it may be supposed that the equilibrium potential of the faster rubro-motoneuronal (and group 1 a) EPSPs might be closer to the sodium equilibrium potential than that of cortico-motoneuronal EPSPs.

4. Correlation between Electrophysiological, Anatomic, and Model Findings

Although the slow time course of the cortico-motoneuronal EPSPs suggests a dendritic distribution of the corresponding synapses (PORTER and HORE, 1969), the anatomic evidence, based largely on studies using the Nauta method, indicates preterminal degeneration in the vicinity of spinal motoneuron somata after lesions in the motor cortex (KUYPERS, 1960; LIU and CHAMBERS, 1964; PATTON and AMASSIAN, 1960). On the other hand, although the anatomic study of the terminal degeneration of rubrospinal fibers (KUYPERS et al., 1962; MILLER and STROMINGER, 1973) presents no evidence consistent with a somatic distribution of rubro-motoneuronal contacts, it does not eliminate the possibility of dendritic terminations. Taken together with polarization experiments, these observations contradict the belief that the time course of the EPSP provides an accurate index of the synaptic distribution. This apparent contradiction may be explained if one takes account of the fact that calculations on Rall's theoretical model are based on the assumption that a motoneuron with the whole dendritic tree can be approximated to the equivalent cylinder. The localization of any particular input in such a model is rather uncertain. The measurement of electrical parameters of simian motoneurons stained with procion yellow dye made it possible to construct an analog model in which synaptic inputs were simulated by short-acting shunts switched on different points of the "membrane" (KURCHAVYI et al., 1973) (Fig. 20). It was found that the wave form of the artificial EPSP depends not only on the distance from the soma (Fig. 20D), but also on its localization on the particular dendrite (Fig. 20C). These results might possibly account for the data obtained in real motoneurons.

B. Polysynaptic Actions

Brainstem-spinal and corticospinal influences are also correlated at interneuronal level. In the cat a systematic comparison has been made of the effects on spinal interneurons from the corticospinal and rubrospinal tract (LUNDBERG, 1969; BAVEY and KOSTYUK, 1972).

In most interneurons very similar synaptic effects were evoked from both projections (Fig. 21). On the other hand, different effects were found in some

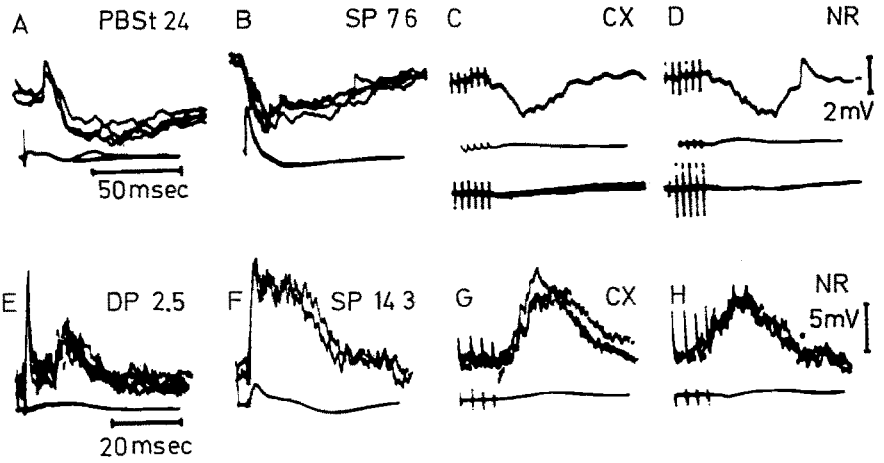


Fig. 21. Similarity of synaptic actions from the red nucleus and from the sensorimotor cortex. Intracellular records (upper traces) from two interneurons in L_7 (A—D and E—H). (LUNDBERG, 1969)

interneurons. At present it is unknown whether the same reflex action can be facilitated from the corticospinal and rubrospinal systems, one controlled mainly from the cerebral cortex, the other mainly from the cerebellum, or whether the differences sometimes observed indicate that the two pathways are used to control slightly different reflex performances (LUNDBERG, 1969).

IX. General Comments on Supraspinal Synaptic Mechanisms

Available data indicate that the fibers of the supraspinal decending systems are synaptically connected with all the spinal elements participating in motor control.

One of the oldest and most universal mechanisms of supraspinal motor control is concerned with direct monosynaptic linkage between certain groups of large brain neurons and spinal motoneurons. The most ancient direct line is represented by the reticulo-motoneuronal input, which is present in all classes from cyclostomes to mammals. This projection is not subject, it seems, to great variations from one species to another, and occupies a similar position in the brain and cord. The evolutionary antiquity of this input and its persistence in most advanced vertebrates, including primates, strongly suggest its continuing importance. As the organization of supraspinal control advanced and was built up in a progressive fashion, system after system was superimposed upon the others, and new descending projections established direct articulations with alpha-motoneurons. This process is paralleled by an increase in the conductance speed of the relevant fibers relative to the conduction velocity of the fastest reticulospinal axons. The new cortico-motoneuronal projections that appeared in primates are in many respects similar to the old nonpyramidal systems, although some properties of cortico-motoneuronal EPSPs (time course, frequency poten-

tiation) differ from those of brainstem-motoneuronal actions. But the amplitudes of cortico-motoneuronal EPSPs in primates and brainstem-motoneuronal EPSPs in subprimates fall within the same range of values. It is thus suggested that there may be some correspondence between the functional roles of direct pyramidal and nonpyramidal synaptic projections to alpha-motoneurons.

Another evolutionary trend is represented by the progressive development of supraspinal control of different systems subserving motor mechanisms that appeared in the course of phylogenetic differentiation of the interneuronal apparatus of the spinal cord. Thus gamma-cells, which are absent in amphibian cord, in mammals receive direct monosynaptic input from the brainstem and the motor cortex. Renshaw cells, which are reported only in mammals, are effectively facilitated or inhibited by many supraspinal structures. This ability to control different spinal elements means that practically the whole synaptic scale of motoneurons is controlled by descending projections. This may provide the basis for the increasing dependence of spinal motor centers on upper levels.

It should be stressed that each descending tract connecting a given brain level with segmental level does not represent a single unity but is composed of different functional systems with certain specific properties of their conducting component and different synaptic organization. Many of these functional systems remain to be elucidated. At present it may be stated that direct cerebro-motoneuronal systems formed by fast-conducting fibers of brainstem-spinal and cortico-spinal pathways are those which are most clearly related to the central motor programming.

References

ABBIE, A. A., ADEY, W. R.: Motor mechanisms in the anuran brain. J. comp. Neurol. **92**, 241–291 (1950).

AGNEW, R. F., PRESTON, J. B., WHITLOCK, D. G.: Patterns of motor cortex effects on ankle flexor and extensor motoneurons in the "pyramidal" cat preparation. Exp. Neurol. **8**, 248–263 (1963).

ASANUMA, H., STONEY, S. D., THOMPSON, W. D.: Characteristics of cervical interneurones which mediate cortical motor outflow to distal forelimb muscles of cat. Brain Res. **27**, 27–79 (1971)

AUERBACH, A. A., BENNET, M. V. L.: A rectifying electrotonic synapse in the central nervous system of a vertebrate. J. Gen. Physiol. **53**, 211–237 (1969).

BALDISSERA, F., TEN BRUGGENCATE, G.: Rubrospinal effects on spinal border cells. Acta Physiol. Scand. **119**, Suppl. 330 (1969).

BALDISSERA, F., TEN BRUGGENCATE, G., LUNDBERG, A.: Rubrospinal monosynaptic connexion with last-order interneurons of polysynaptic reflex paths. Brain Res., **27**, 390–392 (1971).

BALDISSERA, F., LUNDBERG, A., UDO, M.: Stimulation of pre- and postsynaptic elements in the red nucleus. Exp. Brain. Res. **15**, 151–167 (1972).

BALDISSERA, F., WEIGHT, F.: Descending monosynaptic connection to spinal border cell. Acta Physiol. Scand., **76**, 28A (1969).

BANNISTER, C. M., PORTER, R.: Effects of limited direct stimulation of the medullary pyramidal tract on spinal motoneurons in the rat. Exp. Neurol. **17**, 265–275 (1967).

BARNES, C. D., POMPEIANO, O.: Dissociation of presynaptic and postsynaptic effects produced in the lumbar cord by vestibular volleys. Arch. ital. Biol. **108**, 295–334 (1970).

BATUEVA, I. V.: Suprasegmental synaptic effects on the tortoise lumbar motoneurons. Sechenov physiol. J. USSR **58**, 1673–1684 (1972).

BATUEVA, I. V., SHAPOVALOV, A. I.: Electrical activity of reticulospinal neurones of the lampey, *Lampetra fluviatilis*. J. Evol. Biochem. Physiol. USSR **4**, 498–508 (1968).

BATUEVA, I. V., SHAPOVALOV, A. I.: Supraspinal and intraspinal descending synaptic actions on the segmental motoneurons in the lamprey. Neurofiziologia (USSR) **6**, 1974.

BAVEY, K. V., KOSTYUK, P. G.: Investigation of the modes of connection of cortico- and rubrospinal tracts with neuronal elements of the cervical spinal cord in the cat. Neurofiziologia USSR **4**, 158–167 (1972).

BERNHARD, C. G., BOHM, E., PETERSEN, J.: Investigation on the organization of the corticospinal system in monkeys. Acta Physiol. Scand., **31**, 104–112 (1953).

BERNSTEIN, N.: Outlines on the physiology of movements. Moscow: Medicine 1966.

BOGATYREVA, E. S., SHAPOVALOV, A. I.: Synaptic actions evoked by intracortical microstimulation in lumbar motoneurons of the rat. Neurofiziologia (USSR) **5**, 174–180 (1973).

BREMER, F.: Strychnine tetanus of the spinal cord. Ciba Found. Symp. The Spinal Cord, pp. 78–83, London: Churchill 1963.

BRODAL, A.: The Reticular Formation of the Brain Stem. Edinburgh: Oliver and Boyd 1957.

BRODAL, A., POMPEIANO, O., WALBERG, F.: The Vestibular Nuclei and Their Connections, Anatomy and Functional Correlations. Edinburgh: Oliver and Boyd 1962.

BROWN, L. T.: Projections and termination of the corticospinal tract in rodents. Exp. Brain Res. **13**, 432–450 (1971).

TEN BRUGGENCATE, G., BURKE, R., LUNDBERG, A., UDO, M.: Interaction between the vestibulospinal tract, contralateral flexor afferents and 1a afferents. Brain Res. **14**, 529–532 (1969).

BUXTON, D. F., GOODMAN, D. C.: Motor function and corticospinal tracts in the dog and raccoon. J. comp. Neurol. **129**, 341–360 (1967).

CARPENTER, D., ENGBERG, I., LUNDBERG, A.: Primary afferent depolarization evoked from the brain stem and the cerebellum. Arch. ital. Biol. **104**, 73–85 (1966)

CARPENTER, D., LUNDBERG, A., NORSELL, U.: Effects from the pyramidal tract on primary afferents and on spinal reflex actions to primary afferents. Experientia **18**, 337 (1962).

CARPENTER, M. B., STEVENS, G. M.: Structural and functional relationship between the deep cerebellar nuclei and the brachium conjunctivum in the rhesus monkey. J. comp. Neurol. **107**, 109–163 (1957).

CLOUGH, J. F. M., KERNELL, D., PHILLIPS, C. G.: The distribution of monosynaptic excitation from the pyramidal tract and from primary spindle afferents to motoneurons of the baboon's hand and forearm. J. Physiol. **198**, 145–166 (1968).

COOK, W. A., CANGIANO, A., POMPEIANO, O.: Vestibular control of transmission in primary afferents to the lumbar cord. Arch. ital. Biol. **107**, 296–320 (1969).

CORAZZA, R., FADIGA, E., PARMEGGIANI, P. L.: Pathways of pyramidal activation of cat's motoneurones. Arch. ital. Biol. **101**, 337–364 (1963).

CROSBY, E. C., WOODBURNE, R. T.: The comparative anatomy of the preoptic area and the hypothalamus. Res. Nerv. Ment. Dis. Proc. **20**, 52–169 (1940).

CURTIS, D. R.: Pharmacological investigations upon inhibition of spinal neurons. J. Physiol. **145**, 175–192 (1959).

DOW, R. S., MORUZZI, G.: The Physiology and Pathology of the Cerebellum. Minneapolis: Univ. Minnesota Press 1958.

ECCLES, J. C.: The Physiology of Nerve Cells. Baltimore: Johns Hopkins Press 1957.

ECCLES, J. C.: The Physiology of Synapses. Berlin-Göttingen-Heidelberg-New York: Springer 1964.

ECCLES, J. C.: The development of the cerebellum of vertebrates in relation to the control of movement. Naturwissenschaften **56**, 525–534 (1969).

ECCLES, J. C., NICOLL, R. A., SCHWARZ, D. W. F.: Tabariková, E., Cerebello-spinal pathway via the fastigial nucleus and the medial reticular nucleus. Brain Res. **66**, 525–530 (1974).

ECCLES, J. C., SCHMIDT, R., WILLIS, W. D.: Pharmacological studies on presynaptic inhibition. J. Physiol. **168**, 500–530 (1963).

ENGBERG, I., LUNDBERG, A., RYALL, R. W.: Reticulospinal inhibition of transmission in reflex pathways. J. Physiol. **194**, 201–223 (1968a).

ENGBERG, I., LUNDBERG, A., RYALL, R. W.: Reticulospinal inhibition of interneurons. J. Physiol. **194**, 225–296 (1968b).

FADIGA, E., BROOKHART, J.: Monosynaptic activation of different portions of the motor neurone membrane. Amer. J. Physiol. **198**, 683–703 (1960).

FANARDJAN, V. V., SARKISJAN, D. S.: Intracellular investigation of antidromic and synaptic activation of red nuclear neurons in the cat. Sechenov physiol. J. USSR **55**, 121–131 (1969).

FETZ, E. E.: Pyramidal tract effects on interneurons in the cat lumbar dorsal horn. J. Neurophysiol. **31**, 69–80 (1968).

FLUMERFELT, B. A., COURVILLE, J., OTABE, S.: Cerebello-rubral projection in the monkey. Anat. Rec. **169**, 318 (1971).

FOX, J. E.: Reticulospinal neurons in the rat. Brain Res. **23**, 35–40 (1970).

FRITSCH, G. T., HITZIG, E.: Über die elektrische Erregbarkeit des Großhirns. Arch. Anat. Physiol. wiss. Med. (Leipzig) **37**, 300–332 (1870).

FUORTES, M. G. F., NELSON, P. G.: Strychnine, its action on spinal motoneurons in cats. Science **140**, 806–808 (1963).

GERNANDT, B., TERZUOLO, C. A.: Effect of vestibular stimulation on strychnine-induced activity of the spinal cord. Amer. J. Physiol. **183**, 1–8 (1955).

GOLDBY, F., KACKER, G. N.: A survey of the pyramidal system in the coypu rat. J. Anat. (Lond.) **97**, 517–531 (1963).

GRANIT, R.: The Basis of Motor Control. London: Academic Press 1970.

GRANIT, R., BURKE, R.: The control of movement and posture. Conference report. Brain Res. **53**, 1–28 (1973).

GRANTYN, R., MARGNELLI, M., MANCIA, M., GRANTYN, A.: Post-synaptic potentials in the mesencephalic and ponto-medullar regions inderlying descending limbic influences. Brain Res. **56**, 107–121 (1973).

GRILLNER, S.: Supraspinal and segmental control of static and dynamic gamma-motoneurons in the cat. Acta Physiol. Scand., Suppl. 327 (1969).

GRILLNER, S., HONGO, T., LUND, S.: Reciprocal effects between two descending bulbospinal systems with monosynaptic connections to spinal motoneurones. Brain Res. **10**, 477–480 (1968).

GRILLNER, S., HONGO, T., LUND, S.: Descending monosynaptic and reflex control of gamma-motoneurones. Acta Physiol. Scand. **75**, 592–613 (1969).

GRILLNER, S., HONGO, T., LUND, S.: The vestibulospinal tract. Effects on alpha-motoneurones in the lumbosacral spinal cord in the cat. Exp. Brain Res. **10**, 94–120 (1970).

GRILLNER, S., HONGO, T., LUND, S.: Convergent effects on alpha-motoneurons from the vestibulospinal tract and a pathway descending in the medial longitudinal fasciculus. Exp. Brain Res. **12**, 457–479 (1971).

GRILLNER, S., LUND, S.: A descending pathway with monosynaptic action on flexor motoneurones. Experientia **22**, 390 (1966).

GRILLNER, S., LUND, S.: The origin of a descending pathway with monosynaptic action on flexor motoneurones. Acta Physiol. Scand. **74**, 274–284 (1968).

GUREVITCH, N. R., BELOZEROVA, T. V.: Rubrospinal synaptic influences on lumbar motoneurons in the rat. Neurofisiologia USSR **3**, 274–284 (1971).

HAASE, J., VOGEL, B.: Direkte und indirekte Wirkungen supraspinaler Reizungen auf Renshaw-Zellen. Pflügers Arch. **325**, 334–346 (1971).

HERN, J. B. C., PHILLIPS, C. G., PORTER, R.: Electrical thresholds of unimpaled corticospinal cells in the cat. Quart. J. exp. Physiol. **47**, 134–140 (1962).

HOFF, E. C., HOFF, H. E.: Spinal terminations of the projection fibers from the motor cortex of primates. Brain **57**, 454–474 (1934).

HOLMQVIST, B., LUNDBERG, A.: Oscarsson, O.: A supraspinal control system monosynaptically connected with an ascending spinal pathway. Arch. ital. Biol. **98**, 402–422 (1960).

HONGO, T., JANKOWSKA, E.: Effects from the sensorimotor cortex on the spinal cord in cat with transected pyramids. Exp. Brain Res. **3**, 117–134 (1967).

HONGO, T., JANKOWSKA, E., LUNDBERG, A.: The rubrospinal tract. I. Effects on alpha-motoneurones innervating hindlimb muscles in cats. Exp. Brain Res. **7**, 334–364 (1969a).

HONGO, T., JANKOWSKA, E., LUNDBERG, A.: The rubrospinal tract. II. Facilitation of interneuronal transmission in reflex path to motoneurones. Exp. Brain Res. **7**, 365–391 (1969b).

HONGO, T., JANKOWSKA, E., LUNDBERG, A.: The rubrospinal tract. III. Effects on primary afferent terminals. Exp. Brain Res. **15**, 39–53 (1972a).

HONGO, T., JANKOWSKA, E., LUNDBERG, A.: The rubrospinal tract. IV. Effects on interneurones. Exp. Brain Res. **15**, 54–78 (1972b).

HONGO, T., OKADA, Y.: Cortically evoked pre- and post-synaptic inhibition of impulse transmission to the dorsal spinocerebellar tract. Exp. Brain Res. **3**, 163–177 (1967).

ITO, M., HONGO, T., YOSHIDA, M., OKADA, Y., OBATA, K.: Antidromic and trans-synaptic activation of Deiter's neurons induced from the spinal cord. Jap. J. Physiol. **14**, 638–658 (1964).

ITO, M., UDO, M., MANO, N.: Long inhibitory and excitatory pathways converging onto cat reticular and Deiter's neurons and their relevance to reticulofugal axons. J. Neurophysiol. **33**, 210–226 (1970).

ITO, M., YOSHIDA, M.: The cerebellar-evoked monosynaptic inhibition of Deiter's neurons. Experientia **20**, 515–516 (1964).

JANKOWSKA, E., LUND, S., LUNDBERG, A., POMPEIANO, O.: Inhibitory effects evoked through ventral reticulospinal pathways. Arch. ital. Biol. **106**, 124–140 (1968).

JANSEN, J., BRODAL, A.: Aspects of Cerebellar Anatomy. Oslo: Johan Grundt Tanum 1954.

JANZEN, R. W., SPECKMANN, E. J., CASPERS, H.: Responses of lumbar motoneurons to cortical stimulation in the rat. Abstr. II Intern. Symposium on Motor Control, Varna, p. 80, 1972.

JUNG, HASSLER, R.: The extrapyramidal motor system. In: Handbook of Physiology I, Neurophysiology 2 pp. 862–927. Washington: Amer. Physiol. Soc. 1960.

KAPPERS, A. C. U., HUBER, G. C., CROSBY, E. C.: The Comparative Anatomy of the Nervous System of Vertebrates, Including Man. New York: Hafner 1960.

KATO, M., TAKAMURA, H., FUJIMORI, B.: Studies on effects of pyramidal stimulation upon flexor and extensor motoneurones and gamma motoneurones. Jap. J. Physiol. **14**, 34–44 (1964).

KAWAI, I., SASAKI, K.: Effects of strychnine upon supraspinal inhibition. Jap. J. Physiol. **14**, 309–317 (1964).

KIBAKINA, T. V., SHAPOVALOV, A. I.: Vestibulospinal synaptic actions on hindlimb motoneurons of the rat. Sechenov J. Physiol. (USSR) 1975.

KOEZE, T. H.: The independence of corticomotoneuronal and fusimotor pathways in the production of muscle contraction by motor cortex stimulation. J. Physiol. **197**, 87–105 (1968).

KOJANOV, V. M.: Propriospinal monosynaptic actions from ventral descending pathways on lumbar motoneurons of the cat. Sechenov J. Physiol. (USSR) **60**, 171–178 (1974).

KORN, H., SOTELO, C.: Crepel, F., Electronic coupling between neurons in the rat lateral vestibular nucleus. Exp. Brain Res. **16**, 255–275 (1973).

KOSTYUK, P. G.: Neuronal mechanisms of corticospinal motor systems. Sechenov physiol. J. USSR **53**, 1311–1321 (1967).

KOSTYUK, P. G.: Peculiarities of the different types of synaptic influences on spinal motoneurons. In: Synaptic Processes, ed. P. G. KOSTYUK, pp. 169–180, Kiev 1968.

KOSTYUK, P. G., PILYAVSKY, A. J.: A possible direct internal pathway from rubrospinal tract to motoneurones. Brain Res. **14**, 526–529 (1969).

KOSTYUKOV, A. I.: Transmission of activity of "lateral" descending systems by some groups of spinal neurons. Thesis, Kiev, 1974.

KURCHAVYI, G. G., MOTORINA, M. V., SHAPOVALOV, A. I.: Investigation of distribution of synaptic inputs on analogous model of motoneurons. Neurofiziologia USSR **5**, 289–297 (1973).

KURCHAVYI, G. G., SHAPOVALOV, A. I.: Interaction of monosynaptic effects evoked from supraspinal structures and muscle afferents on cat alpha-motoneurons. Rep. Acad. Sci. USSR **200**, 1246–1248 (1971).

KUYPERS, H. C. J. M.: Central cortical projections to motor, somatosensory and reticular cell groups. In: Structure and Function of the Cerebral Cortex, eds. E. Tower and J. Shade pp. 138–143. New York: Elsevier 1960.

KUYPERS, H. C. J. M.: The descending pathways to the spinal cord, their anatomy and function. Progr. in Brain Research. **11**, 178–202 (1964).

KUYPERS, H. C. J. M., FLEMING, W. R., FARINHOLT, J. M.: Subcortical projections in the rhesus monkey. J. comp. Neurol. **118**, 107–137 (1962).

LANDGREN, S., PHILLIPS, C. G., PORTER, R.: Minimal synaptic actions of pyramidal impulses on some alpha-motoneurones of the baboon's hand and forearm. J. Physiol. **161**, 91–111 (1962).

LAWRENCE, D. G., KUYPERS, H. G. J. M.: Pyramidal and nonpyramidal pathways in monkeys: anatomical and functional correlations. Science **148**, 973–975 (1965).

LAWRENCE, D. G., KUYPERS, H. G. J. H.: The functional organization of the motor system in the monkey. II. The effects of lesions of the descending brainstem pathways. Brain **91**, 15–38 (1968).

LENKOV, D. N., SHAPOVALOV, A. I.: Reticulospinal and vestibulospinal synaptic influences in the kitten. Bull. Exp. Biol. Med. **67**, 12–16 (1969).

LIU, C. N., CHAMBERS, W. W.: An experimental study of the cortico-spinal system in the monkey (*Macaca mulatta*). J. comp. Neurol. **123**, 257–284 (1964).

LLINAS, R., TERZUOLO, C. A.: Mechanisms of supraspinal actions upon spinal cord activities. Reticular inhibitory mechanisms upon flexor motoneurons. J. Neurophysiol. **28**, 413–422 (1965).

LLOYD, D. P. C.: Activity in neurons of the bulbospinal correlation system. J. Neurophysiol. **4**, 115–134 (1941 a).

LLOYD, D. P. C.: The spinal mechanisms of the pyramidal system in cats. J. Neurophysiol. **4**, 525–546 (1941 b).

LLOYD, D. P. C.: Note on convergence of pyramidal and primary afferent impulses in the spinal cord of the cat. Proc. nat. Acad. Sci. **59**, 381–384 (1968).

LUND, S., POMPEIANO, O.: Descending pathway with monosynaptic actions on motoneurones. Experientia **21**, 602–608 (1965).

LUND, S., POMPEIANO, O.: Monosynaptic excitation of alpha-motoneurones from supraspinal structures in the cat. Acta physiol. Scand. **73**, 1–21 (1968).

LUNDBERG, A.: Integration in the reflex pathway. In: Muscular Afferents and Motor Control. Nobel Symp. I (ed. R. Granit) pp. 275–305. Stockholm: Almqvist and Wiksell 1966.

LUNDBERG, A.: The supraspinal control of transmission in spinal reflex pathways. EEG Clin. Neurophysiol. Suppl. **25**, 35–46 (1967).

LUNDBERG, A.: Convergence of excitatory and inhibitory action on interneurones in the spinal cord. The Interneurone (ed. M. A. B. Brazier) pp. 231–265 UCLA Forum Med. Sci., Berkeley: Univ. California Press, 1969.

LUNDBERG, A., NORSELL, U., VOORHOEVE, P.: Pyramidal effects on lumbosacral interneurones activitated by somatic afferents. Acta physiol. Scand. **56**, 220–229 (1962).

LUNDBERG, A., NORSELL, U., VOORHOEVE, P.: Effects from the sensorimotor cortex on ascending spinal reflex. Acta physiol. Scand. **59**, 462–473 (1963).

LUNDBERG, A., VOORHOEVE, P.: Effects from the pyramidal tract on spinal reflex arcs. Acta physiol. Scand. **56**, 201–219 (1962).

MCCOMAS, A. J., WILSON, P.: Some properties of pyramidal tract cells in the rat somatosensory cortex. J. Physiol. **188**, 35–36 p (1966).

MCLAUGHLIN, B. J.: A light and electronmicroscopic study of propriospinal and supraspinal projections to cat spinal motoneuronal nuclei. Anat. Rec. **169**, 372 (1971).

MAGNI, F., WILLIS, W. D.: Cortical control of brainstem reticular neurons. Arch. ital. Biol. **102**, 418–433 (1964).

MAGOUN, H. W.: Caudal and cephalic influences of the brain stem reticular formation. Physiol. Rev. **30**, 459–474 (1950).

MASSION, J.: The mammalian red nucleus. Physiol. Rev. **47**, 383–436 (1967).

MILLER, R. A., STROMINGER, N. L.: Efferent connections of the red nucleus in the brainstem and spinal cord of the rhesus monkey. J. Comp. Neur. **152**, 327–346 (1973).

MORTIMER, E. M., AKERT, K.: Cortical control and representation of fusimotor neurons. Amer. J. phys. Med. **40**, 228–248 (1961).

MOTORINA, M. V.: Distribution and types of rubro-motoneuronal synapses in lumbar segments of cat. J. Evol. Biochem. Physiol., USSR **10**, 88–95 (1974).

NIEUWENHUYS, R.: Comparative anatomy of the spinal cord. In: Progress in Brain Research (eds. J. C. Eccles and J. P. Schade) **II**, 1–57. Amsterdam: Elsevier 1964.

NOBACK, C. R., SHRIVER, J. E.: Phylogenetic and ontogenetic aspects of the lemniscal systems and the pyramidal system. In: Evolution of the Forebrain, eds. R. Hassler and H. Stephan, pp. 316–325. Stuttgart: Thieme Verlag 1966.

NYBERG-HANSEN, R.: Sites and mode of termination of reticulospinal fibers in the cat. J. comp. Neurol. **124**, 71–100 (1965).

NYBERG-HANSEN, R.: Do cat spinal motoneurons receive direct supraspinal fibre connections? Arch. ital. Biol. **107**, 67–78 (1969).

NYBERG-HANSEN, R., BRODAL, A.: Sites of termination of corticospinal fibers in the cat. J. comp. Neurol. **120**, 369–391 (1963).

NYBERG-HANSEN, R., BRODAL, A.: Sites and mode of termination of rubrospinal fibers in the cat J. Anat. **98**, 235–253 (1964).

NYBERG-HANSEN, R., MASCITTI, T. A.: Sites and mode of termination of fibers of the vestibulospinal tract in the cat. J. comp. Neurol. **122**, 369–388 (1964).

ORLOVSKY, G. N.: Activity of reticulospinal neurons during locomotion. Biofizika **15**, 728–737 (1970a).

ORLOVSKY, G. N.: Cerebellar influence of the reticulospinal neurons during locomotion. Biofizika **15**, 894–901 (1970b).

ORLOVSKY, G. N.: The effect of different descending systems on flexor and extensor activity during locomotion. Brain Res. **40**, 359–371 (1972a).

ORLOVSKY, G. N.: Activity of vestibulospinal neurons during locomotion. Brain Res. **46**, 85–98 (1972b).

ORLOVSKY, G. N.: Activity of rubrospinal neurons during locomotion. Brain Res. **46**, 99–112 (1972c).

ORLOVSKY, G. N.: Neuronal mechanism of locomotion. Thesis, Moscow University 1973.

PADEL, Y., ARMAND, J., SMITH, A. M.: Topography of rubrospinal units in the cat. Exp. Brain Res. **14**, 363–371 (1972).

PATTON, H. D., AMASSIAN, V. E.: The pyramidal tract, its excitation and functions. Handbook Physiology, Sect. I, Neurophysiology, Vol. **9**, pp. 837–861. Washington: Am. Physiol. Soc. 1960.

PETRAS, J. M.: Some efferent connections of the motor and somatosensory cortex of simian primates and felid, canid and procyonid carnivores. Ann. N.Y. Acad. Sci. **167**, 469–505 (1969).

PHILLIPS, C. G.: Motor apparatus of the baboon's hand. Proc. Roy. Soc. B. **173**, 141–174 (1969).

PHILLIPS, C. G., PORTER, R.: The pyramidal projection to motoneurones of some muscle groups of the baboon's forelimb. In: Physiology of Spinal Neurons (eds. J. C. Eccles and J. P. Schade) pp. 222–245. Amsterdam: Elsevier 1964.

POMPEIANO, O.: Discussion of Dr. Shapovalov's paper. In: „Muscular afferents and Motor Control. Nobel Symp. I" (ed. R. Granit) p. 349, Stockholm: Almqvist and Wiksell, 1966.

PORTER, R.: Early facilitation at corticomotoneuronal synapses. J. Physiol. **207**, 733–745 (1970).

PORTER, R., HORE, J.: Time course of minimal corticomotoneuronal excitatory postsynaptic potentials in lumbar motoneurons of the monkey. J. Neurophysiol. **32**, 443–451 (1969).

PRESTON, J., WHITLOCK, D.: Intracellular potentials recorded from motoneurones following precentral gyrus stimulation in primate. J. Neurophysiol. **24**, 91–100 (1961).

RALL, W.: Distinguishing theoretical synaptic potentials computed for different soma-dendritic distribution of synaptic input. J. Neurophysiol. **30**, 1138–1168 (1967).

RALL, W., BURKE, R. E., SMITH, T. G., NELSON, P. G., FRANK, K.: Dendritic location of synapses and possible mechanisms for the monosynaptic EPSP in motoneurons. J. Neurophysiol. **30**, 1169–1193 (1967).

REPINA, Z. A., SHAPOVALOV, A. I., NIKITIN, P. A.: Pyzamidal and extrapyzamidal synaptic influences on chronically deafferented motoneurons. Neurofiziologia (USSR) **1**, 35–46 (1969).

RESTINAUX, N. J., SATCHELL, G. H.: An unitary study of the reticulomotor system of the dogfish, *Squalus, ebruini*. J. comp. Neurol. **109**, 391–415 (1958).

ROBINSON, L. R.: Bulbospinal fibres and their nuclei of origin in *Lacerta viridis* demonstrated by axonal degeneration and chromatolysis respectively. J. Anat. **105**, 59–88 (1969).

ROSSI, G. F., BRODAL, A.: Corticofugal fibers to the brain stem reticular formation. J. Anat. **90**, 42–67 (1956).

ROSSI, G. F., ZANCHETTI, A.: The brainstem reticular formation. Anatomy and physiology. Arch. ital. Biol. **95**, 203–435 (1957).

ROVAINEN, C. M., JAHNSON, P. A., ROACH, E. S.: MANKOVSKY, J. A.: Projections of individual axons in lamprey spinal cord determined by tracing through serial sections. J. comp. Neurol. **149**, 193–202 (1973).

RUSHTON, W. A. H.: A theory of the effects of fibre size in medullated nerve. J. Physiol. **115**, 101–122 (1951).

SASAKI, K., NAMIKAWA, A., HASHIRAMOTO, S.: The effect of midbrain stimulation upon alpha-motoneurons in lumbar spinal cord of the cat. Jap. J. Physiol. **10**, 303–316 (1960).

SASAKI, K., TANAKA, T.: Effects of stimulation of cerebellar and thalamic nuclei upon spinal alpha-motoneurons of the cat. Jap. J. Physiol. **13**, 64–83 (1963).

SCHOEN, J. H. R.: Comparative aspects of the descending fibre systems in the spinal cord. In: Organization of the spinal cord. (ed. J. C. ECCLES and J. P. SCHADE), Progress in Brain Research **11**, pp. 203–222. Amsterdam: Elsevier 1964.

SECHENOV, I. M.: About mechanisms in frog's brain inhibiting reflexes of spinal cord, 1863. Selected Works of I. E. M. of USSR. Moscow 1935.

SHAPOVALOV, A. I.: Excitation and inhibition of spinal neurons during supraspinal stimulation. In: Muscular Afferents and Motor Control. Nobel Symposium I (ed. GRANIT E.) pp. 331–348, Stockholm: Almqvist and Wiksell 1966.

SHAPOVALOV, A. I.: Post-tetanic potentiation of monosynaptic and disynaptic actions from supraspinal structures on lumbar motoneurons. J. Neurophysiol. **32**, 325–348 (1969).

SHAPOVALOV, A. I.: Extrapyramidal monosynaptic and disynaptic control of mammalian alpha-motoneurons. Brain Res. **40**, 105–115 (1972a).

SHAPOVALOV, A. I.: Evolution of neuronal systems of supraspinal motor control. Neurofiziologia (USSR) **4**, 453–470 (1972b).

SHAPOVALOV, A. I.: Neuronal mechanisms of motor control. Bull. Acad. Sci. USSR **4**, 43–54 (1972c).

SHAPOVALOV, A. I.: Extrapyramidal control of primate motoneurons. In: New Developments. in EMG and Clinical Neurophysiology. (ed. J. E. DESMEDT) Vol. 3, pp. 145–158. Basel: Karger 1973a.

SHAPOVALOV, A. I.: Pyramidal and extra-pyramidal control on mammalian alpha-motoneurones. In: Motor Control (eds. A. A. GYDIKOV, N. T. TANKOV, D. S. KOSAPKOV) pp. 95–111. New York-London: Plenum Press 1973b.

SHAPOVALOV, A. I., ARUCHANJAN, E. B.: Effect of strychnine on activity of spinal motoneurons and interneurons during the stimulation of anterior lobe of cerebellum. Bull. exp. Biol. Med. (USSR) **56**, 3–10 (1963).

SHAPOVALOV, A. I., ARUCHANJAN, E. B.: Effect of brainstem and motor cortex stimulation on activity of spinal neurons. Sechenov physiol. J. USSR **51**, 670–680 (1965).

SHAPOVALOV, A. I., ARUTYUNYAN, B. A.: The activity of spinal neurons during stimulation of the hypothalamus and hippocampus. Bull. exp. Biol. Med. **58**, 3–8 (1964).

SHAPOVALOV, A. I., GRANTYN, A. A.: Suprasegmental synaptic influences on chromatolyzed motoneurons. Biofizika USSR **13**, 260–269 (1968).

SHAPOVALOV, A. I., GRANTYN, A. A., KURCHAVYI, G. G.: Short-latency reticulospinal projections to alpha-motoneurons. Bull. exp. Biol. Med. **64**, 3–15 (1967).

SHAPOVALOV, A. I., GUREVITCH, N. R.: Monosynaptic and disynaptic reticulospinal actions on lumbar motoneurons of the rat. Brain Res. **21**, 249–263 (1970).

SHAPOVALOV, A. I., KARAMJAN, O. A., KURCHAVYI, G. G., REPINA, Z. A.: Synaptic actions evoked from the red nucleus on the spinal alpha-motoneurons in the rhesus monkey. Brain Res. **32**, 325–348 (1971a).

SHAPOVALOV, A. I., KARAMJAN, O. A., TAMAROVA, Z. A., KURCHAVYI, G. G.: Cerebello-rubrospinal effects on hindlimb motoneurons in the monkey. Brain Res. **47**, 49–59 (1972).

SHAPOVALOV, A. I., KURCHAVYI, G. G.: Properties of corticospinal and afferent synaptic influences on monkey lumbar motoneurons. Rep. Acad. Sci. USSR **192**, 694–697 (1970).

SHAPOVALOV, A. I., KURCHAVYI, G. G.: Effects of trans-membrane polarization and TEA injection on monosynaptic actions from motor cortex, red nucleus and group 1a afferents on lumbar motoneurons in the monkey. Brain Research **82** (1974).

SHAPOVALOV, A. I., KURCHAVYI, G. G., KARAMJAN, O. A., REPINA, Z. A.: Extrapyramidal pathways with monosynaptic effects upon primate α-motoneurons. Experientia **27**, 522–524 (1971).

SHAPOVALOV, A. A., KURCHAVYI, G. G., STROGANOVA, M. P.: Synaptic mechanisms of vestibulospinal influences on alpha-motoneurones. Sechenov J. Physiol. (USSR) **52**, 1401–1409 (1966).

SHAPOVALOV, A. I., SAFIANTS, V. I.: Potentiation of supra-segmentary synaptic influences on alpha-motoneurones. Sechenov J. Physiol. (USSR) **54**, 1261–1270 (1968).

SHAPOVALOV, A. I., SHAPOVALOVA, K. B.: Activity of alpha-motoneurons during rhythmical stimulation of red nucleus and effects of strychnine of rubrospinal influences. Rep. Acad. Sci. USSR **168**, 1430–1433 (1966).

SHAPOVALOV, A. I., SHIRYAEV, B. I.: Reticulospinal and propriospinal monosynaptic actions on frog motoneurons. Neurofiziologia (USSR) **5**, 164–173 (1973).

SHAPOVALOV, A. I., TAMAROVA, Z. A., KARAMJAN, O. A., KURCHAVYI, G. G.: Reticulospinal and vestibulospinal synaptic actions on the monkey lumbar motoneurons. Neurophysiologia (USSR) **4**, 408–417 (1971).

SHERRINGTON, C. S.: The Integrative Action of the Nervous System. New Haven: Yale University Press 1906.

SHERRINGTON, C. S.: Man on his Nature. 1940. Cambridge: University Press 1963.

SHIRJAEV, B. I.: Descending synaptic influences on frog motoneurons. Thesis, Leningrad 1972.

SHIRJAEV, B. I.: Synaptic actions from the ventral columns on lumbar motor neurons in frog. Bull. exp. Biol. Med. **75**, 7–11 (1973).

SPERRY, R. W.: A modified concept of consciousness. Psychol. Rev. **76**, 532–536 (1969).

SPRAGUE, J. M., HA, H.: The terminal fields of dorsal root fibres in the lumbosacral cord of the cat, and the dendritic organization of the motor nuclei. In: Progress in Brain Research. Organization of the spinal cord. (ed. J. C. ECCLES and J. P. SCHADE) **11**, pp. 120–154. Amsterdam: Elsevier 1964.

STEFANELLI, A., CARAVITA, S.: Ultrastructural features of the synaptic complex of the vestibular nuclei of *Lampetra planeri* (Bloch.). Z. Zellforsch. **108**, 282–296 (1970).

SZENTAGOTHAI, J.: Propriospinal pathways and their synapses. Progr. Brain Res. Organization of the spinal cord. (eds. J. C. ECCLES and J. P. SCHADE) **11**, pp. 155–177. Amsterdam: Elsevier: 1964.

TABER, E.: The cytoarchitecture of the brain stem of the cat. J. Comp. Neurol. **116**, 27–71 (1961).

TAMAROVA, Z. A.: Activity of rubrospinal neurons in the rhesus monkey. Journal of Evolut. Biochem. Physiol. 1975, in press.

TAMAROVA, Z. A., SHAPOVALOV, A. I., KARAMJAN, O. A., KURCHAVYI, G. G.: Cortico-pyramidal and cortico-extrapyramidal synaptic effects on the monkey lumbar motoneurons. Neurofiziologia USSR **4**, 587–596 (1972).

TEROVAINEN, H., ROVAINEN, C. M.: Fast and slow motoneurons to body muscle of the sea lamprey. J. Neurophysiol. **34**, 990–998 (1971).

TERZUOLO, C. A.: Influences supraspinales sur le tétans strychnique de la moëlle èpinière. Arch. ital. Physiol. **62**, 179–196 (1954).

TERZUOLO, C. A., LLINAS, R.: Distribution of synaptic inputs in the spinal motoneurone and its functional significance. In: Muscular Afferents and Motor Contol. Nobel Symp. I (ed. R. GRANIT) pp. 373–384. Stockholm: Almqvist and Wiksell 1966.

TOYAMA, K., TSUKAHARA, N., KOSAKA, K., MATSUNAMI, K.: Synaptic excitation of red nucleus neurons by fibers from interpositus nucleus. Exp. Brain Res. **II**, 187, 198 (1970.)

TSUKAHARA, N., KOSAKA, K.: The mode of cerebral activation of red nucleus neurons. Experientia **22**, 193–194 (1966).

TSUKAHARA, N., TOYAMA, K., KOSAKA, K.: Electrical activity of red nucleus neurons investigated with intracellular microelectrodes. Exp. Brain Res. **4**, 18–33 (1967).

VALVERDE, F.: The pyramidal tract in rodents. Arch. Zellforsch. **71**, 297–363 (1966).

VASILENKO, D. A., KOSTYUKOV, A. I., PILYAVSKY, A. I.: Cortico- and rubrofugal activation of propriospinal interneurons sending axons into the dorsolateral funiculus of the cat spinal cord. Neurofiziologia (USSR) **4**, 489–500 (1972).

VASILENKO, D. A., VUCHO, I.: Synaptic processes in lumbar motoneurons produced by stimulation of the sensorimotor area of the cerebral cortex. Pavlov J. higher Nerv. Activ. **16**, 52–61 (1966).

VOOGD, J.: The Cerebellum of the Cat. Structure and Fibre Connections. Leiden: Van Gorcum 1964.

WALBERG, F., JANSEN, J.: Cerebellar corticovestibular fibers in the cat. Exp. Neurol. **3**, 32–52 (1961).

WALBERG, F., POMPEIANO, O., WESTRUM, L. E., HAUGLIE-HANSSEN, E.: Fastigoreticular fibers in the cat. J. comp. Neurol. **119**, 187–199 (1962).

WILLIS, W. D., WILLIS, J. C., THOMPSON, W. M.: Synaptic actions of fibers in the ventral spinal cord upon lumbosacral motoneurons. J. Neurophysiol. **30**, 382–397 (1967).

WILSON, V. J., YOSHIDA, M.: Comparison of effects of stimulation of Deiter's nucleus and medial longitudinal fasciculus on neck, forelimb and hindlimb motoneurons. J. Neurophysiol. **32**, 743–758 (1969).

WILSON, V. J., YOSHIDA, M., SCHOR, R. H.: Supraspinal monosynaptic excitation and inhibition of thoracic back motoneurons. Exp. Brain Res. **11**, 282–295 (1970).

WOLSTENCROFT, J. N.: Reticulospinal neurones. J. Physiol. **174**, 91–108 (1964).

YASARGIL, G. M., DIAMOND, J.: Startle-response in teleost fish: an elementary circuit for neural discrimination. Nature **220**, 241–243 (1968).

ZADOROZHNY, A. G., VASILENKO, D. A., KOSTYUK, P. G.: Pyramidal influences on interneurons of spinal reflex arcs in cat. Neurofiziologia (USSR) **2**, 17–25 (1970).

ZIMMERMAN, E. A., CHAMBERS, W. W., LIU, C. N.: An experimental study of the anatomical organization of the cortico-bulbar system in the albino rat. J. comp. Neurol. **123**, 301–324 (1964).

Rev. Physiol. Biochem. Pharmacol., Vol. 72

Evidence for Reentry as a Mechanism of Cardiac Arrhythmias

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Contents

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I. Introduction

Until recently, most of the accumulated knowledge of cardiac arrhythmias was derived by clinical cardiologists from the painstaking analysis of endless strips of electrocardiographic tracings. This knowledge was supplemented by a number of now classical studies on experimental animals, also conducted primarily by clinicians. The only viable theories of the genesis and maintenance of arrhythmias are now many decades old, but three major advances have generated a renewed interest in mechanisms among both clinical and basic scientists.

The first of these advances was the development of intracellular recording techniques due to the invention and widespread application of the Ling-Gerard microelectrode. The second was the development of coronary care facilities, and the realization that continuous monitoring and prompt resuscitation could save lives. The third, primarily a clinical application of physiologic methodology, was the introduction of intracardiac electrode probes, particularly as adapted for recording activity in the bundle of His, and for intracardiac stimulation. Physicians, physiologists, and pharmacologists now share the podium on mutually understandable terms. The ghosts of Einthoven, Rothberger, and Thomas Lewis might say that little more than i-dotting and t-crossing has resulted from the union, that the treatment of arrhythmias is still largely empirical, the diagnosis of mechanism is still uncertain, and no new theories have been developed, only old ones confirmed. Nevertheless, it is heartening that mechanisms observed in experimental animals can now be confirmed in the clinic, and there is hope that more specific prophylaxis and therapy may result. It is the purpose of this essay to discuss some experimental arrhythmias in which a reentrant mechanism has been established, and in which a similar mechanism has been more or less confirmed in man.

1. Conditions Necessary for Reentry

With the exception of randomly spaced premature beats and parasystolic rhythms, almost all forms of ectopic activity have been ascribed to reentrant loops. They have all also been attributed to either the occasional or repetitive discharge of an ectopic focus, and the age-old controversy about which unitarian view is correct can be concluded by recognizing that both mechanisms are possible, both are experimentally demonstrable, and surely both must occur in nature.

Completion of a reentrant loop demands: (1) block of an impulse at some site within the conducting network, (2) slow conduction over an alternate pathway, (3) delayed activation of tissue beyond the block, and (4) re-excitation of tissue proximal to the block. These conditions were clearly recognized by Mines in 1913 and carefully analyzed by Schmitt and Erlanger in 1929, but experimental demonstration under conditions which conclusively define such a circuit pathway in the intact heart is still almost totally lacking. Nevertheless, circumstantial evidence is strong enough to be convincing in many cases, and we shall proceed to analyze some representative models in detail.

II. Arrhythmias Attributed to Reentry

A. Atrial Flutter

Not long after MINES demonstrated a circuitous sequence of activation in an excised ring of tortoise heart, the circus-movement mechanism was invoked to explain atrial flutter and fibrillation. LEWIS, FEIL, and STROUD (1920) succeeded, with admitted difficulty, in inducing atrial flutter by faradic stimulation of the right atrium in the anesthetized dog. In a few instances, regular rhythmic activity persisted for long enough to permit partial mapping of the excitation sequence. They concluded that rhythmic activity in the range of 345–580/min was best explained as a self-sustained circuit around one of the naturally occurring orifices in the atria: in one example the presumed circuit circumscribed the caval openings in the right atrium, in another the superior cava, and in yet another (largely hypothetical) the route was assumed to be the mitral orifice.

Although limited in number, these observations exerted a profound influence on subsequent studies, but it was not until the much later experiments of ROSENBLUETH and GARCIA-RAMOS (1947) that a reliable and reproducible method for inducing flutter in the dog heart was described. On the assumption that the difficulty encountered by LEWIS and his associates in inducing a long-lasting, self-sustained circus movement was due to the short perimeter of the naturally occurring obstacles in the dog heart, ROSENBLUETH and GARCIA-RAMOS increased the dimensions of the inferior caval orifice by crushing an adjacent area of atrial tissue. By this means they could regularly induce a stable rhythmic tachycardia that lasted long enough to permit a number of experimental observations. In at least one experiment they recorded simultaneously from six pairs of electrodes spaced around the presumed circuit pathway, and concluded that the mechanism was indeed a circus movement. Confirmation of that conclusion was achieved in more elaborate experiments by HAYDEN et al. (1967), and there can be little doubt that the interpretation is valid, at least in the experimental model; there is still no evidence to confirm that the same mechanism applies to all cases of flutter in man.

1. Genesis of Flutter

If flutter is indeed reentrant (i.e. a circus movement) we must consider how the necessary conditions for reentry are fulfilled. WIENER and ROSENBLUETH (1946) developed a mathematical model to explain propagation of impulses in a matrix perforated by obstacles. The maintenance of flutter in such a matrix poses no problem: the perimeter of an obstacle must exceed the product of conduction velocity and refractory period (RP), but little has been published about the initiating event. In every experimental study, flutter was induced by a brief period of rapidly repetitive stimulation; chance alone determined whether the electrically induced turbulent activity was replaced by sinus rhythm or by flutter.

The primary condition for the initiation of reentry, the *sine qua non*, is block. The block must be at least temporarily unidirectional. In a simple cable of conducting tissue, a one-way block can easily be achieved by a stimulus applied immediately following the expiration of the absolutely RP of a prior activation. In a biological system it is unlikely that conductivity will recover symmetrically; propagation in one direction from the stimulated site is therefore more than probable. The intact atrium is not a simple cable; neither is it a two-dimensional sheet of tissue, but it is obvious that, while a circus-movement flutter can be established in a ring-like cable, it could not exist in a homogeneous sheet of tissue. Topologically, the atrium is an irregular perforated sphere, with bands of conducting tissue separating the orifices. For a self-sustained circuit to be generated, one band of tissue, regardless of its breadth, must fail to conduct at some strategic moment. This favorable point in time could be encountered by a single premature stimulus, provided that: (1) the isthmus is narrow; (2) the isthmus has a slightly longer RP than adjacent tissue; and (3) the initiating stimulus is applied close to the origin of the isthmus.

These constraints can best be exemplified by considering what would happen if they did *not* apply. (1) If the isthmus were broad, it is unlikely that its whole breadth would remain refractory long enough to exclude an approaching wave front. (2) If the refractory period of the isthmus were briefer than that of surrounding tissue, the isthmus would of course be entered. (3) If the premature stimulus were applied at a distance, the initially slow conduction through relatively refractory tissue would give time for recovery of excitability in the isthmus, and unidirectional block would not occur. We can now examine why a period of repetitive re-excitation is necessary to initiate flutter, as in the experiments of Lewis, Feil and Stroud, and of Rosenblueth and Garcia-Ramos. The temporally disorganized activity characteristic of fibrillation, extending throughout the atria during the period of stimulation, could leave even a broad band of tissue refractory. The last propagated response would thus be forced to circumscribe one of the obstacles, as illustrated in Fig. 1. Given the appropriate relationships between obstacle size, conduction velocity, and RP, the circuit would continue indefinitely. This possibility was demonstrated in a computer model in which self-sustained "fibrillation" was initiated by a brief period of simulated repetitive stimulation. When obstacles of suitable dimensions were created by deleting strips of excitable elements from the fibrillating matrix, a rhythmic circus-movement flutter supervened (Moe et al., 1964). It was possible to show in the model that no single premature response could have resulted in flutter without a prior period of fibrillation. These conclusions do not, of course, exclude the possibility that flutter could be initiated by a single premature atrial beat in a diseased heart, in which a strategically placed lesion could result in a broad region of unidirectional block.

The emphasis on the importance of obstacles, common to most of the analyses of flutter, has been challenged recently by Allessie et al. (1973). They were able to produce circus movement flutter in small areas of left atrium excised from the rabbit heart; no anatomic obstacles were present in the sheet of tissue. These observations do not, of course, deny the necessity of one-way block; their maps of the excitation sequence clearly demonstrate the initial block. In effect, the

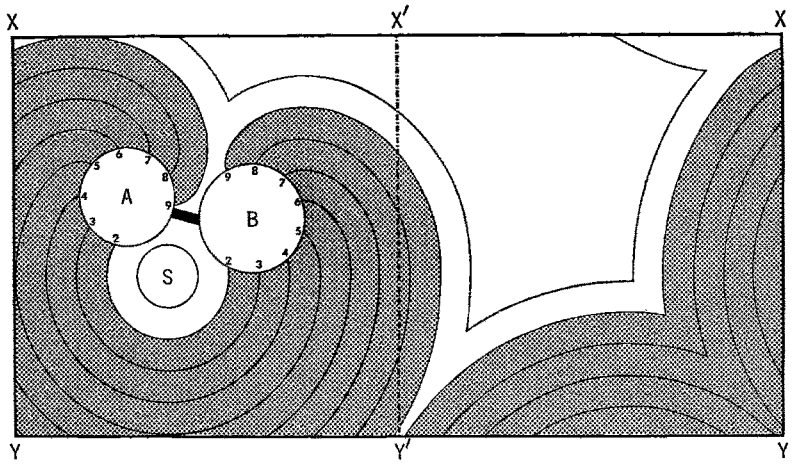

Fig. 1. Initiation of a circus movement flutter by temporary conduction block at an isthmus between two obstacles, A and B. The two areas depicted represent opposite surfaces of a square envelope, cut and opened along the margins XY, XX′, and YY′, to illustrate the spread of activation of a premature response initiated at S. The curved lines represent isochrons assumed to be separated by 20 msec. The black bar connecting A and B represents a band of tissue which is still refractory from a preceding response at the time isochron 2 would have occurred. Conduction of the premature response is therefore channeled around the obstacles: clockwise around A, counterclockwise around B. The shaded area represents the absolutely refractory period as it might exist when the advancing wavefront reaches isochron 9. By this time, the black barrier would have recovered, and a self-sustained circuit would continue. (Reproduced from MOE, 1972, in Pathophysiology, Altered Regulatory Mechanisms in Disease, by permission of the publishers)

original site of refractoriness constitutes the "obstacle". A comparable result was obtained in the computer model described by MOE et al. (1964). Recent observations by G. PASTELIN (unpublished) suggest that loops of the specialized conducting pathways of the right atrium may provide a circus movement pathway in the dog heart; anatomic obstacles are present within such loops, but it can be shown that a circus movement would be theoretically possible even without the caval orifices or the membranous septum.

2. Maintenance of Flutter

Once initiated, flutter can be quite stable, and often difficult to treat. If it is a circus movement, it must obey certain rules. Some of these are self-evident: a specific reduction of conduction velocity should decrease the flutter frequency; a prolongation of the refractory period should terminate the process; and stimulation of the atrium at a higher frequency than that of the automatic process should capture the atria, occlude the circuit pathway, and arrest the arrhythmia. In the clinical situation, the response of flutter to therapeutic measures is not inconsistent with these rules; analysis of the responses may help to explain the mechanisms of conduction in a closed circuit loop. In the following three sections (3, 4, and 5)

we shall consider: the effects of alteration of RP; the obligatory relationship between conduction velocity and RP; and the effect of extrinsic, i.e. atrial stimulation.

3. Refractory Period (RP)

In experimental flutter, the conduction velocity was estimated by Lewis et al. (1920) to be about one half to one third of the "normal" velocity in fully recovered tissue. In other words, the wavefront coursed through relatively refractory tissue. It follows that any agency which reduces the duration of RP (e.g. vagal stimulation) should accelerate the flutter, and prolongation of RP should reduce the frequency. These results are easily demonstrable in the laboratory; when carefully sought, they can also be shown in many clinical cases. Digitalis, which enhances vagal activity in animals under chloralose anesthesia, causes an abbreviation of the atrial RP (Mendez and Mendez, 1953). Acceleration of the flutter and conversion to fibrillation may result (Farah and Loomis, 1950) (see below). Quinidine, which has long been known to prolong the atrial RP, reduces the flutter frequency before sudden conversion to sinus rhythm occurs (Brown and Acheson, 1952).

4. Relationship between Refractory Period and Conduction Velocity

As indicated above, these variables are not independent as long as propagation proceeds through relatively refractory tissue. A prolongation of RP must lead to a slowing of conduction, which in turn increases the cycle length, which further prolongs RP. A "pure" depressant effect on conduction would, of itself, increase the cycle length and, in turn, prolong RP. Why, then, should quinidine, which both depresses conduction and prolongs refractoriness, terminate a flutter? In doses which can be considered "therapeutic," quinidine prolongs the atrial RP at any given frequency by no more than about 20% (Rodriguez and Mendez, 1954). In a mathematical model, gradual prolongation of the RP by 20% leads to a 20% increase in conduction time, but not to arrest of the circuit. In other words, the "excitable gap" does not close. The answer is probably determined by the minimal conduction velocity at which propagation can continue. The minimal value surely does not approach zero; when conduction is impaired to a critical point at which excitation of the next element in an excitable chain fails, the process must extinguish itself. Based on these assumptions, the constructions in Fig. 2 define a zone within which flutter is possible; alteration of any parameter to a degree that crosses the boundaries will arrest the circus movement. The graphic relationships also demonstrate that a slow flutter (i.e. a circus movement about a large obstacle) should be resistant to arrest by agents which prolong RP; a 20% increase would not cross the boundaries. In fact, if the obstacle were so large that the circulating impulse could course about it at full speed, a specific prolongation of RP need not even reduce the frequency.

It has been proposed that the common denominator of most effective antiarrhythmic drugs is a specific depressant effect on conductivity (Szekeres and

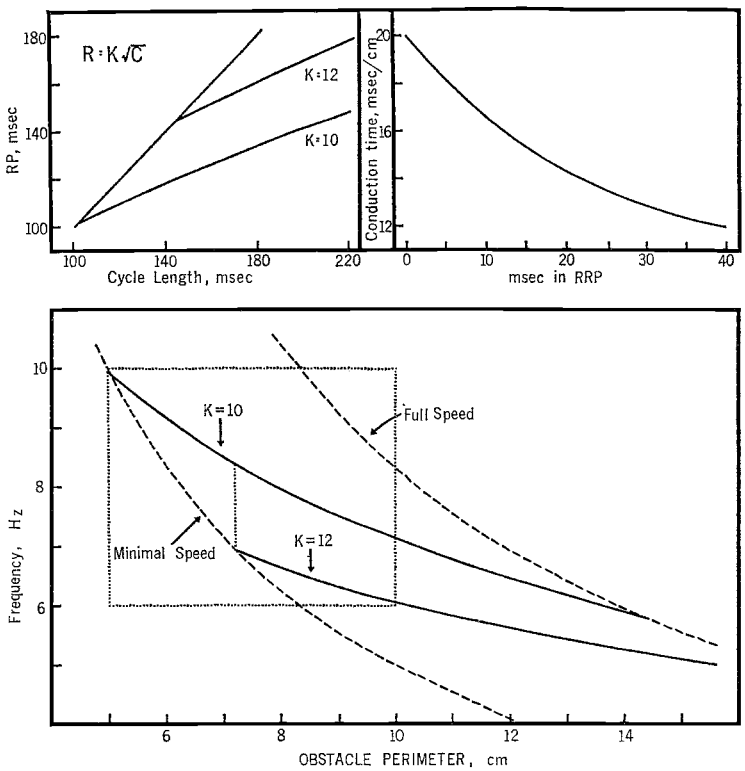

Fig. 2. A schematic representation of the behavior of a circus movement flutter in relation to refractory period and obstacle size. Upper left: the refractory period (RP) is plotted as a function of the cycle length, assuming the empirical relation $R = K\sqrt{C}$, where K is a constant having the dimension of the square root of time, C = cycle duration, and R = refractory period. The diagonal line at 45° represents the limiting cycle length at various values of K. Upper right, the conduction time is plotted as a function of the relatively refractory period (RRP), assuming the RRP to be 40 msec, and assuming full speed conduction to be 83 cm/sec. Below: Given these assumptions, a circus movement flutter could be sustained between the limits of the broken lines in the lower graph. If we assume that the obstacle has a perimeter (wavelength) of 7.2 cm, and a K value of 10, a 20% prolongation of the RP ($K = 10 \rightarrow K = 12$) would arrest the flutter, as indicated by the vertical dotted line. The rectangle outlined by dotted lines indicates the reasonable wavelengths and frequencies which might exist, either naturally or created, in a dog heart. The smaller the obstacle (i.e. the higher the frequency), the less the change in RP necessary to arrest a flutter

VAUGHAN-WILLIAMS, 1962). Whether or not this is a correct appraisal, it is clear that arrest of a circus-movement flutter cannot be divorced from a concomitant depression of conduction.

In a study of the antiarrhythmic properties of the antihistaminic drug, clemizole [1-p-chlorobenzyl-2-(1-pyrrolidinylmethyl)benzimidazole], R. MENDEZ et al. (1969) emphasized that the ideal anti-flutter (i.e. anti-*circus movement*) drug should prolong the wavelength (loosely defined as velocity times RP) without specifically decreasing velocity. They showed that clemizole, in doses which caused little or no depression of conduction velocity in *fully recovered* atrial tissue,

resulted in a considerable increase of RP. In flutter of the Rosenblueth-Garcia-Ramos type, small doses promptly restored normal rhythm; flutter induced by aconitine (presumably due to a rapidly discharging ectopic focus) was temporarily slowed but never arrested. With these considerations in mind, Cardenas et al. (1967) tested small doses of clemizole in 6 unselected cases of atrial flutter in the clinic; 5 of the 6 were promptly restored to sinus rhythm. The authors were prompted to suggest that the drug could be used to differentiate circus-movement flutter from an ectopic tachyarrhythmia.

5. Response to Atrial Stimulation

Mines observed that a single shock applied at the proper time to his ring of tortoise heart could stop the circus movement. Since then, it has been taken as an article of faith that any arrhythmia which could be terminated by a single stimulus must be a reentrant rhythm. That this is not an absolute dictum will be considered below, but it has been well established that a single high-intensity stimulus ("cardioversion") can indeed arrest flutter in the human heart. Attempts have also been made, usually without success, to arrest flutter by a single atrial stimulus of moderate intensity, delivered through an intracardiac electrode probe (Zipes, 1971). The failure of this technique is not surprising, nor does it eliminate a self-sustained circuit as the basic mechanism. The "excitable gap" between the advancing wave front and its retreating refractory tail probably represents no more than 20% of the cycle length (40 msec, more or less, for a flutter frequency of 300/min). To be effective, the stimulus would have to be applied very close to the circuit pathway, and very soon after the expiration of the RP at the stimulated site. Capture of the flutter, however, can be achieved by repetitive stimulation at moderate intensity, at a frequency higher than the intrinsic rate, but reversion to the original mechanism when stimulation is terminated is common (Zipes, 1971). Interruption of the arrhythmia can only succeed if the pathway is effectively occluded by extrinsic pacing.

6. Action of Digitalis in Flutter

Cardiac glycosides prolong the atrial RP in animals under barbiturate anesthesia, in whom vagal activity is greatly depressed (Mendez and Mendez, 1953). Circus-movement flutter in such animals is readily abolished by digitalis, much as it is by quinidine (Farah and Loomis, 1950). When vagal reflexes are active, digitalis causes a reduction of atrial refractory period. The reduction must be spotty, for not all atrial fibers can be bathed in a uniform concentration of acetylcholine when vagal endings discharge (Alessi et al., 1958). If digitalis caused a uniform abbreviation of RP in all parts of the circuit, acceleration of the flutter would occur, but only to a maximum rate determined by full-speed conduction around the obstacle. Conversion of flutter to fibrillation by digitalis can be readily explained if we assume, as we must, that the RP in some elements of the pathway

is diminished, while that in other elements is not only not abbreviated, but even prolonged by the direct action of the drug. The circulating wave front, encountering areas that are now less refractory, will accelerate. As it more quickly reaches islands that are still refractory, the wave front must detour, divide, or fractionate; fibrillation is the almost inevitable result.

7. Is the Evidence for Reentrant Activity Conclusive?

The evidence, as we indicated at the outset, is more circumstantial than definitive. Most of the antiarrhythmic drugs that prolong the RP and/or depress conduction velocity also suppress pacemaker activity (phase 4 depolarization) in ectopic pacemaker sites; quinidine, for example, might well abort an ectopic tachycardia. We have already suggested that arrest by a single stimulus is no more than presumptive evidence for a reentrant mechanism. Perhaps the strongest evidence is the effect of digitalis, as mediated through a cholinergic mechanism. Pacemaker activity in specialized atrial fibers, even when induced by cardioactive steroids, is suppressed by acetylcholine (HASHIMOTO and MOE, 1973), but digitalis does not usually slow a flutter; it accelerates it. All of the evidence is consistent with a reentrant mechanism; some can be interpreted either way, but none is absolute.

B. Atrial Fibrillation

In the opinion of a number of investigators, notably SCHERF and SCHOTT (1953) and PRINZMETAL et al. (1952), fibrillation differs from flutter only in its expression, not its mechanism. Flutter, viewed as the discharge of an ectopic focus firing at a rate that the atria can follow 1:1 (or for that matter, 1:2) represents a slower intrinsic frequency than fibrillation, in which the pacemaker discharges at a rate that leads to irregular and fractionated activity in the surrounding atrial matrix.

Much of SCHERF's "unitary" hypothesis was based on the observed behavior of an aconitine-induced focus, which, like a stimulating electrode, can evoke either slow and regular or fast and irregular activity, depending on the imposed frequency. It is not our purpose to belabor the point; even SCHERF has stated that he would "rather switch than fight". The fact that flutter can apparently change to fibrillation and back again to flutter is by no means decisive, neither for one mechanism nor the other. We shall attempt to show that a circus-movement flutter can lead to fibrillation, and fibrillation to flutter, even though fundamentally different mechanisms are involved.

Fibrillation, unlike flutter, can be easily induced by a single premature stimulus, provided that a sufficient degree of nonhomogeneity exists in the duration of the RP (ALESSI et al., 1958). The mechanism has been recognized as a disorganized excitation process in which, *because of* the nonhomogeneity, many independent wavelets circulate almost randomly, changing in velocity, number, direction, and breadth as they encounter tissue in various stages of excitability (BROOKS et al., 1955; MOE, 1962). Unlike flutter, no fixed circuit is necessary and no obstacle

is required. Fibrillation can be induced in a mathematical two-dimensional area (MOE et al., 1964) or in a closed surface without holes (MOE, 1969). It is certainly reentrant; it is almost certainly not a simple circus movement. In the clinic, fibrillation can be a stable arrhythmia lasting for many years; it can also be intermittent and paroxysmal. Its stability depends on a number of factors: (1) non-uniform distribution of RP—*irregular* activity cannot exist in a homogeneous matrix; (2) sufficient size (more importantly, sufficient *area*); fibrillation does not persist in small hearts (MOORE et al., 1965); (3) relatively brief RP and/or relatively slow conduction. Its persistence is a matter of statistical probability: if the number of independent wavelets is large, the chance that they may coalesce to a single wave front is small; if the conduction velocity is high, coalescence is certain; if the RP is long, wavelets must be snuffed out. It follows from these considerations that vagal stimulation, which decreases the mean RP as it increases the dispersion of RP, will increase the probability of sustained fibrillation (BURN, 1957; MOE and ABILDSKOV, 1959), and that any agency that prolongs the RP will increase the chance of coalescence. Clinical fibrillation follows the same rules as the experimental variety, and the mathematical model. It is perhaps relevant that drugs which abbreviate the atrial RP, or fail to prolong it (diphenylhydantoin, lidocaine) are of little value in the treatment of atrial fibrillation, even though these drugs are effective in suppressing ectopic pacemaker activity (MANDEL and BIGGER, 1971; DAVIS and TEMTE, 1969; BIGGER et al., 1968).

We have already described how fibrillation (or, at least, disorganized activity) can lead to flutter, and how digitalis can convert flutter to fibrillation. It is not surprising, therefore, that the dysrhythmias can interchange, even if the mechanisms are fundamentally different. The eminent cardiologist, Frank Wilson, often remarked that the conversion of fibrillation to sinus rhythm was a chance phenomenon; quinidine merely increased the probability.

C. Paroxysmal Supraventricular Tachycardia

We have introduced the discussion of reentrant rhythms with a description of flutter, principally because of its historic significance, but the most convincing evidence for reentry as a cause of clinically important tachycardias has been derived, in the laboratory and in the clinic, from studies of paroxysmal supraventricular tachycardias.

1. Reciprocal Beats

In 1915, PAUL D. WHITE described a case of A-V block (or A-V dissociation) in whom occasional idioventricular responses were propagated back to the atria, and were followed in turn by QRS complexes of supraventricular origin. In this and a subsequent case report (1921) WHITE called attention to the time relations of the inverted P-wave "sandwiched" between two QRS complexes. His descrip-

tion of the necessary conditions for reciprocal activation of the ventricles has been amply confirmed by later experimental studies, but there has been no real improvement on his analysis. Subsequent studies have indeed been a matter of i-dotting and t-crossing, but the ability of modern cardiologists to support his concepts by direct recording techniques has resulted in widespread acceptance of reciprocal rhythm as a prime cause of paroxysmal tachycardias.

WHITE demonstrated that reciprocation occurred only when an idioventricular discharge occurred relatively early in the sinus node cycle, i.e. when propagation back to the atrium was slow because of relative refractoriness. He also showed an inverse relation between the retrograde conduction time, "R–P", and the anterograde conduction time, "P–R". He assumed that retrograde conduction occurred at a time when the A-V node was dissociated: one portion was capable of propagating the impulse while another portion was still refractory. (This fulfills condition No. 1 for reentry, i.e. one-way block.) Upon arrival in the atria after slow conduction through the node (condition No. 2), delayed excitation of the now-recovered alternate pathway in the node occurred (condition No. 3). If the total conduction delay was sufficient to clear the refractory period of the final common pathway (lower node or His bundle), reentry of the ventricles occurred.

Some years later, SCHERF (1941) demonstrated the possibility of reciprocal rhythm in the dog heart. Stimulation of the ventricles at appropriate intervals often resulted in retrograde atrial responses, which were returned to the ventricles as "Umkehrextrasystolen", or return extrasystoles. More complete studies of A-V reciprocation ("echoes") in the dog heart were published by MOE et al. (1956) and by ROSENBLUETH (1958).

2. Hypothetical Substrate

MOE et al. (1956) postulated two pathways within the A-V transmission system: one characterized by a briefer RP and a slower conduction velocity than the other. The evidence for intrinsically different conduction velocities, which has since been shown to be erroneous, was based on the observation of an abrupt increase in A-V conduction time as the interval between two stimuli (basic, S_1, and premature, S_2) applied to the atrium was gradually decreased. The error in interpretation occurred because ventricular activation was recorded only from the right ventricle; the abrupt delay was, in fact, due to functional block of the right bundle branch and was not a nodal phenomenon (MOE et al., 1965).

ROSENBLUETH (1958) also postulated two communications between atria and ventricles, but he believed that one of the pathways was "polarized" in a synaptic sense, i. e. it could conduct from atrium to ventricle, but not in the reverse direction. The latter conclusion, also erroneous, was based on his failure to observe atrial echoes. Because his experiments were conducted in animals subjected to thoracic sympathectomy and bilateral ligation of the adrenals, it is probable that the "weaker" of two pathways was incapable of retrograde conduction; atrial echoes would have been impossible.

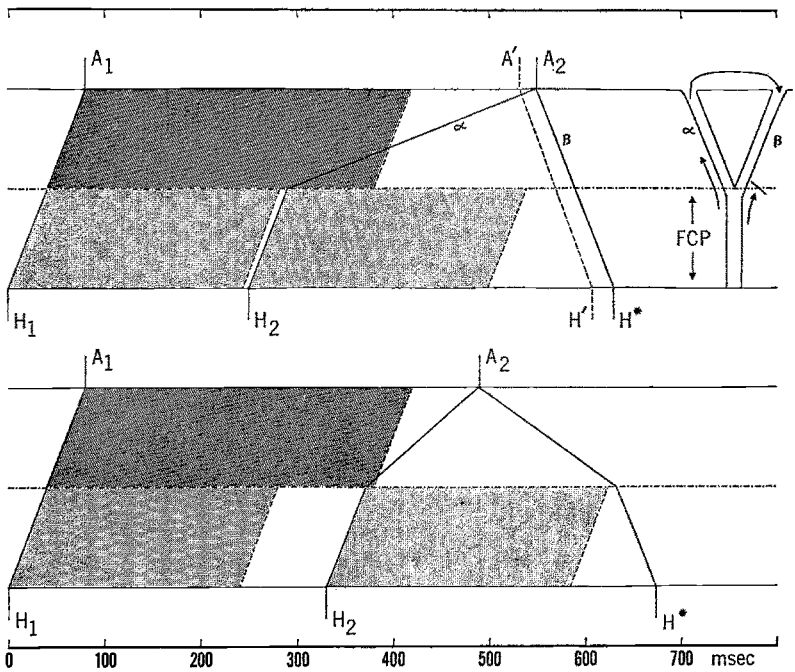

Fig. 3. Schematic representation of ventricular echoes induced by premature stimulation of bundle of His. H_1A_1 = last of a series of rhythmic paced responses propagated from His bundle (H) to atrium (A). At right, diagram of dissociated pathways in upper AV node (α and β), and final common pathway in lower node (FCP). Upper diagram illustrates earliest premature response, which is propagated to atrium and returns as an echo. Propagation to atrium is slow, due to delayed conduction in α pathway; return to His bundle (A_2H^*) clears RP of FCP and is propagated at full speed. Note that pre-excitation of atrium (A'H') could lead to earlier response of His bundle. Lower diagram: latest premature response, which would be blocked in β pathway and therefore yield an echo. H_2A_2 conduction time is now faster, but A_2H^* is delayed by limiting RP in FCP. No *earlier* response of atrium could reach the His bundle. Shading indicates RP in β pathway (upper quadrangle) and in FCP (lower quadrangle) in each diagram. (Based on time relations recorded by C. MENDEZ et al., 1965)

The basic model for intranodal dissociation, reproduced in Fig. 3, differs only in minor details from all previous and subsequent models of reentry, including the verbal description of the necessary conditions for reciprocation as detailed by WHITE (1915), and it appears to fit the temporal characteristics of both atrial and ventricular echoes in dog, rabbit, and human hearts (MOE and MENDEZ, 1966). As shown in Fig. 3, a premature response initiated in the His bundle (or below it) penetrates the lower node and arrives at a junctional site at a time when only one (α) of two possible pathways has recovered sufficiently to permit propagation back to the atrium. Propagation over the α pathway, still relatively refractory, will be slow, thus allowing additional time for recovery of the β pathway and for its activation from above. Provided sufficient time elapses during the round trip, re-excitation of the final common pathway must now occur, and an echo will be observed in the His bundle.

3. Corollaries

A number of corollary conclusions are suggested by the model. (1) Any agency that will unequally depress conductivity at the critical junction or in one of the upper nodal pathways may lead to dissociation. (Most experimental studies have relied on dissociation exposed by premature excitation.) (2) A premature atrial response may also encounter longitudinal dissociation in the upper node, leading to reciprocal excitation of the atria. (3) An atrial echo could conceivably occur without an intervening ventricular response, i.e. the final common pathway below the confluence of the upper nodal tracts could fail to propagate the premature response to the His bundle. (4) As shown in Fig. 3, and as proposed by ROSENBLUETH (1958) and confirmed by MENDEZ et al. (1965), a ventricular echo could not occur without an intervening atrial response. (5) The reentrant circuit could sustain itself as a repetitive tachycardia. (6) An early premature ventricular response should be propagated very slowly on the initial limb of its journey, permitting time for almost complete recovery of conductivity of the parallel pathway for the return trip. Correspondingly, the latest premature response capable of generating an echo should traverse the α pathway more quickly, thus returning to the intranodal junction at a time when the final common pathway, still partially refractory, will retard the final leg of the trip. (This represents the reciprocal relationship of "R–P" versus "P–R" observed by WHITE.)

Because some of the corollary conclusions enumerated above are still controversial, and because there are at least quantitative differences between species, more detailed discussion may be helpful.

i) *Dissociation Exposed by Depression of Conductivity*

Most of the clinical examples of reciprocation, including the cases described by WHITE, have been observed during periods of incomplete A-V dissociation, induced either by disease or by digitalis. In ROSENBLUETH'S experiments, there were occasions when every evoked response of the ventricles was followed by an echo; similar observations were recorded by MOE et al. (1966). The necessary conditions of carefully titrated impairment of conductivity, short of complete block, would be difficult to achieve by design in the laboratory.

ii) *Atrial Echoes*

Spontaneously occurring reciprocal responses of the atria were seldom recorded in the older clinical literature, probably because the recurrent P wave was usually obscured by the QRS complex in body surface electrocardiograms (e.g. KATZ and PICK recorded only one example in their book on arrhythmias, 1956). Several examples were described by KISTIN (1965) who used esophageal leads to expose atrial activity. Since the advent of intracardiac techniques for stimulating and recording, many examples of spontaneous and induced atrial echoes have been recorded (SCHUILENBURG and DURRER, 1968; BIGGER and GOLDREYER, 1970; PUECH, 1970).

It is now generally conceded that, in man, atrial echoes are much easier to induce than ventricular echoes (Puech, 1970); a similar conclusion was reached by Mendez and Moe (1966a) in a study of transmission in the isolated A-V node of the rabbit. The difference is probably dictated by the morphology of the conducting system. If the dissociable upper nodal pathways originate in the atrium, an early premature atrial beat has an excellent chance of encountering one of the two channels in the refractory state. An early premature ventricular response, on the other hand, could be delayed long enough in the lower node (the FCP of Fig. 3) to reach the mid-nodal junction only after both pathways had recovered. Mendez and Moe (1966a) were, however, able to induce ventricular echoes in the rabbit by delivering two sequential premature stimuli to the His bundle; the abbreviation of the RP in the lower node following the first premature response allowed the second response to reach the junction while the β pathway was still refractory. In preparations in which both atrial and ventricular echoes could be elicited, the same "weak" pathway was exposed in each direction.

iii) *Site of Reflection of Atrial Echoes*

Mendez and Moe (1966a) showed examples, in the isolated rabbit heart, of atrial echoes without intervening activation of lower nodal cells, i.e. without a "sandwiched" QRS complex. Similar events have been recorded in the human heart (Kistin, 1965; Puech, 1970) and it is likely that the junction of the α and β pathways must occur well above the His bundle, probably within the N region of the node, as defined by Paes de Carvalho (1961). It follows that a sustained reciprocal tachycardia would be possible with 2:1 A-V block—a clinical syndrome commonly attributed to digitalis intoxication. It also follows that complex patterns of Wenckebach periodicity can occur on the basis of a reentrant circuit in the upper node (Gallagher et al., 1973).

iv) *Site of Reflection of Ventricular Echoes*

It is uncertain whether ventricular echoes can also be reflected from an intra-nodal (sub-atrial) site. In the studies by Rosenblueth (1958) and by Mendez et al. (1965), there was little question that atrial activation served as an essential bridge between retrograde propagation through the α pathway and anterograde activation of β. It is of little practical importance whether this is a universal rule, but because it has become a frequent bone of contention in the cardiological literature, we shall attempt to define the evidence.

The fact that a phenomenon has never been observed does not, of course, exclude its existence. The fact that a ventricular echo without a "sandwiched" atrial response has never been observed in the dog or the rabbit suggests that lateral communications between the α and β pathways are either nonexistent or functionally weak as conduction pathways. The negative evidence surely need not apply to the human heart. Some of the evidence, however, has been adduced in experiments on the dog heart, under conditions similar to those of Mendez et al. (1965); the interpretation of these experiments is not compelling.

MENDEZ et al. showed that when a ventricular echo was induced by the earliest possible premature stimulus applied to the His bundle, the resulting atrial response (recorded at the atrial margin of the node) was propagated back to the ventricle at full speed. The interval between the atrial response (A_2) and the recorded echo in the His bundle (H*) was the same as the A-H interval for a response initiated, at a comparable time, in the atrium. If the echo response had turned around at a sub-atrial site, the A_2H^* interval would have had to be less. In the same experiments it was shown that pre-excitation of the atrium (evoked by a stimulus applied earlier than the expected arrival of the A_2 response generated in the His bundle) resulted in a response which was propagated to the His bundle in advance of the expected arrival of the echo. This could not have happened if the point of reflection had been within the node. MIGNONE and WALLACE (1966), however, described experiments in which pre-excitation of the atrium, within limits, did not result in pre-excitation of the His bundle; the H_2H^* interval remained fixed in spite of atrial stimulation. This result is to be expected within a broad range of H_1H_2 intervals, as shown in Fig. 3. Within this range, the sum of H_2A_2 and A_2H^* intervals can be nearly constant, i.e. H_2H^* is a constant determined by the RP of the FCP. The failure of atrial pre-excitation to prevent the "echo" is not evidence that reflection had occurred within the node: the presumed echo could have been propagated from the evoked atrial response; no atrial response could have reached the His bundle earlier than the echo itself. This concept, documented by MENDEZ et al., has been largely ignored in subsequent studies. It applies to the dog heart under the experimental conditions and also to the rabbit A-V node *in vitro* (MENDEZ and MOE, 1966a). It need not, of course, apply under other circumstances or in other species.

Another possible situation may be relevant to the occurrence of ventricular echoes in the human heart. Anatomical and physiological evidence suggests that specialized conduction pathways emerge from the region of the sinus node and converge at the atrial margin of the A-V node. Physiologically, the specialized fibers are more resistant to depolarization by increased external $[K^+]$ than atrial muscle (WAGNER et al., 1966; HOGAN and DAVIS, 1968). They are also more resistant to the corresponding effect of reduction of internal $[K^+]$ as induced by digitalis. It follows that a response propagated from the ventricles could reach the atrial margin of the node at a time when the specialized fibers, but not atrial muscle, could conduct. In cases of digitalis intoxication, there could thus be a ventricular echo without an intervening P wave.

v) *Self-Sustained Tachycardia*

Although the physiological substrate for repetitive reciprocal rhythm exists in the dog heart (MOE et al., 1956), it has been uncommonly difficult to demonstrate such a tachycardia experimentally. One of a very few successful attempts in our laboratory was published as a "case report" (MOE et al., 1963). The conditions necessary for induction of the self-sustained rhythm in this case were compatible with A-V nodal dissociation and, indeed, difficult to explain in other terms. Self-sustained reentrant activity has subsequently been repeatedly demonstrated in isolated preparations of the rabbit A-V node (MENDEZ and MOE, 1966a;

Janse et al., 1971; Wit et al., 1971). It has also been demonstrated in a number of clinical cases. In individuals subject to spontaneous bouts of reciprocal tachycardia, it has proved possible to initiate identical episodes by premature stimulation of the atria (Coumel et al., 1967; Bigger and Goldreyer, 1970; Puech, 1970).

One of the features of the "paroxysmal" supraventricular tachycardia produced in the dog (Moe et al., 1963) was the ease with which the episodes could be terminated by atrial stimulation. Under the conditions of the experiment, no single atrial stimulus could be delivered early enough to enter the A-V node and occlude the excitable circuit, but two successive stimuli were regularly successful. Properly timed single stimuli applied to the atria through transvenous electrodes have been routinely successful in terminating attacks in man (Coumel et al., 1967; Puech, 1970; Bigger and Goldreyer, 1970).

The efficacy of atrial stimulation with only moderately suprathreshold stimuli has been taken, in the clinical situation, as evidence for the circus-movement nature of paroxysmal atrial tachycardia. It is unlikely (but surely not impossible) that an ectopic pacemaker could be more or less permanently turned off by a single stimulus. On the basis of such evidence, it now appears that most of the cases of paroxysmal supraventricular tachycardia observed in the clinic are examples of transnodal reciprocation. Because single reciprocal responses of the atrium can be readily induced in normal hearts, both in laboratory animals and man, it is also apparent that reciprocation does not require an "anomalous" A-V conduction pathway. Many examples of paroxysmal tachycardia involving anomalous pathways, associated with the WPW syndrome, have been described; these cases do not differ in concept from intranodal dissociation and are not discussed further.

4. Sinus Node Reentry

In their discussion of the mechanism of paroxysmal auricular tachycardia, Barker, Wilson, and Johnston (1943) described three classes, based on the configuration of the P wave. In approximately 40% of their series, the P-wave vector during episodes of tachycardia was the same as during normal sinus rhythm. They proposed that in these cases the tachycardia was due to a circuit involving the S-A node. On the assumption that vagal stimulation would depress conductivity within the sinus node, as it does in the A-V node, the termination of attacks by direct or indirect cholinergic mechanisms could be readily explained.

Direct proof of reentry within the sinus node is difficult to achieve; unlike the A-V node, for which both input and output events can be recorded, the right atrium serves as the only indicator. Nevertheless, it is known that propagation within the node is slow (Sano and Yamagishi, 1965) and probably nonuniform; it is also known that block can occur between the node and the atrium in either direction. These characteristics support the possibility of S-A nodal reentry (Hoffman and Cranefield, 1960; Wallace and Daggett, 1964).

Han, Malozzi and Moe (1968) studied the propagation of premature responses in isolated preparations of rabbit S-A node. They obtained presumptive evidence

for reentrant activity. Early premature responses initiated in the atria resulted in prompt responses in some nodal cells; others, however, were excited only after a noticeable delay followed by a recurrent atrial response which was presumably an echo.

Recent evidence has accumulated suggesting that sinus node echoes can be induced by premature atrial stimulation in the dog and in man (PAULAY et al., 1973a, b; CHILDERS et al., 1973). The sequence of atrial activation recorded during the coupled atrial responses is compatible with sinus node reentry, and incompatible with activity reflected within the A-V node.

D. Intraventricular Echoes

Functional block of one or another of the main branches of the intraventricular conduction system frequently occurs in the human heart. During atrial fibrillation, when the ventricular rate is irregular, "aberrant" QRS complexes often terminate a short cycle preceded by a long cycle. Similar events have been analyzed in the dog heart by MOE et al. (1965). When early atrial premature responses were initiated at a slow basic heart rate, the response pattern of the ventricles was sometimes characteristic of block in the right bundle branch, suggesting that at slow heart rates the RP of the right branch exceeded that of the left bundle, the His bundle, and the A-V node. In animals in which the pattern of right bundle branch block could not, because of refractoriness in the A-V node, be evoked by premature excitation of the atrium, aberrant intraventricular conduction could often be demonstrated by stimuli applied to the His bundle. At more rapid basic heart rates, the functional RP of the His bundle and its major branches diminished and converged.

When block of the right bundle was demonstrable, it appeared to occur high in the bundle, probably near the site of its origin from the common bundle. This circumstance clearly sets the stage for reentry; block at the junctional site, coupled with slow propagation down a parallel pathway (the left bundle branches) and retrograde excitation of the right bundle, could lead to reentry of the His bundle.

The possibility of an intraventricular echo traversing the bundle branches as a reentrant loop was tested by MOE, MENDEZ, and HAN, and in two experiments reentrant responses were recorded in the His bundle. The reentrant activation pattern required two sequential premature stimuli to be applied to the His bundle. The first premature response evoked the pattern of bundle branch block, including retrograde activation of the right bundle, but without a recurrent response of the His bundle. The second response also encountered a refractory right bundle, was propagated slowly down the left, and returned to the junction late enough to excite the now-recovered His bundle. Activation of the His bundle was followed by retrograde activation of the atria. The atrial "echo" in this case occurred considerably later than atrial echoes resulting from dissociation within the A-V node in the same animals. Recently, DAMATO (1973) demonstrated a similar phenomenon in patients: premature atrial responses traversed the A-V node, resulted in aberrant intraventricular conduction, and returned to the His bundle and the atria as an echo.

In the experiments described by MOE et al., it appeared possible that a reentrant loop involving the bundle branches could sustain itself as a ventricular flutter, i.e. reentrant excitation of the His bundle could be followed by re-excitation of the left bundle and repetition of the circus pathway. Under their experimental conditions this was not achieved, yet it is probable that at least some cases of ventricular tachycardia may be due to a bundle branch circus movement (WELLENS et al., 1972; PATTON et al., 1970). The conditions necessary to sustain the circuit would be: (1) intrinsically depressed conduction velocity within the His–Purkinje system, and (2) an abbreviated refractory period within the circuit. Both of these conditions can occur with hyperkalemia, and both can occur with digitalis intoxication.

E. Reentry as a Possible Mechanism for Premature Ventricular Depolarizations

When premature ventricular beats are closely coupled to a preceding normal beat in a bigeminal pattern, they are commonly assumed to be reentrant, but definitive proof of the mechanism is lacking; convincing models cannot be easily constructed even in the experimental situation, and plausible hypotheses for automatic discharge also exist (SCHAMROTH, 1971).

1. "Micro-Reentry"

In the long-lasting circus movement versus ectopic focus controversy, it has sometimes been suggested that an ectopic pacemaker represents a miniature reentrant circuit too small to be demonstrated within the spatial resolution of the recording techniques. Given the constraints of conduction velocity and RP duration in normal tissue, microcircuits would indeed be impossible. For example, a tachycardia at a frequency of 300/min would require 200 msec to complete one circuit. At a conduction velocity of as little as 200 mm/sec, the circuit would have to be 40 mm long—miniature perhaps, but not a microcircuit.

Two recent observations on the cellular physiology of cardiac tissue indicate that reentry is indeed possible within narrowly circumscribed loops. Although thought to be physically impossible, mean conduction velocities of less than 50 mm/sec have been recorded in isolated preparations of Purkinje tissue (WIT et al., 1972). Furthermore, refractory periods of less than 40 msec have been observed in similar preparations (SASYNIUK and MENDEZ, 1971). In both cases, reentry has been demonstrated within loops no more than a few mm in circumference.

2. Depressed Conduction

WIT et al. (1972) produced profound depression of conduction by exposing all or part of their isolated preparations to perfusion media that resulted in reduction

of the resting membrane potential: high [K^+]; high [K^+] plus epinephrine; low [K^+] plus elevated Ca^{++}; low [Na^+]). Reentry was observed in small loops, and documented at several intracellular recording sites, when the apparent conduction velocity was reduced below about 50 mm/sec. The slow conduction was attributed to a "slow response", probably mediated by calcium ions, in contrast to the rapid, Na^+-dependent depolarization of the normal action potential (ARONSON and CRANEFIELD, 1973). The action potentials recorded from depressed areas of Purkinje tissue resemble the normal responses observed in A-V nodal cells, in which a Ca^{++}-mediated depolarization has also been suggested (ZIPES and MENDEZ, 1973) and in which very slow conduction is the rule.

It is possible that the apparently slow conduction velocity is partly due to stepwise delays in excitation at one or more "weak" junctions. Junctional delays of considerable magnitude in the A-V node have been observed by MENDEZ and MOE (1966b) and recent studies by BILLETTE et al. (1973) demonstrate, by means of multiple simultaneous recordings, that a wave front traversing the A-V node during its relatively RP may be arrested for 50 msec or more before propagation is resumed. Whether propagation is slow and continuous, or apparently slow but discontinuous, does not change the end result: if the elapsed time around a circuit exceeds the RP of the elements proximal to a site of one-way block, reentry must occur, and it could conceivably occur repetitively as the fundamental mechanism of an "ectopic" tachycardia. A "microcircuit", with an average conduction velocity of 50 mm/sec and RP of 200 msec, need be no more than 10 mm in circumference.

The model of WIT et al. (1972) is appropriate to the clinical situation of coronary heart disease, in which idioventricular beats are common: ischemic damage to cardiac tissue could result in local areas of elevated external [K^+], with corresponding depression of excitability and conductivity.

3. Abbreviation of Refractory Period

The experiments of SASYNIUK and MENDEZ (1971) emphasized the importance of RP abbreviation in the development of intraventricular reentry. It had been shown, both in A-V nodal cells and at Purkinje fiber-muscle junctions (MENDEZ and MOE, 1966b; MENDEZ et al., 1970) that the action potential duration of cells just proximal to a site of block was greatly curtailed, and evidence was obtained that the RP of these cells was correspondingly abbreviated. SASYNIUK and MENDEZ studied slow propagation and localized block occurring during the relatively RP in isolated false tendon-papillary muscle preparations excised from dog hearts. In "normal" tissue (i.e. not exposed to an abnormal ionic environment), block was produced at Purkinje-muscle junctions by premature stimulation of the false tendon. Properly timed, the premature response was blocked at some, but not all of the junctions; slow propagation over relatively refractory collateral junctions was still possible. In terminal Purkinje fibers just proximal to a site of block, the action potential duration was extraordinarily brief, often no more than 30 msec. Successful propagation to muscle at nearby junctions could, under these conditions, lead to reentry of the terminal Purkinje fiber and retrograde

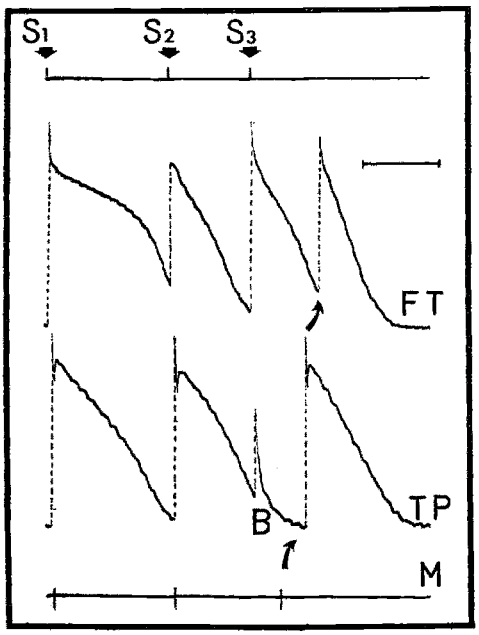

Fig. 4a. Reentry induced at a Purkinje fiber-muscle junction in an isolated preparation of dog ventricular tissue. (Based on experiments reported by Sasyniuk and Mendez, 1971.) Stimuli S_1, S_2, and S_3, applied to central end of a false tendon-papillary muscle preparation, represent the last of a series of rhythmic driving pulses (S_1), and two sequential premature stimuli (S_2 and S_3). Transmembrane action potentials recorded from the false tendon (FT) and from a terminal Purkinje fiber (TP) just proximal to its junction with muscle (M). Extracellular electrogram recorded from muscle. At B, the third evoked response is blocked between the terminal Purkinje fiber and subjacent muscle, leading to an extremely brief action potential. Delayed excitation of muscle through collateral Purkinje fibers leads to re-excitation of the Purkinje fiber (arrows). Time calibration, 100 msec. (Reproduced from Moe and Mendez (1973), New Engl. J. Med., by permission of the publishers)

propagation into the ventricular conduction system (Fig. 4). Again, the circuit need be no more than a few mm in circumference. This mechanism can account for closely coupled premature ventricular beats (bigeminal rhythm), but it is unlikely to sustain itself as a tachycardia. The abbreviated RP that permits reentry is a feature of block at the Purkinje-muscle junction, but once a single circuit has been completed, the conditions that lead to short action potentials will no longer be present. Repetition of the circuit would require, as in the experiments of Wit et al. (1972), a major depression of conduction velocity.

The relevance of the observations of Sasyniuk and Mendez to spontaneously occurring bigeminal rhythm may be questioned: reentry at the Purkinje-muscle junction followed application of two successive premature stimuli. This procedure was chosen for two purposes: (1) to effect a frequency-dependent abbreviation of the RP in the central Purkinje strand, and (2) to permit the third driven response to encroach upon RP at the Purkinje-muscle junction. Clearly, any agency that causes sufficient depression of excitability and conductivity at the junctions will lead to block (and abbreviated action potentials) at some sites while slow pro-

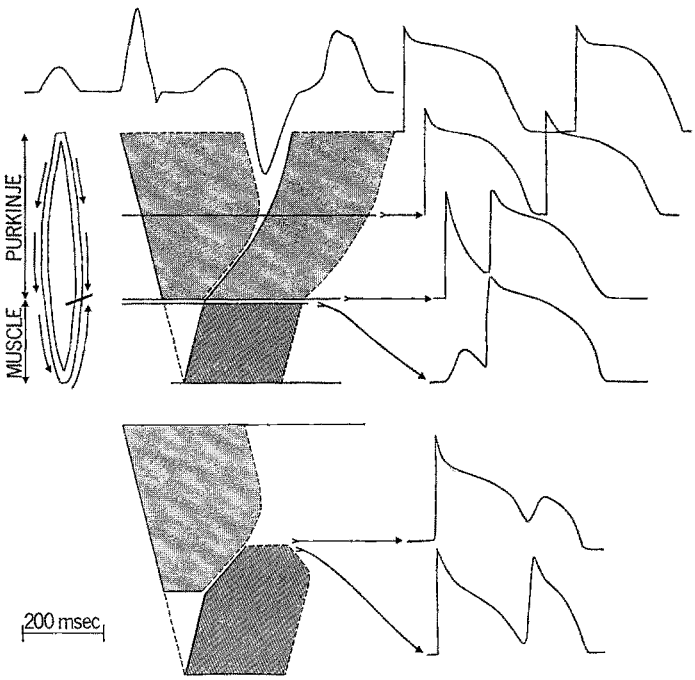

Fig. 4b. Construction illustrating how block at a terminal Purkinje-muscle junction would lead to a bigeminal beat (above), or to a "concealed" or abortive reentry (below). Refractory periods at various levels in Purkinje tissue and muscle are indicated by shading. Simulated action potentials above and below the site of block at a Purkinje-muscle junction are indicated on the right

pagation is still possible at others. Elevated potassium, as shown by MENDEZ et al. (1970), is one such agency; digitalis intoxication is another; and focal ischemia could surely cause local block and impaired conductivity.

Abortive ("silent" or "concealed") reentry can also occur by the mechanism described by SASYNIUK and MENDEZ. Abbreviation of the action potential would be most prominent in the fibers immediately proximal to the blocked junction, where the repolarizing current flowing from the cells beyond would be most intense. The electrotonic interaction would be dissipated exponentially in more proximal fibers, depending upon the membrane impedance and space constant in the cable. In other words, RP of cells a few mm proximal to the junction would scarcely be affected. Accordingly, a reentrant response of the terminal fiber would have to propagate "uphill" in progressively more refractory tissue, with the possibility of decrement and extinction within the peripheral Purkinje net. Concealment of impulses travelling such an uphill gradient has been demonstrated under a variety of circumstances (MENDEZ et al., 1969; MOORE, 1967; MYERBURG et al., 1970). An abortive reentry, not apparent with gross recording techniques, could nonetheless influence the local propagation of a subsequent beat. This could account for the observation that, in some clinical cases showing scattered coupled extrasystoles, only odd numbers of normally conducted beats intervene between the ectopic responses (SCHAMROTH and MARRIOTT, 1961).

4. Ectopic Pacemaker Activity: Parasystole

The potentiality for pacemaker activity in cardiac tissue is widespread; it is certainly a property of the specialized conducting tissue of the atria as well as the ventricles. The occurrence of occasional premature beats, not coupled at a fixed interval, is commonly ascribed to such pacemaker activity. It is necessary to make a number of critical assumptions, not easily validated, to explain how an ectopic impulse generator can occasionally lead to propagated responses without itself being repeatedly discharged, reset and suppressed by the normal cardiac impulse. In particular, one must postulate that the pacemaker site is "protected" by a shell of tissue in which conductivity is depressed to the point of one-way block, i.e. an impulse generated within the site can usually (but not necessarily always) be propagated into the surrounding excitable tissue, but entrance block prevents discharge of the pacemaker from outside. These conditions adequately explain the phenomenon of parasystole; indeed, no other feasible explanation exists.

Although parasystole is identified when the time intervals separating occasional premature responses are multiples of a common period, presumed to be the intrinsic cycle length of the ectopic pacemaker, it is possible that the same basic mechanism can also be responsible for fixed coupling, as in bigeminal rhythm. The "insulation" that protects the pacemaker is obviously not a high impedance electrical barrier: the electrotonic effects of an action potential in the surrounding tissue can be sensed within the pacemaker region as a further partial depolarization superimposed upon the slow diastolic depolarization that characterizes pacemaker activity. Accordingly, if the intrinsic period of such an ectopic focus is slightly longer than the period of the S-A node, or an approximate multiple of it, the pacemaker could be brought to threshold in advance of its expected spontaneous discharge, yielding closely coupled premature beats. Idioventricular discharges would be locked to the normal pacemaker. These hypothetical possibilities have been considered in detail by Schamroth (1971). If the mechanism does indeed exist, then fixed coupling of premature beats can no longer be considered as *prima facie* evidence for reentry.

5. Ventricular Tachycardia

It is impossible to determine from the electrocardiographic record whether a ventricular tachycardia represents a self-sustained reentrant circuit or an ectopic pacemaker. In general, it has been assumed that if a single stimulus, applied through external or intracardiac electrodes, can terminate an episode of tachycardia, then the mechanism must have been a circus movement; an ectopic pacemaker would not be expected to turn off, more or less permanently, following a single stimulus (Wellens et al., 1972). Unfortunately, even this diagnostic criterion is not definitive.

Purkinje fibers exposed to acetylstrophanthidin or ouabain develop afterpotentials following a series of rhythmic driven responses. These have been called

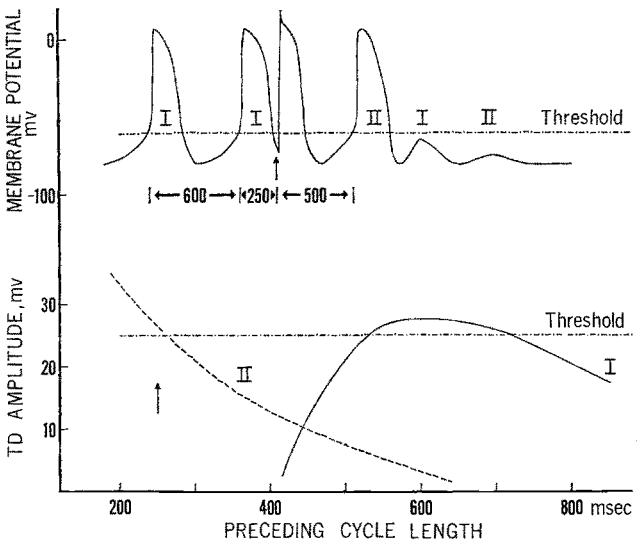

Fig. 5. Termination of a digitalis-induced automatic rhythm in dog Purkinje tissue by a single premature response; schematic representation based on experiments reported by FERRIER et al. (1973). The lower portion of the figure indicates the amplitude of coupled transient depolarizations (TD), I and II, as a function of the preceding cycle length; threshold amplitude is assumed to be 25 mV. The coupling interval of I would be about equal to the preceding cycle length; that of II, approximately double the cycle length. Upper portion: simulated recording of transmembrane potentials in a Purkinje fiber. Spontaneous automatic activity, induced by cardiac glycoside, sustains itself at a cycle length of 600 msec because each discharge is followed by a suprathreshold TD-I. A premature response initiated by stimulation at 250 msec (arrows) is followed by a suprathreshold TD-II (no TD-I would be apparent at that cycle length), occurring at 500 msec, i.e. twice the preceding cycle. The spontaneous response, with a preceding cycle of 500 msec, is followed by subthreshold TD-I and II, and the automatic rhythm therefore ceases

transient depolarizations (FERRIER et al., 1973) or low-amplitude potentials (ROSEN et al., 1973). The coupling interval and amplitude of the depolarizations are frequency-dependent; they may reach threshold, resulting in spontaneous discharges. After a single spontaneous response, one or more transient depolarizations occur; if the preceding pacing frequency is appropriate, cessation of the stimulus train will be followed by a self-sustained spontaneous rhythm. Simultaneous records from various sites in a free-running false tendon reveal no evidence for reentrant activity. If, however, the Purkinje strand is attached to muscle, the further complication of the conduction pathways may introduce a complex interaction between ectopic pacemaker activity, limited to the Purkinje tissue, and reentry generated at the junctions between Purkinje fibers and muscle.

When a self-sustained "tachycardia" develops in an isolated false tendon, it can often be terminated by a single premature stimulus. The reason for this event can perhaps be explained by the diagram of Fig. 5. At the cycle length of the automatic activity (600 msec), each spontaneous discharge is followed by a transient depolarization ("TD-I") which reaches threshold. Because the relation-

ship between the amplitude of the transient depolarizations and the cycle length passes through maxima, a premature response initiated at the time indicated by the arrows in the figure will be followed by a depolarization ("TD-II"), which also reaches threshold. The spontaneous response, with a preceding cycle of 500 msec, is followed by two transient depolarizations, neither of which reaches threshold; the "paroxysm" is therefore arrested. Under these conditions, we have the curious situation in which a premature response may "turn on" an ectopic rhythm and a subsequent premature response may turn it off. The importance of this phenomenon is apparent: the fact that a single stimulus can terminate an episode of tachycardia cannot, unfortunately, be taken as absolute proof of reentry.

III. Summary and Conclusions

Although the evidence for reentry as the mechanism of selected cardiac arrhythmias is largely circumstantial, there are a number of situations in which occasional "ectopic" activity and self-sustained dysrhythmias can best be explained by block and reentry. Clinical observations in many cases follow the same rules as experimental models. The following list is not exclusive. Superficially similar disorders of rhythm could of course result from abnormal automatic activity in an ectopic pacemaker site.

1. Atrial flutter can be induced under circumstances which strongly suggest a fixed reentrant circuit. The response of clinical flutter to agencies that alter RP and the response to electrical stimulation support the same hypothesis in man.

2. Atrial fibrillation, although probably not due to a fixed circuit, conforms to the rules of behavior that would characterize multiple reentrant wave fronts, changing continually in position, breadth, conduction velocity, and number.

3. Paroxysmal supraventricular tachycardias, or isolated single "echo" responses of atria or ventricles, are almost certainly the result of reciprocation through either the A-V node or the S-A node.

4. Intraventricular echoes, involving the main branches of the intraventricular conduction system, can be evoked in experimental animals and in man. Self-sustained repetitive reentry by this mechanism would cause ventricular flutter.

5. Premature ventricular beats, particularly when characterized by close and fixed "coupling", may be the result of reentry through "microcircuits". Solid evidence has been adduced in experimental preparations of cardiac tissue, but a compelling case cannot be made in man.

Although the arrest of any tachysystolic rhythm by a single electrical stimulus has been assumed to be evidence for a reentrant mechanism, there is at least one situation, namely, automatic activity induced by digitalis, in which an ectopic focus can also be "turned off" by a single premature stimulus.

References

ALESSI, R., NUSYNOWITZ, M., ABILDSKOV, J. A., MOE, G. K.: Nonuniform distribution of vagal effects on the atrial refractory period. Am. J. Physiol. **194**, 406–410 (1958).

ALLESSIE, M. A., BONKE, F. I. M., SCHOPMAN, F. J. G.: Circus movement in rabbit atrial muscle as a mechanism of tachycardia. Circulation Res. **33**, 54–62 (1973).

ARONSON, R. S., CRANEFIELD, P. F.: The electrical activity of canine cardiac Purkinje fibers in sodium-free, calcium-rich solutions. J. Gen. Physiol. **61**, 786–808 (1973).

BARKER, P. S., WILSON, F. N., JOHNSTON, F. D.: The mechanism of paroxysmal atrial tachycardia. Am. Heart J. **26**, 435–445 (1943).

BIGGER, J. T., Jr., BASSETT, A. L., HOFFMAN, B. F.: Electrophysiological effects of diphenylhydantoin on canine Purkinje fibers. Circulation Res. **22**, 221–236 (1968).

BIGGER, J. T., Jr., GOLDREYER, B. N.: The mechanism of supraventricular tachycardia. Circulation **42**, 673–688 (1970).

BILLETTE, J., JANSE, M. J., VAN CAPELLE, F. J. L., TOUBOUL, P., DURRER, D.: Frequency-dependent changes in activation of rabbit AV nodal cells. Circulation **48**, Suppl. IV, 62 (1973).

BROOKS, C. McC., HOFFMAN, B. F., SUCKLING, E. E., ORIAS, O.: Excitability of the Heart. New York: Grune and Stratton 1955.

BROWN, B. B., ACHESON, G. H.: Aconitine-induced auricular arrhythmias and their relation to circus-movement flutter. Circulation **6**, 529–537 (1952).

BURN, J. H.: Ion movement and cardiac fibrillation. Proc. Roy. Soc. Med. **50**, 725–736 (1957).

CARDENAS, M., RUIPEREZ, J. A., HERMOSILLO, A., KABELA, E., MENDEZ, R.: El Antihistaminico clemizole en el flutter auricular por movimiento circular. Arch. Inst. Cardiol. Mex. **37**, 380–387 (1967).

CHILDERS, R. W., ARNSDORF, M. F., DE LA FUENTE, D. J., GAMBETTA, M., SVENSON, R.: Sinus nodal echoes. Am. J. Cardiol. **31**, 220–231 (1973).

COUMEL, P., CABROL, C., FABIATO, A., GOURGON, R., SLAMA, R.: Tachycardia permanente par rhythme reciproque. Arch. Mal. Coeur. **60**, 1830–1864 (1967).

DAMATO, A.: Personal communication (1973).

DAVIS, L. D., TEMTE, J. V.: Electrophysiological actions of lidocaine on canine ventricular muscle and Purkinje fiber. Circulation Res. **24**, 639–655 (1969).

FARAH, A., LOOMIS, T. A.: The action of cardiac glycosides on experimental auricular flutter. Circulation **2**, 742–748 (1950).

FERRIER, G. R., SAUNDERS, J. H., MENDEZ, C.: Cellular mechanism for the generation of ventricular arrhythmias by acetylstrophanthidin. Circulation Res. **32**, 600–609 (1973).

GALLAGHER, J. J., DAMATO, A. N., VARGHESE, P. J., LAU, S. H.: Manifest and concealed reentry: a mechanism of A–V nodal Wenckebach in man. Circulation **47**, 752–757 (1973).

HAN, J., MALOZZI, A. M., MOE, G. K.: Sino-atrial reciprocation in the isolated rabbit heart. Circulation Res. **22**, 355–362 (1968).

HASHIMOTO, K., MOE, G. K.: Transient depolarizations induced by acetylstrophanthidin in specialized tissue of dog atrium and ventricle. Circulation Res. **32**, 618–624 (1973).

HAYDEN, W. G., HURLEY, E. J., RYTAND, D. A.: The mechanism of canine atrial flutter. Circulation Res. **20**, 496–505 (1967).

HOFFMAN, B. F., CRANEFIELD, P. F.: Electrophysiology of the Heart, p. 124. New York: McGraw-Hill 1960.

HOGAN, P. M., DAVIS, L. D.: Evidence for specialized fibers in the canine right atrium. Circulation Res. **23**, 387–396 (1968).

JANSE, M. J., VAN CAPPELLE, F. J. L., FREUD, G. E., DURRER, D.: Circus movement within the AV node as a basis for supraventricular tachycardia as shown by multiple microelectrode recording in the isolated rabbit heart. Circulation Res. **28**, 403–414 (1971).

KATZ, L. N., PICK, A.: Clinical electrocardiography: the Arrhythmias, p. 111. Philadelphia: Lea and Febiger 1956.

KISTIN, A. D.: Atrial reciprocal rhythm. Circulation **32**, 687–707 (1965).

LEWIS, T., FEIL, H. S., STROUD, W. D.: Observations upon flutter and fibrillation. Part II: The nature of auricular flutter. Heart **7**, 191–233 (1920).

MANDEL, W. J., BIGGER, J. T., JR.: Electrophysiologic effects of lidocaine on isolated canine and rabbit atrial tissue. J. Pharmacol. exp. Therap. **178**, 81–93 (1971).

Mendez, C., Han, J., Garcia de Jalon, P. D., Moe, G. K.: Some characteristics of ventricular echoes. Circulation Res. **16**, 562–581 (1965).

Mendez, C., Moe, G. K.: Demonstration of a dual A-V nodal conduction system in the isolated rabbit heart. Circulation Res. **19**, 378–393 (1966a).

Mendez, C., Moe, G. K.: Some characteristics of transmembrane potentials of AV nodal cells during propagation of premature beats. Circulation Res. **19**, 993–1010 (1966b).

Mendez, C., Mueller, W. J., Merideth, J., Moe, G. K.: Interaction of transmembrane potentials in canine Purkinje fibers and at Purkinje fiber-muscle junctions. Circulation Res. **24**, 361–372 (1969).

Mendez, C., Mueller, W. J., Urquiaga, X.: Propagation of impulses across the Purkinje fiber-muscle junctions in the dog heart. Circulation Res. **26**, 135–150 (1970).

Mendez, R., Kabela, E., Pastelin, G., Martinez Lopez, M., Sanchez Perez, S.: Antiarrhythmic actions of clemizole as pharmacologic evidence for a circus movement in atrial flutter. Schmiedebergs Arch. exp. Path. Pharmak. **262**, 325–336 (1969).

Mendez, R., Mendez, C.: The action of cardiac glycosides on the refractory period of heart tissues. J. Pharmacol. exp. Therap. **107**, 24–36 (1953).

Mignone, R. J., Wallace, A. G.: Ventricular echoes: evidence for dissociation and reentry within the A-V node. Circulation Res. **19**, 638–649 (1966).

Mines, G. R.: On dynamic equilibrium in the heart. J. Physiol. (Lond.) **46**, 350–383 (1913).

Moe, G. K.: On the multiple wavelet hypothesis of atrial fibrillation. Arch. internat. Pharmacodyn. Thér. **140**, 183–188 (1962).

Moe, G. K.: Computer simulation of cardiac arrhythmias. In: Electrical Activity of the Heart (Manning, G. W. and Ahuja, S. P., eds.) Springfield: Charles C. Thomas 1969.

Moe, G. K.: Cardiac rhythmicity. In: Pathophysiology: Altered Regulatory Mechanisms in Disease (Froehlich, E. D., ed.), Chap. 5. Philadelphia: Lippincott 1972.

Moe, G. K., Abildskov, J. A.: Atrial fibrillation as a self-sustaining arrhythmia independent of focal discharge. Am. Heart J. **58**, 59–70 (1959).

Moe, G. K., Cohen, W., Vick, R. L.: Experimentally induced paroxysmal A-V nodal tachycardia in the dog. Am. Heart J. **65**, 87–92 (1963).

Moe, G. K., Mendez, C.: The physiologic basis of reciprocal rhythm. Prog. Cardiovasc. Dis. **8**, 461–482 (1966).

Moe, G. K., Mendez, C.: Physiologic basis of premature beats and sustained tachycardia. New Engl. J. Med. **288**, 250–254 (1973).

Moe, G. K., Mendez, C., Han, J.: Aberrant A-V impulse propagation in the dog heart: a study of functional bundle branch block. Circulation Res. **16**, 261–286 (1965).

Moe, G. K., Mendez, C., Han, J.: Some features of a dual A-V conduction system. In: Mechanisms and Therapy of Cardiac Arrhythmias (Dreifus and Likoff, eds.), pp. 361–372. New York: Grune and Stratton 1966.

Moe, G. K., Preston, J. B., Burlington, H.: Physiologic evidence for a dual A-V transmission system. Circulation Res. **4**, 357–375 (1956).

Moe, G. K., Rheinboldt, W. C., Abildskov, J. A.: A computer model of atrial fibrillation. Am. Heart. J. **67**, 200–220 (1964).

Moore, E. N.: Microelectrode studies on retrograde concealment of multiple premature ventricular responses. Circulation Res. **20**, 88–98 (1967).

Moore, E. N., Fisher, G., Detweiler, D. K., Moe, G. K.: The importance of atrial mass in the maintenance of atrial fibrillation. In: Proc. International Symposium on Comparative Medicine, Norwich, N.Y.: Eaton Labs. 1965.

Myerburg, R. J., Stewart, J. W., Hoffman, B. F.: Electrophysiological properties of the canine peripheral A-V conducting system. Circulation Res. **26**, 361–378 (1970).

Paes de Carvalho, A.: Cellular electrophysiology of the atrial specialized tissues. In: The Specialized Tissues of the Heart (Paes de Carvalho, A., de Mello, W. C., and Hoffman, B. F., eds.) Amsterdam: Elsevier 1961.

Patton, R. D., Stein, E., Rosen, K. M., Lau, S. H., Damato, A. N.: Bundle of His electrograms: a new method for analyzing arrhythmias. Am. J. Cardiol. **26**, 324–327 (1970).

Paulay, K. L., Varghese, P. J., Damato, A. N.: Atrial rhythms in response to an early atrial premature depolarization in man. Am. Heart J. **85**, 323–331 (1973a).

PAULAY, K. L., VARGHESE, P. J., DAMATO, A. N.: Sinus node reentry. An in vivo demonstration in the dog. Circulation Res. **32**, 455–463 (1973b).

PRINZMETAL, M., CORDAY, E., BRILL, I. C., OBLATH, R. W., KRUGER, H. E.: The Auricular Arrhythmias. Springfield: Charles C. Thomas 1952.

PUECH, P.: La conduction réciproque par la noeud de Tawara. Bases expérimentales et aspects cliniques. Ann. Cardiol. Angiol. (Paris) **19**, 21–40 (1970).

RODRIGUEZ, M. I., MENDEZ, R.: Acción de la quinidina y de la procainamida sobre la excitabilidad, la conducción, y el período refractario de los tejidos del corazón de mamifero. Arch. Inst. Cardiol. Mex. **24**, 456–491 (1954).

ROSEN, M. R., GELBAND, H., HOFFMAN, B. F.: Correlation between effects of ouabain on the canine electrocardiogram and transmembrane potentials of isolated Purkinje fibers. Circulation **47**, 65–72 (1973).

ROSENBLUETH, A.: Ventricular "echoes". Am. J. Physiol. **195**, 53–60 (1958).

ROSENBLUETH, A., GARCIA-RAMOS, J.: Studies on flutter and fibrillation. Am. Heart J. **33**, 677–684 (1947).

SANO, T., YAMAGISHI, S.: Spread of excitation from the sinus node. Circulation Res. **16**, 423–430 (1965).

SASYNIUK, B., MENDEZ, C.: A mechanism for reentry in canine ventricular tissue. Circulation Res. **28**, 3–15 (1971).

SCHAMROTH, L.: The Disorders of Cardiac Rhythm. Oxford: Blackwell 1971.

SCHAMROTH, L., MARRIOTT, H. J. L.: Intermittent ventricular parasystole with observations on its relationship to extrasystolic bigeminy. Am. J. Cardiol. **7**, 799–809 (1961).

SCHERF, D.: An experimental study of reciprocating rhythm. Arch. int. Med. **67**, 372–382 (1941).

SCHERF, D., SCHOTT, A.: Extrasystoles and Allied Arrhythmias. Melbourne-London-Toronto: Heinemann 1953.

SCHMITT, F. O., ERLANGER, J.: Directional differences in the conduction of the impulse through heart muscle and their possible relation to extrasystolic and fibrillary contractions. Am. J. Physiol. **87**, 326–347 (1929).

SCHUILENBURG, R. M., DURRER, D.: Atrial echo beats in the human heart elicited by induced atrial premature beats. Circulation **37**, 680–693 (1968).

SZEKERES, L., VAUGHAN-WILLIAMS, E. M.: Antifibrillatory action. J. Physiol. (Lond.) **160**, 470–482 (1962).

WAGNER, M. L., LAZZARA, R., WEISS, R. M., HOFFMAN, B. F.: Specialized conducting fibers in the interatrial band. Circulation Res. **18**, 502–518 (1966).

WALLACE, A. G., DAGGETT, W. M.: Re-excitation of the atrium. "The echo phenomenon". Am. Heart J. **68**, 661–666 (1964).

WELLENS, H. J., SCHUILENBURG, R. M., DURRER, D.: Electrical stimulation of the heart in patients with ventricular tachycardia. Circulation **46**, 216–226 (1972).

WHITE, P. D.: A study of atrioventricular rhythm following auricular flutter. Arch. int. Med. **16**, 517–535 (1915).

WHITE, P. D.: The bigeminal pulse in atrioventricular rhythm. Arch. int. Med. **28**, 213–219 (1921).

WIENER, N., ROSENBLUETH, A.: The mathematical formulation of the problem of conduction of impulses in a network of connected excitable elements, specifically in cardiac muscle. Arch. Inst. Cardiol. Mexico **16**, 205–265 (1946).

WIT, A. L., GOLDREYER, B. N., DAMATO, A. N.: An *in vitro* model of paroxysmal supraventricular tachycardia. Circulation **43**, 862–875 (1971).

WIT, A. L., HOFFMAN, B. F., CRANEFIELD, P. F.: Slow conduction and reentry in the ventricular conducting system. Circulation Res. **30**, 1–10 (1972).

ZIPES, D. P.: The contribution of artificial pacemaking to understanding the pathogenesis of arrhythmias. Am. J. Cardiol. **28**, 211–222 (1971).

ZIPES, D. P., MENDEZ, C.: Action of manganese ions and tetrodotoxin on atrioventricular nodal transmembrane potentials in isolated rabbit hearts. Circulation Res. **32**, 447–454 (1973).

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Regulation of Intracellular Enzyme Levels by Limited Proteolysis

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Contents

Introduction

It has long been thought that regulation of the intracellular metabolic rate occurs via changes in the activity or amount of an existing enzyme. We take it for granted that a reaction ensues if suitable quantities of substrate and active enzyme are found in the same location. The controlling factors are (KHAIRALLAH and PITOT, 1968) enzyme activity (KATUNUMA, 1973a), enzyme concentration, and (HOLTE et al., 1967) substrate concentration. However, this does not appear to be the case if large substrates with tertiary structures are involved (i.e. proteins or nucleic acids). A further requirement must be satisfied to initiate the subsequent reaction. It is our objective to elucidate the system of intracellular enzymatic degradation under *in vivo* conditions.

Enzymes are constantly undergoing anabolism or catabolism, yet maintaining an equilibrium between the two. What, though, is the trigger mechanism leading to enzyme degradation within the cell? Just as a change in conformation may alter the catalytic activity of an enzyme, so might a protein substrate be rendered more susceptible to the action of proteases. Such a conversion is considered to be the initial step in intracellular enzyme degradation. The proteases can then act via a limited proteolytic mechanism.

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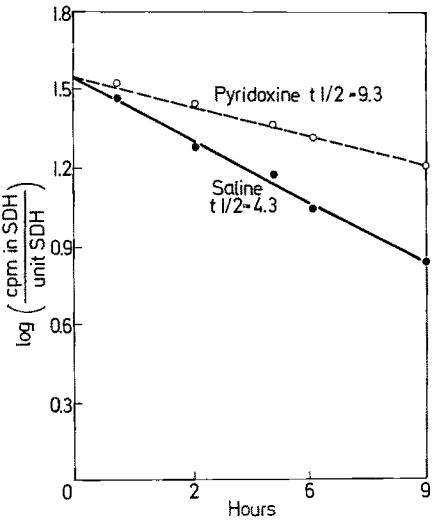

Fig. 1. Effect of pyridoxine on the half-life of serine dehydrase. Pyridoxine administration, half-life $t^{\frac{1}{2}} = 9.3$ and NaCl administration as the control, half-life $t^{\frac{1}{2}} = 4.3$ (KHAIRALLAH and PITOT)

A knowledge of the pathways of both biosynthesis and degradation of enzymes is essential to clarify the regulatory mechanisms at an intracellular level. As yet, little can be said about modes of breakdown. There are many unanswered questions, e.g. what kind of proteases participate in the degradation of an enzyme? more specifically—which protease acts in the initiating step? in which state is the substrate protein most susceptible to the proteases? and what is the initiating trigger?

Pertaining to these questions, several new proteases isolated from various organs of rats, which exhibited the desired substrate specificity for pyridoxal enzymes and which catalyzed a limited proteolysis, were recently discovered in our laboratory (KOMINAMI et al., 1972; KATUNUMA et al., 1973; KATUNUMA, 1973b). We believe that these play the role of initiations in intracellular enzyme degradation.

Some data has been accumulated which indicates that the half lives of the pyridoxal enzymes are prolonged by administration of their coenzyme *in vivo*, particularly in the case of vitamin B_6 deficient animals (KHAIRALLAH and PITOT, 1968). In these studies, pyridoxal enzymes *in vivo* were labelled and the effect of administration of B_6 derivatives on their subsequent degradation was investigated. In a comparison between treated and non-treated animals, the total amounts of pyridoxal enzymes were increased and their half lives prolonged in the former case (Fig. 1). Furthermore the *in vivo* incorporation of ^{14}C or ^{3}H amino acids into the enzyme substrate, using the technique of pulse-labeling, was observed to determine the rate of enzyme synthesis. B_6-administration did not lead to a significant increase in the incorporation of labeled amino acids into the relevant enzyme (Table 1). These results, therefore, imply that the increase in the amount of pyridoxal enzyme is due to the protective ability of the coenzyme against degradation rather than an increase in the rate of synthesis (KATUNUMA, 1973a). This idea of regulation of apoenzyme levels via their

Table 1. Immunochemical analysis of ornithine transaminase increasing by PIN in small intestine of B_6 deficient rats

Treatment	Ornithine TAase Specific activity	Soluble protein (cpm/g tissue)	Ornithine TAase (cpm/g tissue)	$\frac{\text{Ornithine}}{\text{Soluble Protein}} \times 10^{-2}$
NaCl	0.59 ± 0.03	15025 ± 651	681 ± 31	4.5 ± 0.02
PIN	0.85 ± 0.04	14247 ± 2336	617 ± 38	4.6 ± 1.30

stabilization by coenzymes has received much support (HOLTE et al., 1967; GREENGARD and GORDON, 1963) (Fig. 1, Table 1).

In this review, our attention will be focused on the initiating steps of intracellular enzyme degradation and on the importance of conformational changes of substrate enzymes, from the non-susceptible form to one which can be readily attacked by the proteases via limited proteolysis.

New Proteases Participating in Intracellular Enzyme Degradation

The levels of intracellular enzymes are regulated by a careful balance between synthesis and degradation. However, the protease responsible for the latter process is still unknown.

This protease is expected to possess the following characteristics: it reacts in the physiological pH range (neutral or weakly alkaline), it is located in the relevant cells, and it attacks only the susceptible forms of the substrate proteins. Its mode of action is one of limited proteolysis, it exhibits relative substrate specificity for a certain group of enzymes, and its activity or amount is regulated under a variety of physiological conditions.

In our laboratory, a proteolytic activity, capable of inactivating several pyridoxal enzymes, has been detected in various organs in rats. Furthermore, highly purified samples have been obtained from heavy mitochondrial fractions. Proteases of liver, muscle, intestinal muscle and intestinal mucosa layers possess some similar features but differ in molecular weights and electrical charge. A comparison of the properties of these proteases is discussed in this section (KATUNUMA, 1973a; KOMINAMI et al., 1972; KATUNUMA et al., 1973; KATUNUMA, 1973b).

Protease activities were assayed as follows: the substrate apoenzyme was incubated with varying amounts of protease, and its residual activity was determined after stopping the reaction by 10–15 fold dilution with cold buffer.

On the whole, purifications of the four proteases from different organs were similar. Differences occurred in the method of solubilization from the particular membrane, the elution pattern from DE-52, the position of appearance from a Sephadex G-75 column (i.e. molecular weight) and electrophoretic mobility. All the molecular weights appeared to be small. A comparison of the purifications is given in Table 2.

Fig. 2 shows the crystallized protease from intestinal muscle layer. Judging from acrylamide disc gel electrophoresis, the proteases of skeletal muscle and

Table 2. Purification and purity of group-specific protease from various organs

Organ	Muscle	Small intestine		Liver
		muscle layer	mucosa layer	
Location	Membrane bound	Membrane bound	Membrane bound	Membrane bound
Solubilization	Acetone powder	Sonication	Triton X	0.6 M KPB
DE-52	0.05 M	0.005 M	0.25 M	0.1 M (after protamine treatment)
Sephadex G-75	before Cyto. C	after Chymotrypsin	before Chymotrypsin	before Cyto. C
Purification fold	9682	250	1211	8367
Specific activity of purified enzyme (units/mg)	5325	75	545	251
Purity	Single	Crystal		Single

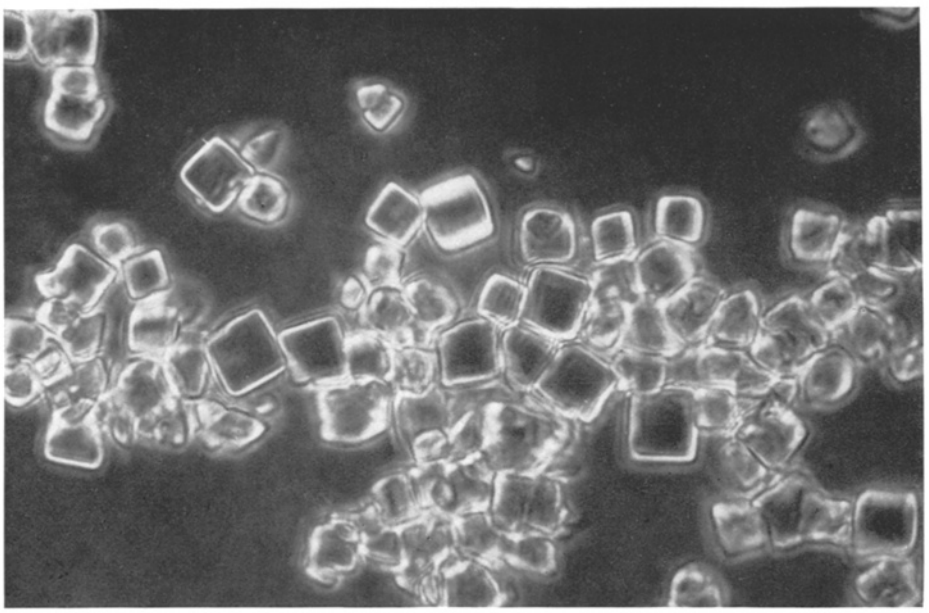

Fig. 2. Crystals of the group-specific protease from the muscle layer of the small intestine (×400)

liver are almost homogeneous. In some stages during the purification, a marked increase in total protease activity is observed. This may be related to the presence of specific inhibitors in the tissues.

Ouchterlony double-diffusion plates containing antibody against crystalline muscle layer protease indicated that this enzyme was immunochemically different from the other isolated proteases and also from lysosomal proteases (sonicated

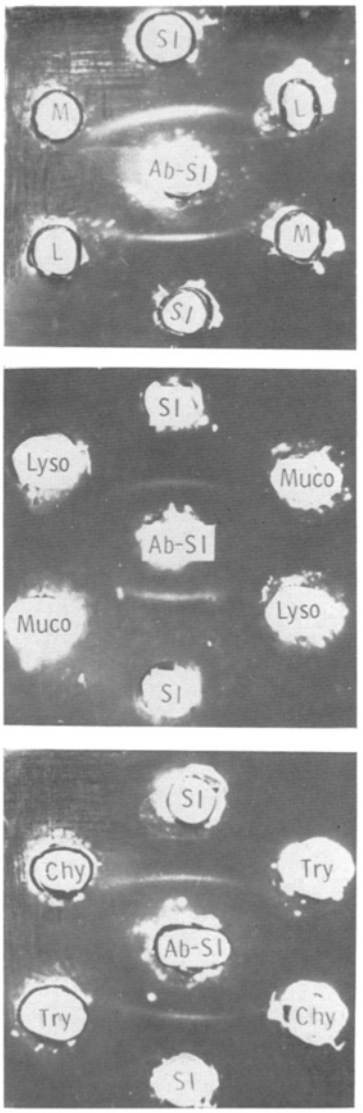

Fig. 3. Identification of differences between the proteases from the intestinal muscle and the mucosa layer of small intestine, liver and skeletal muscle using Ouchterlony double diffusion technique (Ab-SI). The central well contained 5 µl of antiserum (43 mg/ml) for the protease from intestinal muscle and the wells M, L, SI, Lyso and Muco contained 5 µl of skeletal muscle (50 µg. ml), 5 µl of liver enzyme (300 µg/ml) and 5 µl of intestinal muscle enzyme (60 µg/ml), 5 µl of disrupted lysosome, 5 µl of intestinal mucosa enzyme (100 µg/ml) respectively

lysosomes), trypsin and chymotrypsin (Fig. 3). Moreover, on incubation with disrupted lysosomal preparations at pH 8.0, minimal inactivation of apoornithine transaminase (apo-OTA) was observed. Other differences between the new proteases and lysosomal ones (cathepsins) (BARRETT, 1969) are as follows: (1) molecular weights are smaller than cathepsins; (2) optimum pH in alkaline range whereas cathepsins act on the strongly acidic side; (3) the proteases are serine proteases whereas the lysosomal variety are thiol proteases. A summary of the properties of the new proteases is shown in Table 3.

Using the automatic analyzer, the amino acid composition of intestinal muscle protease was determined. Tryptophan was detected by GOODWIN's method

Table 3. Comparison of group-specific protease in various organs

Properties	Skeletal muscle	Liver	Intestine	
			muscle layer	mucosa layer
Common properties				
1. Substrate specificity	B_6 enzymes	B_6 enzymes	B_6 enzymes	B_6 enzymes
2. Susceptibility to trypsin substrate (TAME)	No	No	No	No
3. Susceptibility to chymotrypsin substrate (ATEE[a])	30%	20%	1000%	25%
4. Inhibition by synthetic chymotrypsin inhibitor	No	No	No	No
5. Optimum pH in alkaline	9.0	8.6	9.0	8.6
6. Coenzyme protection (by PALP, PAMP)	Exists	Exists	Exists	Exists
7. SH inhibitor	No effect	No effect	No effect	No effect
8. Histidine modification (photo-oxidation)			loss of activity	
9. Tyrosine modification			loss of activity	
10. Modification of seryl-OH by DFP	loss of activity	loss of activity	loss of activity	loss of activity
Different properties				
1. Molecular weight	13000	16000	25000	21000
2. Elution from DE-52 column	0.05 M	0.1 M	0.005 M	0.25 M
3. Effect by Ca^{++}	Inhibited	No effect	No effect	
4. Catalytic speed	Very fast	Fast	Very slow	Very fast
5. Susceptibility to TEE[a]	0	0	100%	0
6. Reaction with antibody for GSP from iutestine (Muscle layer)	No	No	Yes	No

[a] 100% represent OTA as a substrate.
GSP = Group Specific Protease for B_6 enzymes.

(GOODWIN and MORTON, 1946). The results indicated a rather high content of basic amino acids. This was further shown by the electrophoretic mobility of the enzyme. The photo-oxidation of the protease was carried out in the presence of methylene blue. The subsequent changes in protease activity and amino acid composition were observed (Table 4). Residual activity at pH 6.4 and pH 7.5 were found to be 41% and 5%, respectively. Among the amino acids, only the content of histidine was significantly decreased. When the 4 new proteases were treated with 0.02 M diisopropylfluorophosphate (DFP), all activities were lost with a half-time of about 20 minutes. This observation indicates that these are

Table 4. Amino acid composition of the protease from intestinal muscle

Amino acid	Native	Photooxidized	
Lysine	12.0	12.0	12.0
Histidine	8.4	7.2	4.8
Arginine	11.4	11.4	10.2
Aspartic acid	14.4	13.2	13.8
Threonine	12.0	11.4	10.8
Serine	10.8	10.8	10.8
Glutamic acid	18.0	16.8	17.4
Proline	15.6	15.6	13.2
Glycine	18.0	16.8	18.6
Alanine	16.2	15.0	16.2
Half-cystine	4.2	4.8	3.6
Valine	21.0	18.6	21.6
Methionine	4.8	4.8	3.6
Isoleucine	15.6	15.0	15.6
Leucine	15.6	16.2	15.0
Tyrosine	9.0	9.0	7.2
Phenylalanine	6.0	6.0	6.0
Tryptophan	(7.0)		
% Remaining activity	100	41	5

typical serine proteases. Modification of tyrosine residues with N-acetylimidazole left enzymatic activity unaffected. However, a contradictory result was obtained when tetranitromethane was used. Perhaps tyrosine residues are involved in enzyme activity of the proteases.

Amino acid analysis revealed that 4 half-cystine residues were contained in one molecule of protease. Sulfhydryl residues were not detected, though, when enzymatic titration with 5,5′-dithio-bis-(2-nitrobenzoate) (DTNB) in the presence of sodium dodecyl sulfate was carried out. Moreover, p-chloromercuribenzoate (pCMB) was without effect on the activity of the enzyme. Modifications of the amino groups using acetic anhydride according to TURANO et al. (1962), and the carboxyl groups were performed but did not alter protease activities (Tables 4 and 5).

Chemical modifications imply that serine, histidine and perhaps tyrosine residues are involved in the manifestation of protease activity. Usually, in the case of serine proteases, the serine-OH and histidine labile proton play essential roles in the mechanism of splitting the peptide bond. Furthermore, tyrosine or aspartate are thought to determine specificity. Alteration via chemical means does not always indicate the active center but often affects activity by changes in tertiary structure.

Our proteases show a relative substrate specificity for the pyridoxal enzyme group (Table 6). However, many enzymes of this group are degraded only in the apo form. Thus, we call our proteases group-specific for pyridoxal enzymes (GSP). It is of interest to note that apo forms of tyrosine transaminase and aspartate transaminase were not inactivated by the protease, although they are pyridoxal enzymes.

Table 5. Chemical modification of the protease from intestinal muscle

Residues	Modifying reagents	Enzyme activities
Serine	DFP	↓
Histidine	Photooxidation (Methylene blue)	↓
	TPCK	←
	TLCK	←
Tyrosine	Tetranitromethane	↓
	N-Acetylimidazole	←
Cysteine	PCMB	←
Carboxyl group	1-Ethyl-3 (3-dimethylaminopropyl) carbodiimide	←
Amino group	Acetic anhydride	←

Table 6. Substrate specificity of new intracellular proteases

Substrate	Source	Liver	Small intestine		Skeletal muscle
			muscle	mucosa	
Ornithine transaminase	Rat liver	100	100	100	100
Serine dehydratase	Rat liver	72	19	10	56
Homoserine deaminase	Rat liver	63	59	89	74
Phosphorylase	Rabbit muscle	27	22	70	108
δ-Amino levulinic acid synthetase	Rabbit reticulocyte	6	9	—	+
Aspartate transaminase (s, m)	Rat liver	=0	=0	—	=0
Tyrosine transaminase	Rat liver	=0	=0	—	=0
Malate dehydrogenase	Rat kidney	=0	=0	=0	=0
Glutamate dehydrogenase	Bovine liver	=0	=0	=0	=0
Glyceraldehyde-3-phosphate dehydrogenase	Rabbit muscle	7	2	—	3
Glucose-6-phosphate dehydrogenase	Rat liver	3	—	—	—
Arginase	Rat liver	=0	=0	=0	=0
Glutaminase	Rat kidney	=0	=0	=0	=0
Pyruvate kinase (M type)	Rabbit muscle	=0	=0	=0	=0
Ornithine transcarbamylase	Rat liver	=0	=0	19	=0
Xanthine oxidase	Rat liver	=0	=0	=0	=0
D-amino acid oxidase	Rat kidney	—	=0	—	—

Conformational Changes in Substrate Enzymes

General initiating mechanisms of intracellular enzyme degradation are discussed in this section.

Previous studies by SCHIMKE and coworkers have established that the rate of degradation of a given protein within a cell is not constant, but controllable by

dietary or hormonal conditions (SCHIMKE, 1969). Further, SCHIMKE (1970) and SEGAL (1969) have suggested that protein degradative processes do not constitute a uniform system. Another scheme is required to explain the variability in (relative) turnover rates of individual enzymes. SCHIMKE has obtained evidence that protein degradation, *in vivo*, may occur in a step-wise manner (ARIAS et al., 1969). Thus, proteases which degrade native enzymes probably catalyze a very limited proteolysis. The resulting large fragments are then completely broken down by other proteases. From this point of view, our proteases are very interesting as they act in the first step of pyridoxal enzyme degradation.

At least two conformations of each enzyme exist in the cell. Conversion from a non-susceptible to a susceptible form, via interactions with ligands, i.e. substrates, or coenzymes, phosphorylation or adenylation, may be the starting point in their catabolism. Then an initiating protease, such as our group-specific protease, act on the amenable structure by limited proteolysis. This further enables non-specific proteases to complete the task of total breakdown into amino acids. These processes are thought to occur in our model for intracellular enzyme degradation.

It was mentioned in the introduction that an increase in pyridoxal enzyme activities caused by B_6 administration was due to the ability of pyridoxal phosphate (co-enzyme) to protect these enzymes from degradation and not to an increase in the rate of biosynthesis. A relationship involving the conformational change between holo- and apo-structures of ornithine transaminase and their respective susceptibilites to the protease was studied. It is important to note that the addition of coenzyme (pyridoxal phosphate or pyridoxamine phosphate) to this enzyme exhibited a protective effect against inactivation by the protease. However, neither pyridoxine phosphate or pyridoxamine showed any effect. Likewise, pyridoxamine phosphate which is *not* a coenzyme of homoserine deaminase gave no defence. In the case of δ-aminolevulinic acid synthase, only pyridoxal phosphate allowed inactivation. The effect of vitamin B_6 derivatives on hydrolysis of N-acetyl-L-tyrosine ethyl ester was investigated. However, no change was observed. This suggests that the coenzyme does not act via protease inhibition. Furthermore, it implies that the coenzyme binding site must be vacant when the pyridoxal enzymes are subjected to inactivation. The extent of susceptibility of the substrate is dependent on the tertiary structure of the apo- and holoforms. A comparison of conformation to susceptibility is shown in Table 7.

MATSUZAWA et al. (1972) have reported that the apo form of ornithine transaminase is different from that of the pyridoxal phosphate one from protein structure studies using complement fixation tests and an increase in titratable $-$SH groups on dissociation of pyridoxal phosphate from the enzyme. However, SANADA et al. observed that the latter process is accompanied by dissociation of the enzyme from a tetrameric to a dimeric form. Both the tetra- and dimeric-structures of apo-ornithine transaminase (apo-I and apo-II, respectively) are susceptible to protease activity. Therefore, the degree of agrgegation is not as important as the vacancy of the coenzyme binding site.

Differences between holo-ornithine transaminase and apo-ornithine transaminase II were investigated with respect to the degree of aggregation, the varying amounts of exposed $-$SH groups, tyrosine and tryptophan residues. To confirm

Table 7. Relation between conformation and susceptibility to the protease

	Method	Holo-OTA	Apo-OTA II	Product A	Product B
Molecular conformation	10% TCA	Insoluble	Insoluble	Insoluble	Soluble
	Analysis by UCA, Sephadex, gel electrophoresis (SDS)	Tetramer	Dimer	Dimer	Oligopeptide
	Molecular weight	140000	67000	= 67000	< 10000
Reaction with anti-holo OTA	Ouchterlony method	Bindable	Bindable	Bindable	
Effect of PALP addition	Bindability	Bindable	Bindable	Bindable	
	Maximum absorption	420 mμ	420 mμ	420 mμ	
	430 mμ/280 mμ	1/10	1/10	1/10	
	K_m for PALP	10^{-6} M	10^{-6} M	10^{-6} M	
	Polymerization	Tetramer	Tetramer	Dimer	
	OTA activity	Exists	Appears	No activity	
Amino-terminal	Dansyl chloride method	Masked	Met. or Try.	Gly.	Masked
Exposed —SH	DTNB titration	1.5/Dimer	3/Dimer	3/Dimer	
Exposed — Try	Differential spectra	3/Dimer	7/Dimer	7/Dimer	
Exposed — Try	Differential spectra	5–6/Dimer	11–12/Dimer	9–13/Dimer	
Susceptibility to group specific protease		No	Yes	No	

the data of MATSUZAWA et al., the titration of —SH groups, using Ellman's method, in the isolated apo-ornithine transaminase II and in the pyridoxal phosphate enzyme was performed. The results indicated an increase in reactive —SH groups in the former.

By following the difference spectra of the apo- and holo-enzymes, the extent of exposure of tryptophan residues was studied. Also, difference spectra produced by varying concentrations of ethylene glycol were measured. The positive peaks appearing at 278 and 292 mμ were plotted against concentration of ethylene glycol. The molar extinction of tryptophan ($\Delta\varepsilon$) was estimated as 592 units per 40% ethylene glycol and the ratio of the increments of $\Delta\varepsilon$ for each enzyme form was calculated (Table 7). From the results of amino acid composition, the number of tryptophan residues in ornithine transaminase were calculated as 3.7 per monomer (protomer). It was deduced that the total content of tryptophan in the holo and apo III enzymes were 15 and 7, respectively. As exposed, tryptophan residues in the holo form were calculated as 5–6; one-third are apparently exposed on the surface of the molecule, whereas all seem to be exposed in the case of the apo II form (6–7 in total) (Table 7).

In conclusion, coenzymes of the pyridoxal enzymes showed a protective effect on the apo forms against inactivation by proteases. Furthermore, dissociation of ornithine transaminase caused an increase in the number of sulfhydryl groups and tryptophan residues. These findings suggest that the susceptibility

of the substrate to group-specific proteases is dependent upon the tertiary structures of the apo- and holo-enzymes. These changes in protein structure probably increase the accessibility of the bond susceptible to group-specific proteases.

Mechanisms of Limited Proteolysis

Our hypothesis states that group-specific proteases catalyze the degradation of a native enzyme via limited proteolysis.[1] The products are subsequently broken down in a step-wise manner by other proteases. Evidence is accumulating as to the physiological importance of limited proteolysis—for instance, proinsulin → insulin, zymogen to chymotrypsin, the kinin and plasmin systems and so on. However, in most cases, the mechanism is not clear.

The facts that the purified proteases show relative specificity for pyridoxal enzyme groups and their activities are very low with respect to the holo forms suggest that these proteases attack at or near the coenzyme binding site (Fig. 4).

As shown in Fig. 4 a decrease in transaminase activity was seen to accompany an increase in the acid-soluble, ninhydrin reactive material (product B). On the other hand, there was no detectable decrease in the total amount of protein precipitated by 5% TCA (product A) even after transaminase activity was completely lost. Paper chromatography indicated the release of only a few peptides and a small quantity of ninhydrin reactive substance. These results suggest that the inactivating reaction is associated with a very limited proteolysis.

The products A and B were analyzed by Sephadex column filtration and acrylamide gel electrophoresis. The indication was that product A is similar in molecular weight to the native enzyme.

Using the dansyl chloride method, the amino terminals of apo-ornithine transaminase II and of products A and B were analyzed. Methionine or tryptophan, the original terminal, which is masked was replaced by glycine in product A (Table 7). In ouchterlony double-diffusion, apo-ornithine transaminase II, holo-enzyme, and product A exhibited a line of complete fusion with the antibody against holoenzyme.

The binding ability of pyridoxal phosphate per dimer unit, the coefficient of spectrum (420 mμ/280 mμ) and K_m values were exactly the same between apo-II and product A. However, enzyme activity and the capacity to polymerize on addition of pyridoxal phosphate were lost completely in procut A (Fig. 8, p. 96).

Judging from the binding affinity with coenzyme and antibody and exposure of $-SH$ groups, tyrosine and tryptophan residues, the tertiary structure of product A, compared with the native apo-ornithine transaminase, is not changed by limited proteolysis. This could be the reason why further degradation of product A does not proceed via the same group-specific protease (Fig. 4). Following denaturation of apo-ornithine transaminase II with 8 M urea, a random proteolysis by our proteases was observed (Fig. 5).

The important feature involved in susceptibility, substrate specificity and the limited proteolysis is the tertiary structure of the substrate protein, particularly, that in the region of the coenzyme binding site.

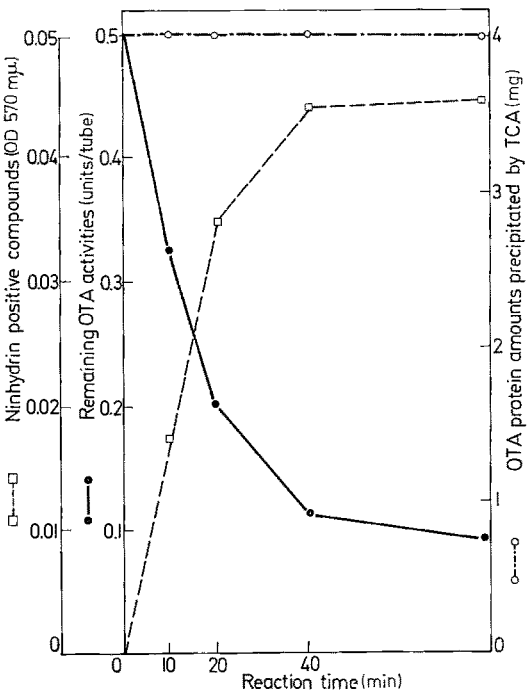

Fig. 4. Relationship between inactivation of ornithine transaminase and breakdown of its protein by the group-specific protease from sekletal muscle. Each reaction mixture contained 4 mg of apo-ornithine transaminase, 6.3 units of group-specific protease and 200 μmoles potassium phosphate buffer, pH 8.5, in a final volume of 1.0 ml. After incubation for 0, 10, 20, 40 and 80 minutes remaining activity of ornithine transaminase, TCA precipitable protein and the amounts of ninhydrin positive substances in the TCA soluble fraction were determined. Following illustrations were used: the remaining ornithine transaminase activity (▪—▪), ninhydrin positive substances in the TCA soluble fraction (□---□), TCA precipitable protein (○—○)

Our hypothesis of the mechanism of limited proteolysis of apo-ornithine transaminase by our group-specific proteases is depicted in Fig. 6.

Matsuda and Fischer studied the degradative kinetics of muscle phosphorylase a and b by the GSP from muscle and the properties of the products (MATSUDA and FISCHER, 1974). These fragments had a strong tendency to aggregate in SDS polyacrylamide gel electrophoresis and precipitate in buffer. After proteolysis, therefore, reduction and alkylation were carried out (Fig. 7).

Holo phosphorylase b, after incubation with protease, produced two main components of molecular weight 63000 and 30000 and smaller amounts of 93000 and 85000 fragments. In the case of holo-phosphorylase a, only the 63000 and 30000 components were seen. Unlike b, though, no further degradation was observed after 10 hours of incubation at 30° C and pH 8.0.

The mixtures, after reduction with NaBH and alkylation, were separated on a hydroxyapatite column. The reduced pyridoxal fragments were identified by their fluorescent properties. Fragment A contained the reduced pyridoxal phos-

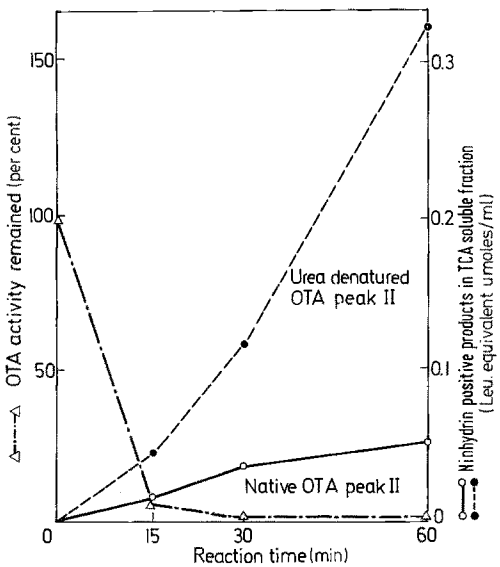

Fig. 5. Proteolysis of urea-denatured apo-ornithine transaminase II. Denatured enzyme was obtained by treatment with 8 M urea and subsequent removal of urea by Sephadex column. Reaction mixtures containing 30 units of muscle layer protease of small intestine, 1.45 mg of apo-ornithine transaminase II or the denatured enzyme and 37.5 µmoles of potassium phosphate buffer, pH 8.0 in a final volume of 0.75 ml were incubated at 37° C for the times indicated, after which an aliquot was removed for assay of ornithine transaminase activity

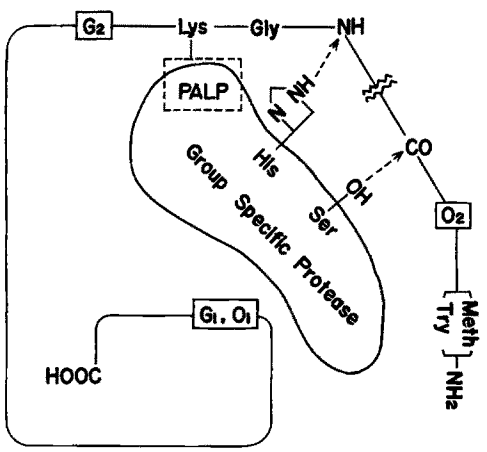

Fig. 6. Hypothesis of limited proteolysis of ornithine transaminase by group-specific protease. Since N-terminal of OTA is marked, Meth or Try as the N-terminal is not sure yet

phate and an amino-terminal of isoleucine. Fragment B was not fluorescent and its amino-terminal was blocked. As the terminal of phosphorylase a (N-acetyl serine) is also blocked, B appears to contain the complete amino-terminal of phosphorylase a. When ^{32}P labelled phosphorylase a was used as substrate, Fragment B was found to be radioactive. Since FISCHER et al. (1972) have shown

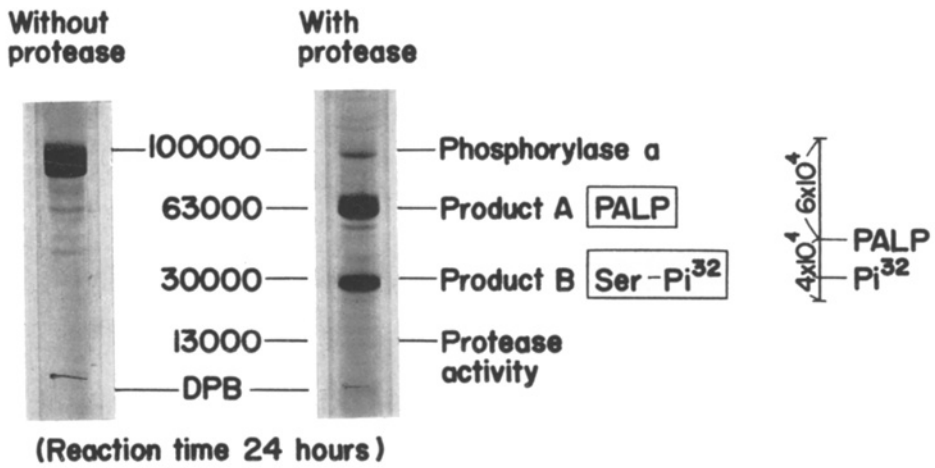

Fig. 7. Degradation of holo-phosphorylase a by group-specific protease. The reaction mixture contained 0.2 ml of 0.2 M Tris-HCl, pH 7.5, 1 mg of ^{32}P-labelled holo-phosphorylase a, 50 µl of group-specific protease and 1 drop of toluene in a final volume of 0.5 ml. It was incubated at room temperature without SDS for 0, 24 and 48 hrs. Subsequently, acrylamide disc gel electrophoresis with SDS was carried out

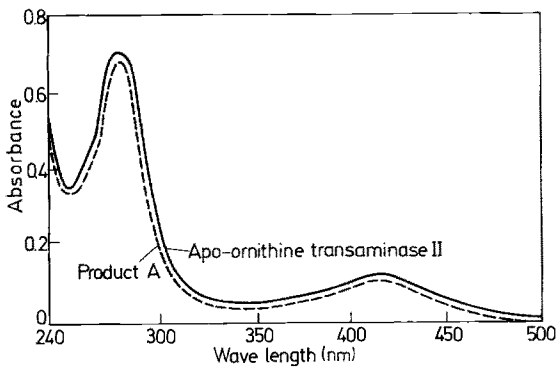

Fig. 8. Absorption spectra of apo-ornithine transaminase II and its inactive product formed by proteolysis after incubation with pyridoxal phosphate. Apoenzyme II or its inactive product (2 mg) was incubated with 10^{-5} M of pyridoxal phosphate for 30 min at 37° C, and then put onto a column of Sephadex G-25 equilibrated with 0.05 M potassium phosphate buffer, pH 7.5. Protein fractions were collected and used for measurements of absorption spectra

the phosphorylated site is 14 residues from the N-terminal in phosphorylase a, this experiment shows clearly that B forms the amino-terminal of the enzyme.

Apo-phosphorylase b is degraded more easily than the holo form but via an identical process. However, as the apo form is unattainable by itself, it is very difficult to demonstrate clear-cut data. It is possible to assume that the splitting position between Fragment B (36000) and A (60000) occurs in the vicinity of the pyridoxal phosphate binding site in view of FISCHER's studies (FISCHER et al., 1963; FISCHER et al., 1958).

The results obtained from ornithine transaminase and phosphorylases as substrates imply that the enzyme is hydrolyzed at a specific point by our proteases, representing the initiation of intracellular enzyme degradation.

Location of Group-Specific Proteases

Activities which inactivate apo-ornithine transaminase could be detected in various organs. Using sonicated preparations of tissue homogenates, these activities were assayed (Table 8). As the amount of inhibitor of protease may differ from one organ to another, the data may not represent the true protease content.

The intracellular location of rat liver protease was studied in subcellular fractions, separated by the standard sucrose method. All the reaction mixtures contained about the same concentration of protein (Fig. 9). All fractions were sonicated. In nuclear, microsomal, light mitochondrial and supernatant fractions, no inactivating activities of apoornithine transaminase were seen. Considerable activity was detected in heavy mitochondria. Thus the protease is probably contained in the pellet of heavy mitochondrial sonicate (membrane). The inhibitor may exist in the supernatant.

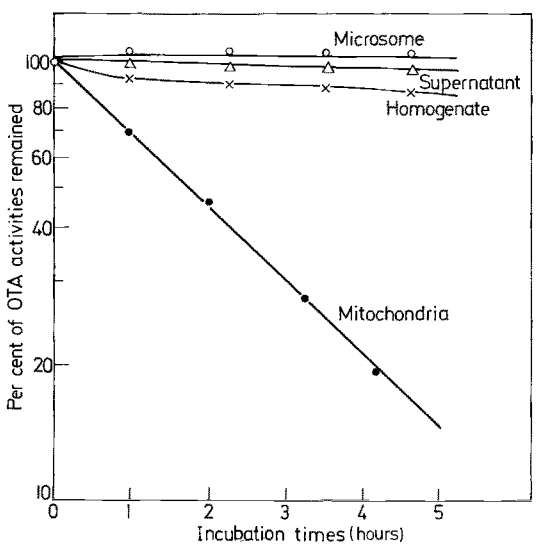

Fig. 9. Decrease of OTA acitivities in subcellular fractions of liver. Subcellular fractionation was performed by the method of Hogeboom et al. Ten percent suspensions of each fraction in 0.05 M potassium phosphate buffer (pH 7.5) were sonicated. 0.2 ml of the resultant mixture, 50 units of apo-ornithine transaminase and 0.2 M potassium phosphate buffer (pH 8.5) (final volume of 0.3 ml) were incubated for 1, 2, 3 and 4 hours at 37° C. The remaining ornithine transaminase activities were determined. Following illustrations were used: Homogenate (×—×, Mitochondria ▪—▪), Microsome (○—○), and Supernatant (△—△)

The amount of protease in various types of muscle differs greatly, activity being higher in red muscle than in white, whereas none could be detected in heart.

Table 8. Distribution of group-specific protease in various organs.

Rats weighing 250 to 300 g were maintained on an ordinary laboratory chow before experiments. Crude preparations were obtained as described in Section 5, and activities are expressed as the means of 4 to 5 rats.

Organs	Activity units/mg protein
Skeletal muscle	0.3
Digestive tract	
Muscle layer of stomach	0.02
Muscle layer of duodenum	0.7
Muscle layer of jejunum	0.6
Muscle layer of ileum	0.6
Muscle layer of colon	0.1
Mucosa layer of duodenum	0.07
Heart	=0
Liver	0.02

In vivo Regulation of Group-Specific Protease Activity

The manifestation of protease activity is controlled by the ratio of the amount of protease to its inhibitor. It should be recalled that a marked increase in total activity was observed during some steps in the purification of proteases from various organs. This observation suggests the existence of a protein-like inhibitor, located in the same subcellular fraction as the protease. HOLZER et al. have reported on similar kinds of proteases and inhibitors in yeast (FERGUSON et al., 1973).

HATA and coworkers found three proteases, A, B and C, in yeast. Furthermore, KATSUNUMA and HOLZER showed that proteases A and B (A = inactivase I and B = inactivase II) but not C, had the ability to inactivate tryptophan synthase by proteolysis. Inactivase I (A) also inactivates threonine dehydratase, whereas inactivates II (B) affects aspartate aminotransferase, as well. However, neither have any effect on the three non-pyridoxal enzymes, alcohol dehydrogenase, hexokinase and glucose-6-phosphate dehydrogenase (KATSUNUMA et al., 1972).

FERGUSON et al. have found and purified two protein inhibitors (I and II) with molecular weights about 10000. The specificity of inhibitor II was investigated. Proteases, such as trypsin, chymotrypsin and pronase from sources other than yeast, were not inhibited. Of the three yeast proteinases A, B and C, only B was affected. The experiments shown in Fig. 10 imply that the inhibitory mechnism is due to the binding of inhibitor to protease (FERGUSON et al., 1973; BETZ and HOLZER, in press).

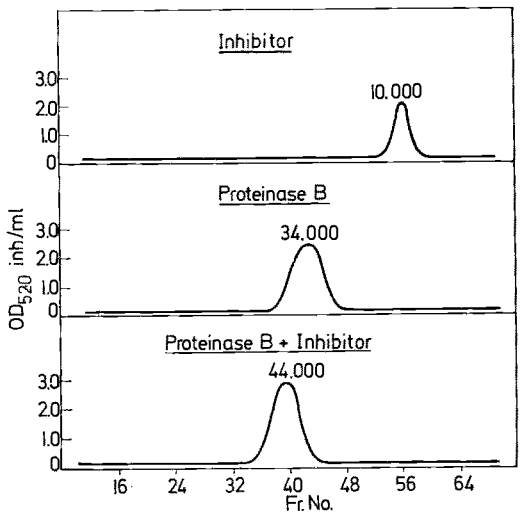

Fig. 10. Filtration of yeast proteinase B and inhibitor on Sephadex G-75 (BETZ and HOLZER)

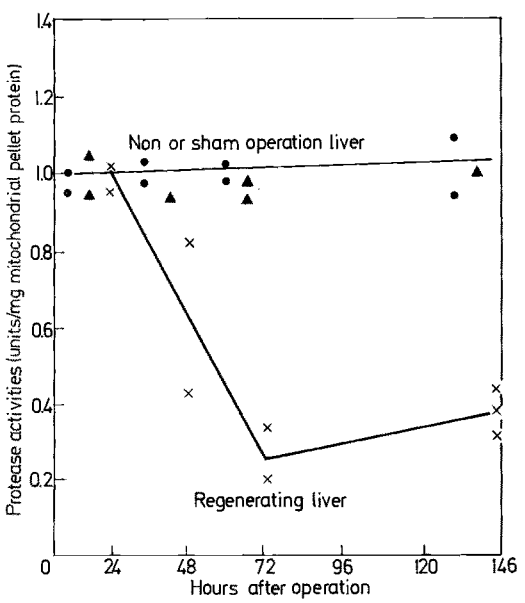

Fig. 11. Changes of group-specific protease activity in regenerating liver. At hours indicated after operation, 10% mitochondrial suspensions in 0.05 M potassium phosphate buffer, pH 7.5 (w/v) were prepared and sonicated. The resultant precipitates were collected by centrifugation at 10000× g and were resuspended in 0.5 M potassium phosphate buffer, pH 8.5 to 10% suspension. The protease activities were assayed according to the method of KATUNUMA et al.

It is possible that this regulation of protease activities by variation in inhibitor concentrations also exists in animal systems. It is well known that many pyridoxal enzyme activities, in liver, change in response to a variety of physiological condi-

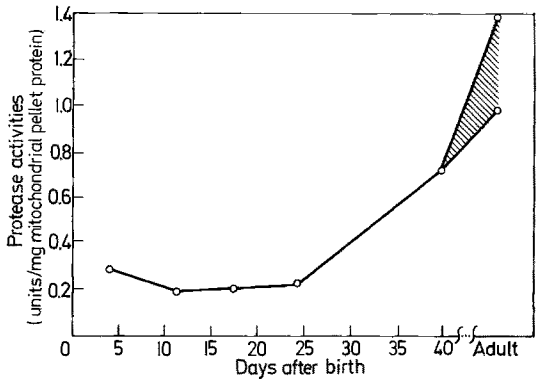

Fig. 12. Changes of the liver protease during development. In succession of days after birth, livers of neonatal rats were collected and divided into two groups. The precipitates of mitochondrial suspension in 0.5 M potassium phosphate buffer (pH 8.5) were obtained by the same method as in Fig. 11. The protease activities were assayed according to the method of KATUNUMA et al.

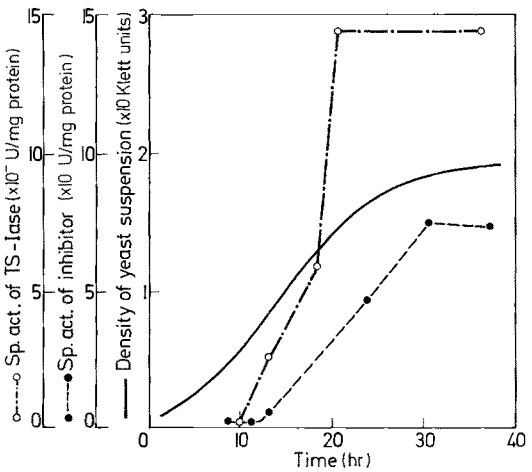

Fig. 13. Dependence of the tryptophan synthase inactivating activity and its inhibitor on the growth time (T. KATSUNUMA et al.)

tions, e.g. dietary, hormonal or neurological (KNOX and GREENGARD, 1965). Regenerated and neonatal liver show very low protease activities (Fig. 11). Two days after partial hepatectomy, a marked decrease in protease activity was observed. The change in activity during development was followed (Fig. 12). In the neonatal period (within two weeks) activity was initially at a very low level but increased rapidly to that of the adult. Assays of activity utilized the pellet, corrected after centrifugation of sonicated mitochondria. Thus the inhibitor was already removed.

There is speculation as to the relationship between the appearance of protease activity and the cell division cycle. This was studied in yeast by Holzer's group.

Table 9. Effect of diet on the group-sepcific protease activities in skeletal muscle

Diet	Group-specific protease activity (units/mg)
0% Casein diet	0.143 ± 0.020
15% Casein diet	0.067 ± 0.004
60% Casein diet	0.079 ± 0.013
Starvation	0.224 ± 0.030

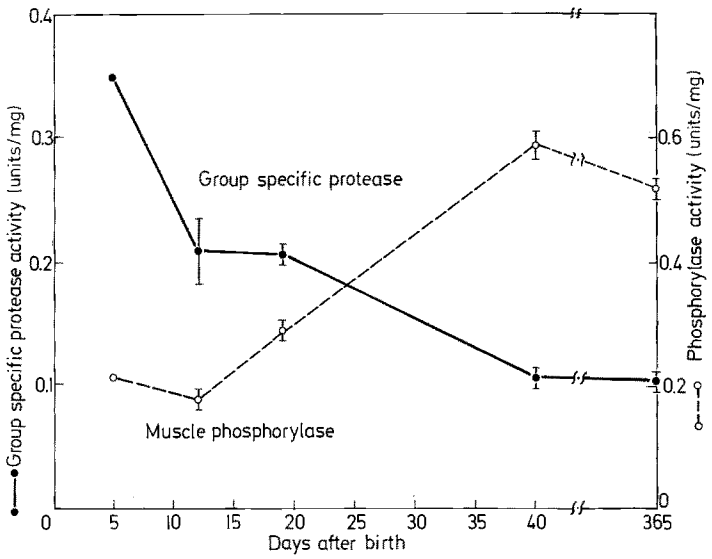

Fig. 14. Changes of activities of the muscle protease and muscle phosphorylase during development in the gastrocnemius and soleus muscle

In the lag and logarithmic phases, the level of tryptophan synthase inactivating activity was low but rose to a high level in the stationary phase. In connection with this, the proteinous inhibitor of the inactivating enzyme was studied. It was interesting to see their simultaneous appearance during the growth of yeast (FERGUSON et al., 1973) (Fig. 13).

The level of protease activity was observed under a variety of dietary conditions. Data in Table 9 show the results. The enhancement of GSP activity in response to starvation or a non-protein diet is consistent with the normal increase in the amount of amino acids released and transported into the blood under these conditions.

The change of protease activity in gastrocnemius muscle during development is shown in Fig. 14. In the neonatal period, activity is high gradually decreasing to the adult level. A reciprocal type of behavior was observed by phosphorylase in the same muscle. This gives rise to speculation as to the role played by the protease in controlling the level of phosphorylase in muscle.

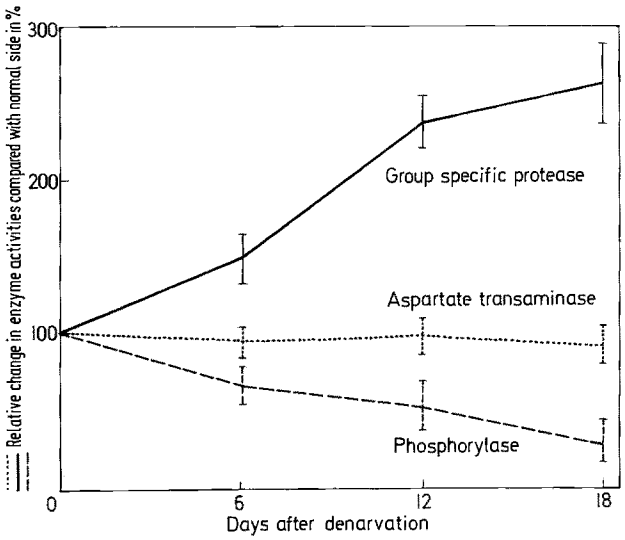

Fig. 15. Changes of the group-specific protease activities and muscle phosphorylase activities in gastrocnemius and soleus muscle by denervation of ischiadicus. The animals, male Wistar strain rats weighing 200 g, were maintained on laboratory chow. Rats were denervated by amputation of the ischiadicus nerve at the position of the foramen infrapiriforme of one side only. 6, 12 and 18 days after the operation, the denervated and innervated sites of the gastrocnemius muscle were prepared for the enzyme assay. Relative changes were compared with innervated side as a 100%

Another role is indicated by the observation that there is a significant increase in the level of protease during atrophy of the gastrocnemius muscle. This was shown by an experiment in which the ischiadicus nerve was amputated only on one side of the body. The level of GSP on the amputated side was three times higher than that on the normal side (Fig. 15). This result agrees with the data showing that amputation of the ischiadicus nerve leads to a marked decrease in the level of muscle phosphorylase activity.

The effects of administering various hormones to the group-specific protease of rat liver were studied. Sufficient quantities of hormones to induce elevated activity in the marked enzymes were used. These enzymes were liver tyrosine transaminase for hydrocortisone (LIN and KNOX, 1957) and insulin (HOLTEN and KENNEY, 1967), liver serine dehydratase for glucagon (PERAINO and PITOT, 1964), kidney ornithine aminotransferase for estrogen (SANADA et al., 1970). A single injection of hydrocortisone (hydrocortisone acetate 2 mg/100 g body weight) or frequent injections of triamcinolone twice daily for 4 days via an intraperitoneal method, left protease activity unaffected. So also did treatment with insulin (4 I.U./100 g body weight every 8 hours for five times), growth hormone (1 unit/100 g body weight via a single injection), glucagon (injection every three hours, eight times) and estrogen (250 μg/100 g body weight, daily for 4 days). No response via movement of the various groupspecific proteases to administration of various hormones was observed. This suggests that protease activity does not participate in the instant regulation of pyridoxal enzyme levels.

Conclusion — General Process of Intracellular Enzyme Degradation

Our hypothesis of the general process of intracellular enzyme degradation is shown in Fig. 16 as a conclusion (KATUNUMA et al., 1974; KOMINAMI et al., 1974; BANNO et al., 1974).

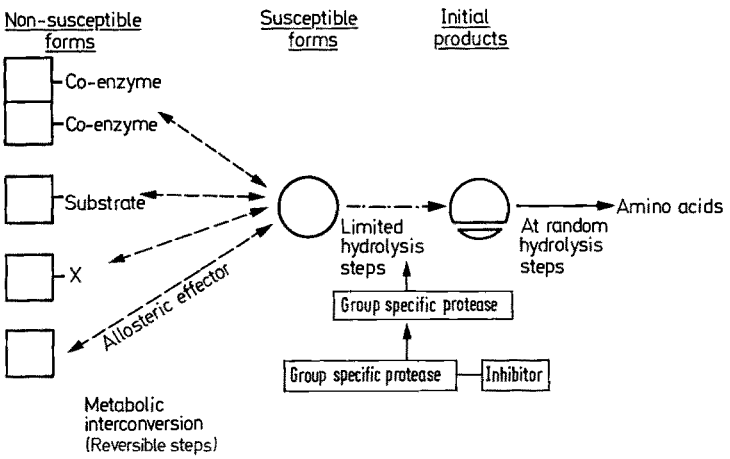

Fig. 16. Hypothesis of general process of intracellular enzyme degradation

(The studies mentioned in this paper were carried out by NOBUHIKO KATUNUMA, EIKI KOMINAMI, KEIKO KOBAYASHI, YOSHITAKA HAMAGUCHI, YOSHIKO BANNO, KENJI CHICHIBU, TSUNEHIKO KATSUNUMA, E. G. AFTING and TAIICH SHIOTANI.)

References

ARIAS, I. M., DOYLE, D., SCHIMKE, R. T.: J. Biol. Chem., 244, 3303 (1969).

BANNO, Y., SHIOTANI, T., TOWATARI, T., KOSHIKAWA, D., KATSUNUMA, N., AFTING, E. G., KATUNUMA, N.: Eur. J. Biochem., in press, 1974.

BARRETT, A. J.: In Lysosome in Biology and Pathology (DINGLE, J. T. and FELL, H. B., eds) Vol. 2, pp. 245–312. Amsterdam: North-Holland 1969.

BETZ, H., HINZE, H., HOLZER, H.: J. Biol. Chem. **249**, 4515 (1974).

FERGUSON, A. R., KATSUNUMA, T., BETZ, H., HOLZER, H.: Eur. J. Biochem., **32**, 444 (1973).

FISCHER, E. H., COHEN, P., FOSSET, M., MUIR, L. W., SAARI, J. C.: In Metabolic Interconversion of Enzymes (WIELAND, O., HELMREICH, E., HOLZER, H., eds.), pp. 11, Berlin-Heidelberg-New York: Springer 1972.

FISCHER, E. H., FORREY, A. W., HEDRICK, J. L., HUGHES, R. O., KENT, A. B., KREBS, E. G.: In Chemical and Biological Aspects of Pyridoxal Catalysis, (SNELL, E. E., FASELLA, P. M., BRAUNSTEIN, A., FANELLI, A. R.) pp. 543–562, Oxford: Pergamon Press 1963.

FISCHER, E. H., KENT, A. B., SNEIDER, E. R., KREBS, E. G.: J. Am. Chem. Soc. **80**, 2906 (1958).

GOODWIN, Y. W., MORTON, R. A.: Biochem. J. **40**, 628 (1946).

GREENGARD, O., GORDON, M.: J. Biol. Chem. **238**, 3708 (1963).

HATA, T., HAYASHI, R., DOI, E.: Agric. Biol. Chem. **31**, 150 (1967).

HOLTE, D., WICKS, W. D., KENNEY, F. T.: J. Biol. Chem. **242**, 1053 (1967).
HOLTEN, D., KENNEY, F. T.: J. Biol. Chem. **242**, 4372 (1967).
KATSUNUMA, T., SCHOTT, S. E., HOLZER, H.: Eur. J. Biochem. **27**, 520 (1972).
KATUNUMA, N.: In Current Topics in Cellular Regulation (HORECKER, B. L., STADTMAN, E. R., eds.) Vol. 7, pp. 175–203. New York-London: Academic Press 1973a.
KATUNUMA, N.: In Metabolic Interconversion of Enzymes (FISCHER, E. H. et al., eds.) pp. 313–324. Berlin-Heidelberg-New York: Springer 1973b.
KATUNUMA, N., KATSUNUMA, T., KOMINAMI, R., SUZUKI, K. lHAMAGUCHI, Y., CHICHIBU, K., KOBAYASHI, K., SHIOTANI, T.: In Advances in Enzyme Regulation, (WEBER, G., ed.) Vol. 11, pp. 37–51. Oxford-New York: Pergamon Press 1973.
KOMINAMI, E., BANNO, Y., CHICHIBU, K., SHIOTANI, T., HAMAGUCHI, Y., KATUNUMA, N.: Eur. J. Biochem., in press, 1974.
KHAIRALLAH, E. A., PITOT, H. C.: In Symposium on Pyridoxal Enzymes (YAMADA, K., KATUNUMA, N., WADA, H., eds.) pp. 159–164. Tokyo: Maruzen 1968.
KNOX, E. W., GREENGARD, O.: In Advances in Enzyme Regulation (WEBER, G., ed.) Vol. 3, pp. 247–313. Oxford-New York: Pergamon Press 1965.
KATUNUMA, N., KOMINAMI, E., KOBAYASHI, K., BANNO, Y., SUZUKI, K., CHICHIBU, K., HAMAGUCHI, Y., KATSUNUMA, T.: Eur. J. Biochem., in press, 1974.
KOMINAMI, E., KOBAYASHI, K., KOMINAMI, S., KATUNUMA, N.: J. Biol. Chem., **247**, 6848 (1972).
LIN, E. C. C., KNOX, E. W.: Biochim. Biophys. Acta **26**, 85 (1957).
MATSUDA, Y., FISCHER, E. H.: In Intracellular Protein Turnover (SCHIMKE, R. T. and KATUNUMA, N., eds.) New York: Academic Press, in press, 1974.
MATSUZAWA, T., NISHIYAMA, M.: J. Biochem. **73**, 481 (1972).
PERAINO, C., PITOT, H. C.: J. Biol. Chem. **239**, 4308 (1964).
SANADA, Y., SUEMORI, I., KATUNUMA, N.: Biochim. Biophys. Acta **220**, 42, 1970.
SEGAL, H. L., MATSUZAWA, T., HAIDER, M., ABRAHAM, G. J.: Biochem. Biophys. Res. Commun. **36**, 764 (1969).
SCHIMKE, R. T.: In Current Topics in Cellular Regulations (HORECKER, B. L., and STADTMAN, E. R., eds.) Vol. 1, pp. 77–120. New York: Academic Press 1969.
SCHIMKE, R. T.: In Mammalian Protein Metabolisms (MUNRO, H. N., ed.) Vol. 4, pp. 178–223. New York: Academic Press 1970.
TURANO, C., GIARTOSIO, VECCHINI, P.: Arch. Biochem. **40**, 245 (1962).

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Lactose Biosynthesis

KEITH BREW and ROBERT L. HILL*

Contents

I. Introduction

The disaccharide lactose appears to be synthesized only in mammals, although it has been reported to occur in small amounts in a few plants (KUHN and LÖW, 1949; VENKATARAMAN and REITHEL, 1958). It is the major free carbohydrate of milk and accounts for about 3 to 7 per cent of the weight of milk from those species examined (LING et al., 1961; JENNESS, 1970). It is absent, or present in

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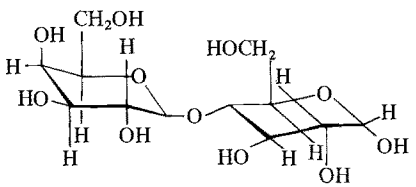
very low amounts, in the milk of some sea mammals including the California sea lion (PILSON and KELLY, 1962) and the Northern fur seal (SCHMIDT et al., 1971). Its synthesis is confined normally to adult females and occurs only in the mammary gland during lactation. Because of these special features, lactose synthesis is one of the few metabolic processes confined uniquely to the female mammal and to a single tissue within this class of higher vertebrates.

It is the purpose of this review to consider our present knowledge of lactose biosynthesis. Emphasis will be placed on studies over the past decade which have given considerable insight into several aspects of the enzyme responsible for the ultimate step in lactose biosynthesis, lactose synthetase (E.C. 2.4.1.22). The two components of the synthetase, α-lactalbumin and the galactosyl transferase, will be considered from several aspects, including their chemical and physical properties, and the unique type of control mechanism provided by the interaction of the two proteins. The evolutionary origins of the synthetase, its hormonal control, and the subcellular organization and control of the synthetase will also be evaluated.

Earlier reviews of lactose synthetase and lactose biosynthesis have been written by HILL et al. (1968), BREW (1970) and EBNER (1970, 1973). Reviews by MCKENZIE (1967) and LYSTER (1972) and the volumes by MCKENZIE (1970a, b) and KON and COWIE (1961a, b) may also be useful references for readers interested in milk and its constituents.

II. Historical Review

Lactose was recognized as a constituent of milk in the 17th century but it was not until 1927 that it was shown to have the following structure (LELOIR and CARDINI, 1961).[1]



The first report that glucose could serve as a precursor of lactose was made in 1936 (GRANT, 1936). Subsequent studies by several workers in the 1950's demonstrated through the use of perfusion studies of the mammary gland with ^{14}C-labeled glucose, that more than 80% of the carbon of lactose was derived from blood glucose (REITHEL et al., 1952; REISS and BARRY, 1953; DIMANT et al., 1953; KLEIBER et al., 1955; BAXTER et al., 1956). The results obtained by WOOD and coworkers[2] from elegantly designed perfusion studies of udders from lactating cows, clearly showed that the percursors for the glucose and galactose moeities of lactose were not the same (WOOD et al., 1957a, 1957b, 1958). These workers recognized the importance of nucleotide sugars in formation of glycosidic

[1] The review by CARDINI and LELOIR (1960) may be consulted for a more detailed discussion of lactose synthesis prior to 1960.

[2] For a superb personal account of these pioneering studies, see WOOD (1972).

bonds (CAPUTTO et al., 1950) and suggested that the mammary gland may contain an enzyme system which synthesizes lactose from UDP-galactose and glucose. This proposal accounted for all of the experimental observations in their studies, in contrast to other biosynthetic schemes put forth about the same time (GANDER et al., 1956). WATKINS and HASSID (1962) later confirmed the suggestion of WOOD and coworkers by finding that lactating bovine and guinea pig mammary glands catalyzed lactose synthesis according to the following reaction.

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP} \qquad (1)$$

Although the enzyme was found to be associated with a particulate fraction of gland homogenates, BABAD and HASSID (1964, 1966) found it in a soluble form in unpasteurized bovine milk.

Attempts to extensively purify the soluble synthetase from bovine milk (BABAD and HASSID, 1966) were unsuccessful, presumably as the result of its apparent lability during purification. BRODBECK and EBNER (1966a, b) were to show, however, that the lack of success in purification resided in the fact that lactose synthetase is composed of two protein components. They observed that the bovine milk enzyme could be separated by gel filtration into two protein fractions, designated as the A and the B proteins. Neither protein had lactose synthetase activity, but when the two were combined, synthetase activity was regained. After this key observation, further studies by the same workers demonstrated that the B protein was identical to bovine α-lactalbumin (EBNER et al., 1966; BRODBECK et al., 1967), a protein which was first identified 30 years earlier on ultracentrifugal analysis of whey (PEDERSEN, 1936; SVEDBERG, 1937; SVEDBERG and PEDERSEN, 1940)[3]. After its initial discovery, α-lactalbumin was subsequently crystallized (SØRENSEN and SØRENSEN, 1939; GORDON and SEMMET, 1953) and many of its physical and chemical properties were described (MCKENZIE, 1967), but its biological function in lactose synthesis went unrecognized for 30 years.

BREW and CAMPBELL (1967a) noted considerable similarity between the physical and chemical properties of bovine and guinea pig α-lactalbumins and chicken egg-white lysozyme. This observation stimulated BREW et al. (1967a, b) to examine the amino acid sequence of bovine α-lactalbumin and compare it with the known sequence of lysozyme (CANFIELD, 1963; CANFIELD and LIU, 1965; JOLLÉS, 1967). A striking similarity in the sequences of the two proteins was evident even from partial amino acid sequence studies of α-lactalbumin. Because of this structural similarity, these workers concluded that the structural genes for α-lactalbumin and lysozyme were derived from a common ancestor, and proposed that during evolution, as mammals emerged, a structural gene which controlled the sequence of a lysozyme-like enzyme duplicated, and the duplicate genes evolved independently to give rise to the genes for α-lactalbumin and lysozyme. Complete sequence analysis of bovine α-lactalbumin (BREW and HILL, 1970; BREW et al., 1970; VANAMAN et al., 1970) and subsequently guinea pig (BREW, 1972) and human (FINDLAY and BREW, 1972) α-lactalbumins as well as human lysozyme (CANFIELD et al., 1971), established beyond doubt the validity of the proposed evolutionary relationships.

[3] GORDON (1971) has pointed out that α-lactalbumin was first recognized by SEBELEIN in 1885 and probably crystallized by WICHMANN in 1889.

The nature of the A protein of lactose synthetase was first established by BREW et al. (1968) who found it to be a galactosyl transferase, which in the absence of α-lactalbumin catalyzed the following reaction.

$$\text{UDP-galactose} + \text{N-acetylglucosamine} \rightarrow \text{N-acetyllactosamine} + \text{UDP} \qquad (2)$$

This transferase had been described earlier and was known to be widely distributed in animal tissues including the mammary gland (MCGUIRE et al., 1965). Its major function in most tissues is in glycoprotein biosynthesis and it catalyzes the transfer of galactose to a growing oligosaccharide side chain with N-acetylglucosamine at the non-reducing end of the chain (HILL et al., 1968). BREW et al. (1967) further noted that the transferase was virtually devoid of lactose synthetase activity but that in the presence of α-lactalbumin, lactose synthesis was obtained and the rate of synthesis was dependent on the concentration of α-lactalbumin. In addition, α-lactalbumin inhibited or stimulated the rate of synthesis of N-acetyllactosamine. Lactose synthesis was also observed by the galactosyl transferase from rat liver in the presence of bovine α-lactalbumin and substrates. These observations clearly showed that α-lactalbumin served to regulate lactose synthesis and it was suggested that because the specificity of lactose synthetase resides partly in α-lactalbumin, it may be termed a specifier protein. It was also evident that lactose synthesis occurs normally only in the mammary gland, because α-lactalbumin is synthesized only in this organ. The transferase has now been purified and many aspects of its mechanism of action including its interaction with α-lactalbumin have been established.

III. The Galactosyl Transferase

A. Purification

The galactosyl transferase (UDP galactose-N-acetylglucosamine-β4-galactosyl transferase) is found in particulate ("microsomal") fractions of the mammary gland (WATKINS and HASSID, 1962; BRODBECK and EBNER, 1966a) and other tissues (MCGUIRE et al., 1965; DEN et al., 1970). There is general agreement that it is bound to the membranes of the Golgi apparatus (MORRÉ et al., 1972; SCHACHTER et al., 1970) and it has often been used as a marker enzyme for this organelle in liver (FLEISCHER and FLEISCHER, 1970) and mammary tissue (MORRÉ, 1971). Although the enzyme can be extracted from particulate fractions of mammary tissue with the aid of detergents and purified to homogeneity[4] the most convenient source of the enzyme is milk, where it exists in soluble form, as noted first for lactose synthetase (BABAD and HASSID, 1966). Early attempts to obtain pure transferase from milk employed conventional methods of purification (BREW et al., 1968; PALMITER, 1969b; FITZGERALD et al., 1970) but it is now clear that the transferase obtained by these methods was impure and the yields were low. In addition, the apparent molecular weights (70000–75000) reported for some of these preparations (FITZGERALD et al., 1970) were subsequently shown to be incorrect where pure enzyme was obtained. The first successful purification of the enzyme to constant specific activity was achieved by affinity chromatography

[4] R. L. HILL and H. HILL, unpublished observations, 1974.

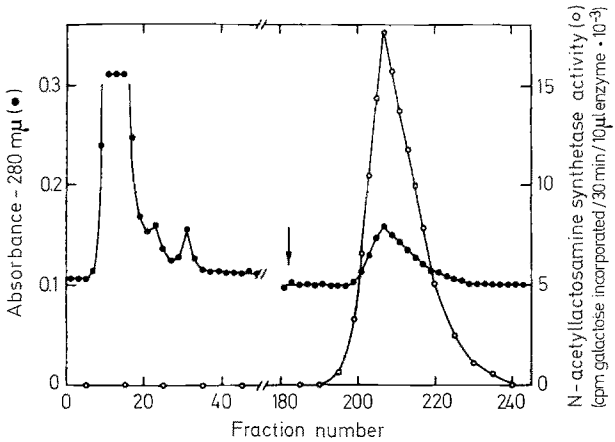


Fig. 1. Purification of the galactosyl transferase of lactose synthetase by affinity chromatography on α-lactalbumin-agarose. Partially purified enzyme was applied to the column in buffer at pH 7.4 containing glucose. Glucose was removed from the elution buffer at the fraction indicated by the arrow. Protein concentration (•); galactosyl transferase activity (○). From TRAYER and HILL (1971)

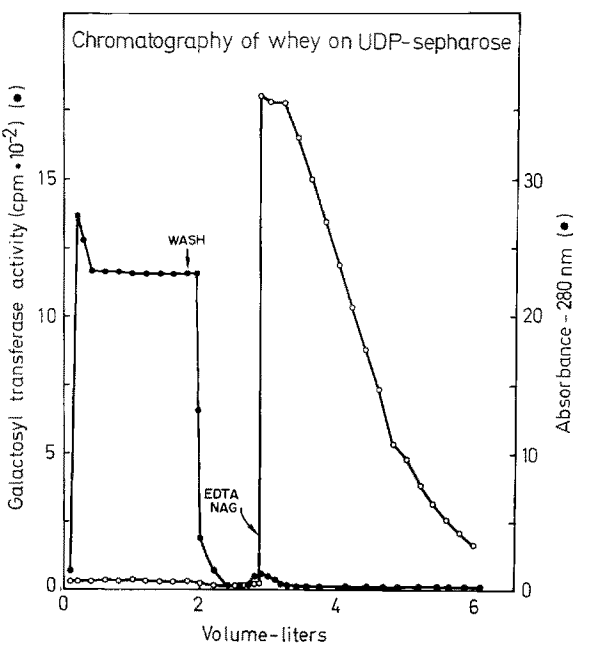


Fig. 2. Purification of the galactosyl transferase of lactose synthetase from bovine whey by affinity chromatography on UDP-hexanolamine-agarose. Whey was applied in buffer, pH 7.4, containing Mn^{2+}. At A, the column was washed with the same buffer and at B, with buffer, pH 7.4, containing EDTA and N-acetylglucosamine. Protein concentration (•); transferase activity (○). From BARKER, et al. (1972)

with columns of bovine α-lactalbumin attached to cyanogen bromide activated agarose (TRAYER et al., 1970; TRAYER and HILL, 1971; ANDREWS, 1970), as shown in Fig. 1. TRAYER et al. (1970) noted that the bovine milk transferase

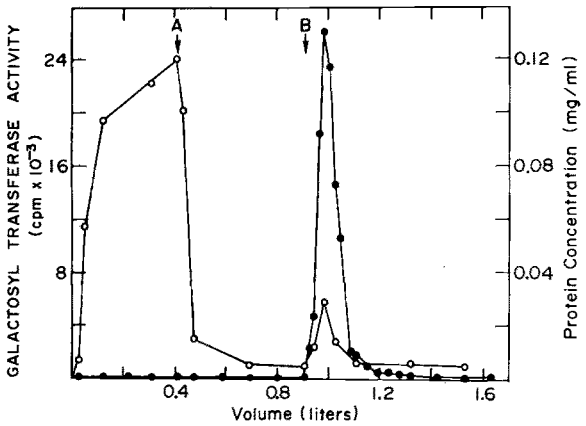
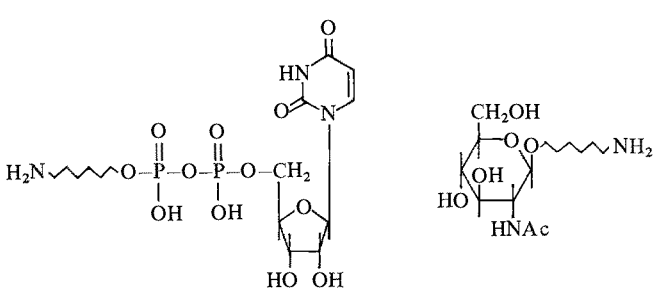


Fig. 3. Purification of galactosyl transferase. The enzyme was partially purified on UDP-hexanolamine-Sepharose (see Fig. 2) and then applied to N-acetylglucosaminyl-hexanolamine-agarose in buffer containing 5×10^{-4} M UMP. At A, the column was washed with the same buffer and at B, the column was washed with buffer containing N-acetylglucosamine but not UMP. Protein concentration (○); Enzyme activity (●). From BARKER et al. (1972)

partially purified by ion-exchange chromatography, was specifically adsorbed to the α-lactalbumin-agarose columns in the presence of glucose and that contaminating proteins emerged unretarded. When glucose was removed from the elution buffer, the transferase emerged. The requirement for glucose in the elution buffer was indicated by earlier studies (BREW et al., 1968) which suggested that binding of the transferase to α-lactalbumin was enhanced by glucose. After rechromatography on the same affinity adsorbent, the transferase had a constant specific activity of 14.1 μmoles galactose incorporated per min per mg enzyme and was obtained in an overall yield of 17%. Similarly, ANDREWS (1970) employed the same affinity adsorbent to purify the human milk transferase and obtained a preparation with a specific activity of 5.7 μmoles galactose incorporated per min per mg enzyme in 37% yield. In addition, Andrews observed that the enzyme was retarded more effectively on columns of α-lactalbumin-agarose in the presence of N-acetylglucosamine than in the presence of glucose. Thus, N-acetylglucosamine is prefered in purification of the transferase with this affinity adsorbent (TRAYER et al., 1974).

BARKER et al. (1972) designed and prepared other affinity adsorbents which proved effective for purification of the galactosyl transferase. These adsorbents



were prepared by coupling hexanolamine derivatives of either UDP or N-acetylglucosamine through the primary amino groups to cyanogen bromide activated agarose to give adsorbents containing ligands with the above structures.

It was found that the enzyme in bovine whey was adsorbed on UDP-agarose in the presence of Mn^{2+} and could be eluted by including EDTA in the elution buffers. This adsorbent has a high capacity for the transferase and the enzyme in 5–10 liters of whey can be quantitatively adsorbed by about 100 ml of UDP-agarose, the exact amount depending upon the extent of incorporation of UDP-hexanolamine in the agarose. The purification of the transferase is about 150 fold by this single step, but is never obtained in homogeneous form because of non-specific adsorption, presumably through ionic interactions between inert proteins and the phosphate groups on the adsorbent (Fig. 2). The enzyme eluted from UDP-Sepharose can then be adsorbed more specifically and thus obtained in near pure form by affinity chromatography on either α-lactalbumin-agarose (Fig. 1) or N-acetylglucosamine-agarose (Fig. 3). Binding of the enzyme to N-acetylglucosamine-agarose occurs only when UDP or UMP are included in the solutions of enzyme applied to the adsorbent. The enhanced binding may be explained by kinetic studies (MORRISON and EBNER, 1971a; KHATRA et al., 1974) which indicate that in the course of the catalytic mechanism, N-acetylglucosamine binds to a galactosyl transferase-Mn^{2+}-UDP-galactose complex. Inert protein emerges unretarded from columns of N-acetylglucosamine-agarose and the transferase is eluted by removal or UDP or UMP from the elution buffers. The preparation and properties of nucleotide-agarose and monosaccharide-agarose adsorbents and their use for purification of the transferase and other enzymes or proteins has been described in recent reports (SHAPER et al., 1973; BARKER et al., 1974a, 1974b).

B. Physical and Chemical Properties

It is now clear that the galactosyl transferase isolated from bovine milk may be heterogeneous in size, and species with molecular weights of about 42000, 48000 and 54000 have been observed as shown in Fig. 4 (BARKER et al., 1972; MAGEE et al., 1973). The first pure preparations of galactosyl transferase (TRAYER and HILL, 1971) from bovine milk were isolated by a combination of ion-exchange and affinity chromatography and a single major species was obtained. This enzyme was homogeneous as judged by ultracentrifugation and electrophoretic analysis. Its molecular weight determined by gel electrophoresis in sodium dodecyl sulfate was 44000 and by sedimentation-equilibrium analysis in 6 M quaindine-HCL was 42900. Ultracentrifugal analyses by sedimentation equilibrium in non-denaturing solvents revealed species with molecular weights between about 40000 and 160000. At very low enzyme concentrations, sucrose density gradient sedimentation revaled a single enzymically active species with a molecular weight of about 42900. These analyses indicate that the native enzyme has a tendency to agregate in dilute salt solutions at neutral pH, but that the catalytically active species has a molecular weight of about 42000–44000. The enzyme is not dissociated into lower molecular weight components in denaturing solvents, thus it is devoid of subunits despite its tendency to aggregate in non-denaturing solvents.

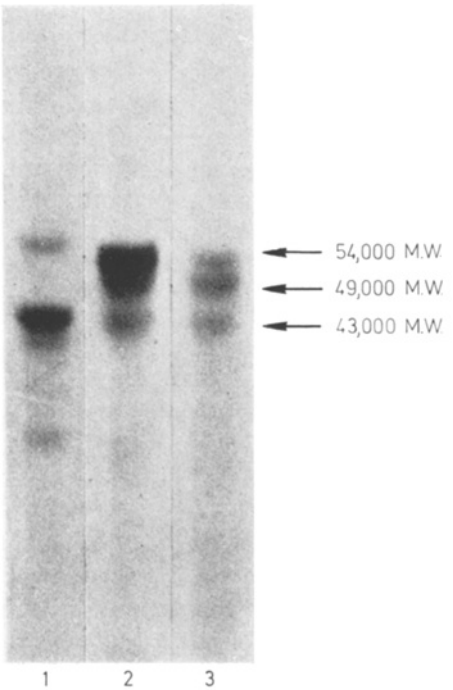


Fig. 4A—C. Electrophoretic patterns of purified galactosyl transferase on polyacrylamide gels in sodium dodecyl sulfate. (A) Enzyme prepared by the method of TRAYER and HILL (1970). (B) Enzyme purified on UDP-hexanolamine-agarose and then on N-acetylglucosamine-agarose. (C) Enzyme purified on UDP-hexanolamine-agarose and then on N-acetylglucosamine-agarose. From BARKER et al. (1972)

The transferase prepared solely by affinity chromatography of bovine whey shows multiple molecular weight species as judged by gel electrophoresis in sodium dodecyl sulfate (BARKER et al., 1972). There appears to be variation in the amounts of the three major species which appear from one preparation of milk to another, and in the purification procedure of TRAYER and HILL (1971) it is possible that the higher molecular weight forms were removed by the ion-exchange chromatographic steps used prior to affinity chromatography. MAGEE et al. (1972) also noted only two different electrophoretic species with apparent molecular weights of 45000 and 58000, which could not be separated by affinity chromatography on α-lactalbumin-agarose. Each of the species with different molecular weights appears to be enzymically active.

MAGEE et al. (1973) have presented evidence that the lower molecular weight species of transferase may result from proteolysis of the higher molecular weight form. These workers found that trypsin digestion of transferase containing two species (mwt. 58000 and 42000) proceeded with loss of the 58000 molecular weight form and an increase in the 42000 molecular weight form. The ratio of the two forms before proteolysis was 4:1 (58000:42000) but after brief periods of exposure to trypsin at 0°, the ratio was about 1:3. During this same period

only 20% of the enzymic activity was lost. On more prolonged digestion, species with lower molecular weights appeared and was accompanied by loss of transferase activity. One of the forms of the transferase to appear during proteolysis had molecular weights between 37000 and 39000, bound weakly to α-lactalbumin-agarose and apparently had little or no transferase activity. The active, degraded form with a molecular weight of 42000 was not characterized sufficiently to judge whether it was identical to the active species with about the same molecular weight which is isolated from milk (TRAYER and HILL, 1971). It is unlikely that trypsin acts to degrade the transferase *in vivo* but, as suggested by MAGEE et al. (1973), plasmin or other proteolytic enzymes in milk may do so. It is of interest that cytochrome b_5 reductase, which is also membrane bound, is degraded by proteolysis to give lower molecular weight forms which retain activity (SPATZ and STRITTMATTER, 1973).

It is clear that the transferase is a glycoprotein (TRAYER and HILL, 1971) and each of the different forms contains oligosaccharide prosthetic groups (BARKER et al., 1972) containing sialic acid, glucosamine, galactosamine and neutral sugars. The 42000 molecular weight species from bovine milk contains about 12% total carbohydrate by weight (sialic acid, 3%; glucosamine, 1.1%; galactosamine 1.1% and neutral sugars 8.1%). Galactose and fucose are the major neutral sugar components. The transferase preparations containing two forms in a ratio of about 4:1 (58000:42000) are also reported to contain between 12 and 13.8% carbohydrate including the same monosaccharides reported for the 42000 form (TRAYER and HILL, 1971). In addition, the two forms did not appear to differ in carbohydrate content, in accord with the view that the active forms with different molecular weights have polypeptide chains of different lengths but similar, if not identical, oligosaccharide prosthetic groups.

The amino acid composition of the bovine transferase (TRAYER and HILL, 1971) does not reveal any particularly notable features although it contains low amounts of half-cystine and a rather high content of non-polar, compared to polar residues. The compositions listed in Table 1 for the two different forms with molecular weights of 40800 and 50000 show no striking differences (POWELL and BREW, 1974).

Galactosyl transferase isolated by affinity chromatography from bovine, ovine and porcine colostrum, and also from human milk is a homogeneous glycoprotein which corresponds in size to the highest molecular weight form from bovine milk (POWELL and BREW, 1974). Treatment of this enzyme with trypsin generated the typical three-banded pattern found in milk enzyme. The absence of degraded forms in colostrum was attributed to the presence of protease inhibitors. Following controlled proteolysis of the colostrum or bovine milk enzyme with trypsin, an apparently homogeneous form of the enzyme (galactosyl transferase-T) corresponding to the lowest molecular weight species from bovine milk was indicated by gel filtration and affinity chromatography. Although the molecular weights of the colostrum galactosyl transferase and galactosyl transferase-T appeared from gel electrophoresis in sodium dodecyl sulfate to be 53000–54000 and 43000–44000 respectively, more rigorus determinations of the sizes by sedimentation equilibrium ultracentrifugation in buffered water and D_2O (as a means of measuring the partial specific volume), gave values of 50000 and

Table 1. The amino acid composition of the two different molecular weights forms of bovine galactosyl transferase

Amino acid	High molecular weight form (residues per molecule)	Low molecular weight form (residues per molecule)
Carboxymethylcystine	2.8 (3)	2.0 (2)
Aspartic acid	30.2 (30)[b]	30.1 (30)[b]
Threonine	34.6 (35)[c]	28.5 (29)[c]
Serine	47.6 (48)[c]	43.5 (44)[c]
Glutamic acid	48.4 (48)[b]	41.6 (42)[b]
Proline	23.4 (23)	16.7 (17)
Glycine	40.9 (41)	34.8 (35)
Alanine	42.6 (43)	23.2 (23)
Valine	42.8 (43)[aa]	37.4 (37)[aa]
Methionine	2.6 (3)	2.7 (3)
Isoleucine	11.3 (11)	11.4 (11)
Leucine	34 (34)	31 (31)
Tyrosine	15 (15)	8.9 (9)
Tryptophan	8 (8)	6 (6)
Phenylalanine	10.6 (11)	9.8 (10)
Lysine	26.9 (27)	23.2 (23)
Histidine	8 (8)	6.2 (6)
Arginine	14.8 (15)	14.2 (14)
Total No. residues	432	372
Molecular weight glycoprotein[a]	50000	41200

[a] From sedimentation-equilibrium.
[b] Includes asparagine in aspartic acid and glutamine in glutamic acid.
[c] Extrapolated to zero time hydrolysis.
[aa] From 72 hour hydrolysates. From POWELL and BREW, 1974b.

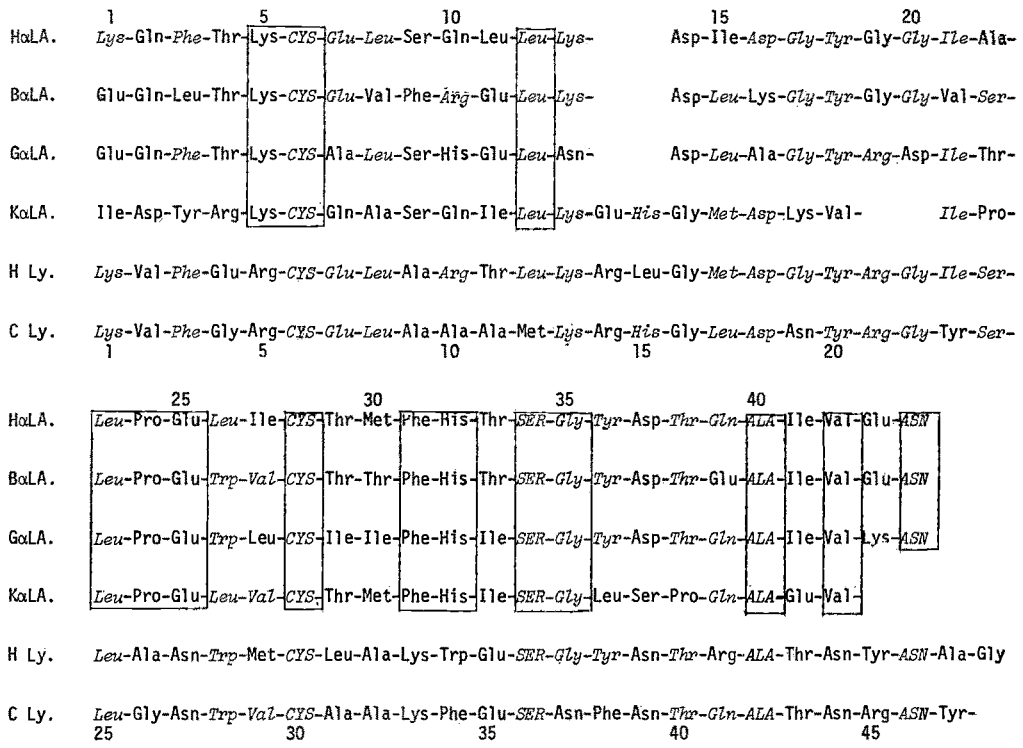
40800 for the two forms of the bovine enzyme. Porcine colostrum glactosyl transferase appeared to be slightly larger, with a molecular weight of 51200. The specific activity of the colostrum enzyme was about twice as high as that of the trypsin-degraded form (18.5 and 9.0 μmoles/min/mg protein respectively). A disulphide-bonded dimeric form was also isolated from colostrum with a specific activity of about 5% of that of the monomer. Hydrodynamic measurements indicated that the colostrum enzyme is rather asymmetric ($f/f\,\mathrm{min} = 1.49$). Amino acid analysis of the two forms indicates that the fragment removed on trypsin digestion has a high content of proline, and may thus be a somewhat nonglobular region of the polypeptide (see Table 1).

Kinetic studies of the two enzyme forms revealed that the binding of UDP-galactose by galactosyl transferase-T is different from its binding by the colostrum enzyme. The binding of UDP-galactose to the latter is virtually irreversible (dissociation constant for UDP-galactose from $E \cdot Mn^{2+} \cdot$ UDP-galactose $\cong O$) whereas UDP-galactose binding to galactosyl transferase-T is at thermodynamic equilibrium during the catalytic cycle, clearly indicating a dramatically increased rate constant for its dissociation, possibly as a result of a more open structure of the binding site in this enzyme form.

IV. α-Lactalbumin

A. Primary Structure and Evolution

Bovine α-lactalbumin was first isolated over 30 years before the discovery of its function as a component of lactose synthetase and several isolation procedures as well as many of its physical and chemical properties were subsequently established (for review of earlier studies see GORDON, 1971). The α-lactalbumin molecule was found to consist of a single polypeptide chain of molecular weight approximately 15000, containing eight half-cystinyl residues joined in disulphide bonds and with an NH_2-terminal glutamyl residue and a COOH-terminal leucine (for reviews see MCKENZIE, 1967; GORDON, 1971). On the basis of this information, and the characteristics of α-lactalbumin from guinea-pig milk, BREW and CAMPBELL (1967a) were able to point out a remarkable general similarity in the chemical characteristics of α-lactalbumin and chicken lysozyme. It is interesting to note that the similarity had been previously mentioned by YASUNOBU and WILCOX (1958). The determination of the complete amino acid sequence of bovine α-lactalbumin (BREW et al., 1967a, b; BREW and HILL, 1970; BREW et al., 1970) confirmed the close structural similarity and common genetic origin of α-lactalbumin and lysozyme. More recently, the sequences of α-lactalbumins from two other mammals have been determined, those from human (FINDLAY and BREW, 1972) and guinea-pig (BREW, 1972) milks, together with the sequence of the amino-terminal 44 residues of an α-lactalbumin from the grey kangaroo (BREW et al., 1973). These structures are all shown in Fig. 5 in comparison with the sequences of human and chicken lysozymes (CANFIELD, 1967; JOLLES, 1967; CANFIELD et al., 1971). The α-lactalbumins are somewhat smaller than the lysozymes (123 as opposed to 129 residues and 130, respectively, in chicken and human lysozymes) and as shown in this figure, a maximum level of similarity between the proteins is obtained only when three gaps; two of 2 residues and one of 1 residue are inserted in the sequences of the α-lactalbumins from the placental mammals (i.e. human, bovine and guinea-pig). Only one of these gaps is present in a region where a comparison with the marsupial α-lactalbumin (kangaroo) is possible, and it can be seen that in this protein the gap (also of 2 residues) must be placed in a different position to maintain the homology with lysozyme. On the basis of the structural similarity of α-lactalbumins and lysozymes, it is clear that the genes which control the sequences of the two proteins must have a common ancestor. As lysozymes are more widely distributed than α-lactalbumins, in both avian and mammalian species, it appears likely that the ancestral gene controlled the structure of a lysozyme. Indeed, the presence of several gaps in the α-lactalbumin sequences in the above alignment, and their absence from the lysozyme sequence confirms that the ancestor of the group was closer to the modern lysozymes in size, as gaps reflect the deletion of base triplets as a result of mutational events during phylogeny. It can be proposed that the ancestral gene for the group underwent a complete duplication. One duplicate gene gave rise to α-lactalbumin by a series of mutational events, whereas the other duplicate continued to control the structure of lysozyme.


1 5 10 15 20
HαLA. Lys-Gln-Phe-Thr-Lys-CYS-Glu-Leu-Ser-Gln-Leu-Leu-Lys- Asp-Ile-Asp-Gly-Tyr-Gly-Gly-Ile-Ala-
BαLA. Glu-Gln-Leu-Thr-Lys-CYS-Glu-Val-Phe-Arg-Glu-Leu-Lys- Asp-Leu-Lys-Gly-Tyr-Gly-Gly-Val-Ser-
GαLA. Glu-Gln-Phe-Thr-Lys-CYS-Ala-Leu-Ser-His-Glu-Leu-Asn- Asp-Leu-Ala-Gly-Tyr-Arg-Asp-Ile-Thr-
KαLA. Ile-Asp-Tyr-Arg-Lys-CYS-Gln-Ala-Ser-Gln-Ile-Leu-Lys-Glu-His-Gly-Met-Asp-Lys-Val- Ile-Pro-
H Ly. Lys-Val-Phe-Glu-Arg-CYS-Glu-Leu-Ala-Arg-Thr-Leu-Lys-Arg-Leu-Gly-Met-Asp-Gly-Tyr-Arg-Gly-Ile-Ser-
C Ly. Lys-Val-Phe-Gly-Arg-CYS-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-His-Gly-Leu-Asp-Asn-Tyr-Arg-Gly-Tyr-Ser-
1 5 10 15 20
25 30 35 40
HαLA. Leu-Pro-Glu-Leu-Ile-CYS-Thr-Met-Phe-His-Thr-SER-Gly-Tyr-Asp-Thr-Gln-ALA-Ile-Val-Glu-ASN
BαLA. Leu-Pro-Glu-Trp-Val-CYS-Thr-Thr-Phe-His-Thr-SER-Gly-Tyr-Asp-Thr-Glu-ALA-Ile-Val-Glu-ASN
GαLA. Leu-Pro-Glu-Trp-Leu-CYS-Ile-Ile-Phe-His-Ile-SER-Gly-Tyr-Asp-Thr-Gln-ALA-Ile-Val-Lys-ASN
KαLA. Leu-Pro-Glu-Leu-Val-CYS-Thr-Met-Phe-His-Ile-SER-Gly-Leu-Ser-Pro-Gln-ALA-Glu-Val-
H Ly. Leu-Ala-Asn-Trp-Met-CYS-Leu-Ala-Lys-Trp-Glu-SER-Gly-Tyr-Asn-Thr-Arg-ALA-Thr-Asn-Tyr-ASN-Ala-Gly
C Ly. Leu-Gly-Asn-Trp-Val-CYS-Ala-Ala-Lys-Phe-Glu-SER-Asn-Phe-Asn-Thr-Gln-ALA-Thr-Asn-Arg-ASN-Tyr-
25 30 35 40 45
45 50 55 60 65
HαLA. Asp-Gln-Ser-Thr-Glu-TYR-GLY-Leu-Phe-GLN-ILE-Ser-Asn-Lys-Leu-Trp-CYS-Lys-Ser-Ser-Gln-Val-Pro-Gln-
BαLA. Asn-Gln-Ser-Thr-Asp-TYR-GLY-Leu-Phe-GLN-ILE-Asn-Asn-Lys-Ile-Trp-CYS-Lys-Asn-Asp-Gln-Asp-Pro-His-
GαLA. Ser-Asn-His-Lys-Glu-TYR-GLY-Leu-Phe-GLN-ILE-Asn-Asn-Lys-Asp-Phe-CYS-Glu-Ser-Ser-Thr-Thr-Val-Gln-
H Ly. Asp-Arg-Ser-Thr-Asp-TYR-GLY-Ile-Phe-GLN-ILE-Asn-Ser-Arg-Tyr-Trp-CYS-Asn-Asp-Gly-Lys-Thr-Pro-Gly-
C Ly. Asp-Gly-Ser-Thr-Asp-TYR-GLY-Ile-Leu-GLN-ILE-Asn-Ser-Arg-Trp-Trp-CYS-Asn-Asp-Gly-Arg-Thr-Pro-Gly-
50 55 60 65 70
70 75 80 85 90
HαLA. Ser-Arg-Asn-Ile-CYS-Asp-Ile-Ser-CYS-Asp-Lys-Phe-LEU-Asn-Asp-Asn-Ile-THR-Asn-Asn-Ile-Met-CYS-Ala-
BαLA. Ser-Ser-Asn-Ile-CYS-Asn-Ile-Ser-CYS-Asp-Lys-Phe-LEU-Asn-Asn-Asp-Leu-THR-Asn-Asn-Ile-Met-CYS-Val-
GαLA. Ser-Arg-Asp-Ile-CYS-Asp-Ile-Ser-CYS-Asp-Lys-Leu-LEU-Asn-Asp-Asn-Leu-THR-Asn-Asn-Ile-Met-CYS-Val
H Ly. Ala-Val-Asn-Ala-CYS-His-Leu-Ser-CYS-Ser-Ala-Leu-LEU-Gln-Asp-Asn-Ile-Ala-Asp-Ala-Val-Ala-CYS-Ala
C Ly. Ser-Arg-Asn-Leu-CYS-Asn-Ile-Pro-CYS-Ser-Ala-Leu-LEU-Ser-Ser-Asp-Ile-Thr-Ala-Ser-Val-Asn-CYS-Ala
75 80 85 90 95

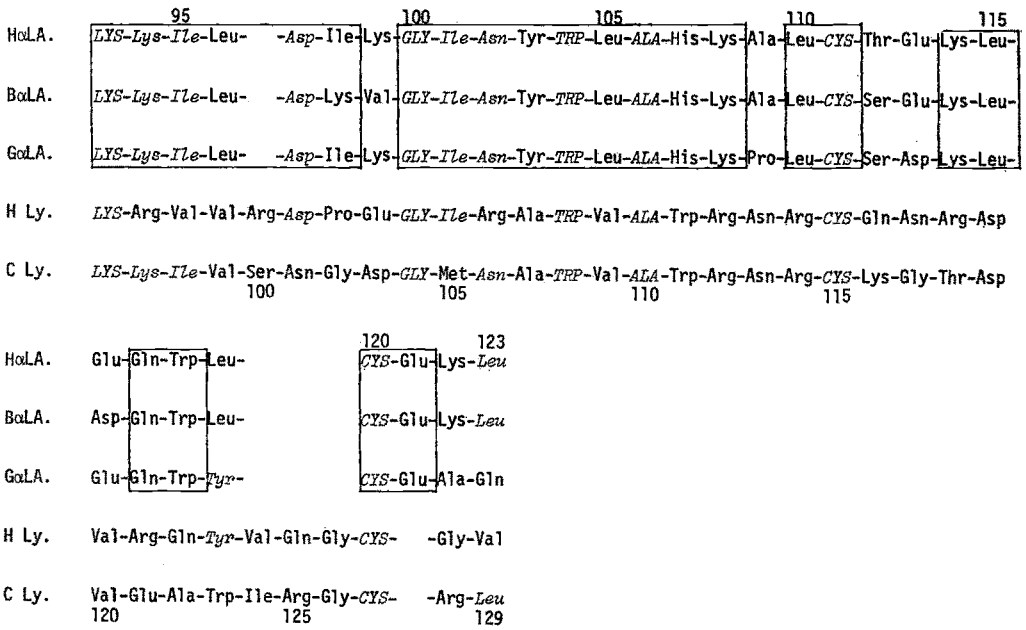


Fig. 5. Comparison of the complete sequences of bovine (BαLA), guinea pig (GαLA) and human (HαLA) α-lactalbumins and the partial sequence of kangaroo α-lactalbumin (KαLA) with one another and with the complete sequences of human (H Ly) and chicken (C Ly) lysozymes. Residues in lower case italics (e.g., residue 1) are identical in at least one species of α-lactalbumin and one species of lysozyme. Residues in upper case italics (e.g., residue 6) are identical in all sequences compared. Residues which are identical in all α-lactalbumins are enclosed in boxes. The numbers above the sequence of human α-lactalbumin are the positions of residues in the α-lactalbumins, each of which contains 123 residues. The numbers below the chicken lysozyme sequence are the positions of residues in this molecule and are exactly the same from 1–47 in the human sequence but correspond to the number shown plus 1 for the human sequence from residue 47–130, because human lysozyme contains one more residue than chicken lysozyme. The gaps in sequence are included to obtain maximum homology

As one of the deletions in the α-lactalbumin sequences is differently placed in the placental and marsupial proteins, it must represent an event that has occurred since the divergence of these two mammalian lines.

The galactosyl transferase component of lactose synthetase is a widely-distributed enzyme, being present in non-mammalian species, and it must therefore have preceded the development of lactose synthesis during phylogeny. The evolutionary development of α-lactalbumin was therefore the crucial step in the development of lactose biosynthesis. Indeed, even the unique subcellular organization of lactose synthetase can be seen as the logical outcome of the evolutionary development of α-lactalbumin (*vide infra*).

Although several other groups of functionally divergent homologous proteins are known, for example the serine proteases (SHOTTON and WATSON, 1970) and

the globins (PERUTZ et al., 1968), α-lactalbumin and lysozyme are the most functionally divergent group yet discovered. Despite their structural similarity neither is able to replace the other in their respective functions as a regulatory protein and an antimicrobial enzyme. It is to be hoped that a study of their structural similarities and differences will give some insight into the way in which new biochemical functions arise during the course of evolution. Clearly the development of α-lactalbumin and thus lactose synthesis helped in the production of a balanced secretion, milk, for the nutrition of young mammals.

B. The Conformation of α-Lactalbumin

The close similarity in the amino acid sequences of α-lactalbumin and lysozyme suggests that their three-dimensional structures may also show some similarity. This is made more probable by the fact that the pairing of the eight half-cystinyl residues into disulphide bonds is identical in the two proteins (I–VIII; II–VII; III–V; IV–VI) (VANAMAN et al., 1970). BROWN and coworkers (1969) therefore attempted to build a model of the three-dimensional structure of α-lactalbumin to see if the primary structure is compatible with a conformation similar to that of chicken lysozyme as determined by Phillips and coworkers (BLAKE et al., 1967). Despite the fact that about 60% of the amino acids in corresponding positions in the sequences are not identical, the conservative nature of many of the substitutions and the mutually-compensating nature of other groups of substitutions permitted a good fit. The deletions in the α-lactalbumin sequence were accomodated without major conformational changes.

This work has been recently refined and extended by applying to the α-lactalbumin sequence placed in a lysozyme conformation, computational procedures designed to minimize the conformational energy (WARME et al., 1974).

Although it appears likely, on the basis of a considerable body of indirect evidence, that α-lactalbumin and lysozyme will prove to have similar conformations, no three-dimensional structure of a protein has been successfully predicted by either computation or model building. The outcome of current crystallographic studies of α-lactalbumin is therefore eagerly awaited, to test the validity of the predictions.

Some difficulty was encountered in the model building study at the carboxyl terminus of α-lactalbumin, where it can be seen from Fig. 5 there is some considerable difference from lysozyme (see footnote to Fig. 5). A change in conformation appears to have occurred in the region and is probably associated with a reversal of the chirality of the disulphide bond between the first and last $^1/_2$ cysteines in the sequence (see BROWNE et al., 1969). In their computational studies, WARME et al. (1974) found three energetically-equivalent structures for the C-terminus of the molecule, and suggest that its conformation may change under different conditions. The main features of α-lactalbumin predicted by these studies are:

1. The molecule is generally similar in size and shape to lysozymes, but may be slightly flattened ($23 \times 37 \times 32$ Å and $28 \times 38 \times 33$ Å for lysozyme).

2. The molecule is divided into two wings separated by a cleft region (the active site in lysozyme). The cleft is however shortened by the presence of a

tyrosyl residue in α-lactalbumin position 104 (alanine in lysozyme), which projects into the cleft and may form a new hydrophobic core with other residues in the region.

3. The front right-hand surface of the molecule (neighboring the cleft region) contains few polar side chains and a considerable number of hydrophobic residues: Phe 31, Tyr 36, Trp 118, His 32 (WARME et al., 1974).

Clearly the only satisfactory test of the predicted conformation of α-lactalbumin will be the determination of the three-dimensional structure by X-ray crystallography. Some aspects of the structure are, however, open to testing by more rapid techniques, for example the size and shape of the molecule and the character of its secondary structure can be investigated by various physical techniques and the microenvironments of chemically-reactive amino acid side chains studied by chemical modification. Initial results from low angle X-ray scattering experiments (KRIGBAUM and KUGLER, 1970) suggested that the size and shape of α-lactalbumin and lysozyme molecules in solution are widely different. ACHTER and SWAN (1971) reassessed the data, and showed that they can be interpreted in terms of closely similar sizes and shapes for the two proteins combined with a low degree of dimerization in the α-lactalbumin preparation. PESSEN et al. (1971) have more recently reported a study of the low angle X-ray scattering of α-lactalbumin and lysozyme which indicates a close similarity in their size and shape.

RAWITCH (1972), from measurements of the fluorescence polarization of the dansyl-protein conjugates, obtained rotational relaxation times that are consistent with a larger effective volume or increased asymmetry for α-lactalbumin over lysozyme. The propensity for aggregation observed in dansyl-α-lactalbumin may however have contributed to this result, as in confirmation of the X-ray scattering studies, BAREL et al. (1972) have found a close similarity in the shapes and sizes of human α-lactalbumin and human lysozyme by hydrodynamic procedures.

Other experiments have been designed to compare the conformations of the polypeptide backbones of α-lactalbumin and lysozyme (secondary structure). The optical rotatory dispersion and circular dichroism curves for the two proteins are compatible with a overall similarily in their polypeptide conformations, although differences in the numbers and environments of aromatic chromophores complicates the comparison at wavelengths in the region of aromatic absorption (AUNE, 1968; KRONMAN, 1968; COWBURN et al., 1972). COWBURN et al. (1970) have also found that the proton magnetic resonance spectrum of bovine α-lactalbumin can be interpreted in terms of the lysozyme-derived conformation.

A considerable amount of evidence indicates that the accessibility and reactivities of the disulphide bonds of α-lactalbumin differ considerably from those of lysozyme. ATASSI and coworkers (1970) and HABEEB and ATASSI (1971) have found that more than 2 disulphide bonds of α-lactalbumin are reduced by 2-mercaptoethanol at neutral pH, whereas under the same conditions none of the disulphide bonds of lysozyme react. A similar difference in the reactivities of the disulphides of the two proteins to dithiotreitol has been reported by IYER and KLEE (1973). WARME et al. (1974) point out that in their computed structure for α-lactalbumin, the I–VIII and II–VII disulphide bonds are much more exposed

Table 2. Chemical modification studies of bovine α-lactalbumin

Reagent/Conditions	Residues modified	Reactivity	Predicted[a] environ.	Effect on activity	Reference
Iodoacetate pH 6.5	Methionine 90	Fast	E	None	CASTELLINO and HILL (1970)
	Histidine 32	Intermediate	S	Progressive Loss down to 40%	
	68	Fast	E		
	107	Slow	S		
2-hydroxy 5-Nitro-benzyl bromide pH 2.9: 10% acetone or dimethyl (2-hydroxy-5-nitro-benzyl) sulphonyl bromide (pH 6.0)	Tryptophan 26	Fast	I	Not reported	BARMAN (1972), BARMAN and BAGSHAW (1972)
	60	Slow	I		
	104	Intermediate	S		
	118	Fast	S		
Glycine ethyl ester and carbodiimide pH 4.75	Carboxyl Groups (18 per molecule)	Rapid (Compared to lysozyme)	E or S (only 4 are homo-logous	Activity lost	LIN (1970)
Cyanuric Fluoride pH 13	Tyrosine (4 per molecule)	4 react	S	Not reported	GORBUNOFF (1967)
pH 9.3		3 react			
Tetranitromethane	Tyrosine (and tryptophan)	All react	S	Activity lost parallels tyrosine loss, but unfolding occurs	DENTON and EBNER (1971); PRIEEL, J. P. and K. BREW, unpublished 1974
Iodine	Tyrosine (histidine)	All react	S	Activity loss parallels tyrosine loss but unfolding occurs	DENTON and EBNER (1971)
N-acetylimidazole low concentration	Tyrosine	2 quite reactive 1 less reactive 1 weakly reactive	S	Not reported	KRONMAN et al. (1971)
High concentration	Tyrosine and Lysine	6 amino groups	?	(Effects similar to acid de-naturation)	
Tyrosinase	Tryptophan	1 reacts	?	None	DENTON and EBNER (1971)

Table 2 (Continued)

Reagent/Conditions	Residues modified	Reactivity	Predicted[a] environ.	Effect on activity	Reference
Mercaptoethanol	Disulphide bonds	React more rapidly than lysozyme	2 different than in lysozyme (see text)	Not reported	IYER and KLEE (1973)
Photooxidation in presence of proflavine	Tryptophan	2 fast 1 slower 1 unreactive	Residues reacted not identified	Not reported	TANBURRO et al. (1972)

[a] E, exposed to solvent; S, surface; I, internal (buried).

to solvent than the corresponding bonds in lysozyme, a feature that is entirely consistent with the observations.

An interesting correlation with the presence of a surface hydrophobic site in the predicted α-lactalbumin conformation is to be found in studies of the binding of the fluorescent probe 2-p-toludenyl-naphthalene-6-sulfonate to α-lactalbumin and lysozyme (BAREL, TURNEER and DOLMANS, 1972). Both proteins bound the reagent to form 1:1 molar complexes with similar dissociation constants (0.3–0.5 millimolar), but the fluorescent properties of the complex with α-lactalbumin indicated that the reagent is bound in a more hydrophobic environment than in lysozyme.

Other workers have used chemical modification studies with group specific reagents both as a means of testing the model through the microenvironments of individual side chains, and as an approach to studying the relationship of structure and function in α-lactalbumin. The results of such studies are summarized in Table 2. Unfortunately only in two studies (CASTELLINO and HILL, 1972; BARMAN, 1970) were the relative rates of modification of individual residues examined. Other studies showed a rather uncritical approach to the problem, for example the gross reactivity of the carboxylate side chains of a α-lactalbumin to modification with glycine methyl ester and a water soluble carbodiimide was found to be greater than that found for lysozyme (LIN, 1970). The conclusion of differences in conformation was assumed without examination of the sequences of the proteins. Such an examination would have shown that of the 17 side chain carboxyl groups of α-lactalbumin, only 4 correspond to such groups in lysozyme. In general, the results of most chemical modification studies are in good agreement with the environments of the side chains in the model. A striking exception is the high reactivity of tryptophan 26 with 2-hydroxy-5-nitrobenzyl bromide (BARMAN, 1972), as this residue is buried in the α-lactalbumin model. Reaction with this reagent requires conditions unfavorable to the maintenance of the native conformation (pH 2.8, 10% acetone), but the same relatively high reactivity is found when the water soluble derivative dimethyl (2-hydroxy-5-nitrobenzyl) sulphonium bromide is used to modify α-lactalbumin at pH 6.0. Reaction, which occurs with tryptophans 26, 104 and 118, as well as a histidyl residue, is ac-

companied by loss of activity and possibly some unfolding (BARMAN and BAGSHAW, 1972). In contrast, solvent perturbation studies of α-lactalbumin by KRONMAN and HOLMES (1965) suggest that, in agreement with the model, two of the four tryptophan residues of the protein are exposed to solvent. In human α-lactalbumin (see below), tryptophan 26 is replaced by a leucyl residue, and assuming a similarity in conformation between the human and bovine proteins, the presence of a clearly hydrophobic residue in this position would make it appear probable that this residue is buried in α-lactalbumin (although hydrophobic side chains may be occasionally found exposed to solvent in proteins). In contrast to some earlier studies of GUIRE (1970), ROBBINS and HOLMES (1972) find that one tryptophan of α-lactalbumin is sufficiently exposed to be capable of face to face interaction with N-methynicotinamide chloride. A second tryptophan becomes available for interaction at pH 2.

Unfortunately, the side chains which may, from these reports, be directly or indirectly involved in the biological function of α-lactalbumin, i.e., tyrosines and carboxylate groups, were not explored with respect to the roles and reactivities of individual residues. It would appear that further investigations in this direction would be rewarding. A reinvestigation of the modification of the tyrosines of α-lactalbumin by nitration with tetranitromethane[5] has failed to confirm the simple correlation of tyrosine modification with activity loss observed in the previous study (DENTON and EBNER, 1971). Extensive tyrosine modification appears to be accompanied by unfolding of the protein, nevertheless derivatives can be prepared in which the tyrosines are completely modified but which retain a considerable proportion of the ability to specify lactose synthesis. KRONMAN et al. (1971) in their studies of the acylation of α-lactalbumin with N-acetylimidazole find that acetylation of the two most reactive tyrosyl residues increases (reversibly) the fluorescence quantum yield of what is probably a tryptophan residue. Extensive acetylation with this reagent, which results in modification of 6 amino groups, gave rise to effects analogous to those observed on acid denaturation. These effects can be very easily interpreted in terms of the closeness of two tryptophan-tyrosine groups in the model and the proximity of a number of lysyl, glutamyl and aspartyl side chains. SOMMERS et al. (1973) have observed that the quantum yield of the intrinsic tryptophan fluorescence of guinea-pig α-lactalbumin is more than twice that of the bovine, goat or human proteins. Guinea-pig α-lactalbumin, unlike the other three proteins does not have a tryptophanyl residue in position 60, and they propose that the presence of a tryptophan in this position results in quenching of the fluorescence of a second tryptophan in the other three proteins. In the proposed conformations for α-lactalbumin, tryptophan 104 lies within a relatively small distance of Trp 60 (7 Å) which lies within 6 Å of two disulphide bridges. They suggest that transfer of excitation energy from Trp 104 to Trp 60 occurs with subsequent quenching by the disulphides, an explanation that is entirely consistent with the proposed conformational similarity with lysozyme.

Most investigations, therefore, are reasonably consistent with the hypothesis that α-lactalbumin and lysozyme are similar in their 3-dimensional structures.

[5] PRIEELS, J. P., and BREW, K., unpublished observations, 1974.

The roles that specific amino acid side chains play in the regulatory activity of α-lactalbumin is, however, far from clear as in most studies the precise sites of modification have not been investigated and further more rigorous chemical modification studies should be rewarding.

C. Species Variation in α-Lactalbumin Structure and Function

Superficial examination of the sequences in Fig. 5 shows that the α-lactalbumins as a group are more similar to each other than to the lysozymes. Extensive sequence changes in α-lactalbumin have occurred, however, during the course of evolution, and a more quantitative examination of the sequence indicates that the rate of evolutionary change in the α-lactalbumin line is about twice that in the lysozyme stem. The existence of a genetic variant of bovine α-lactalbumin, in which Arg-10 is replaced by glutamine (GORDON et al., 1968) is consistent with this high rate of evolutionary change. This feature may be considered to support the view that α-lactalbumin is a relatively recent evolutionary development.

Many other points of interest can be deduced from the comparison of sequences in Fig. 5. Thus, in a negative sense, the variable residues in α-lactalbumin are obviously not specifically critical for the activity in lactose synthetase. Among these are several residues which correspond to residues in lysozyme which constitute the active site of the enzyme and come into close contact with the substrate during catalysis (BLAKE et al., 1967). Examples of these are residues 33 (Thr or Ile) and 49 (Asp or Glu) which correspond to the pair of residues (Glu and Asp) in lysozyme which are directly involved in the catalytic mechanism of the enzyme. Residue 33 in α-lactalbumin obviously is unlikely to function directly in catalysis, and the variability of residue 49 suggests that it plays a less critical role than the corresponding residue in lysozyme. The change from lysozyme to α-lactalbumin has therefore involved changes in the catalytic site (as opposed to binding sites) which contrasts with the nature of the changes found in other groups of homologous enzymes. In the serine proteases (SHOTTON and WATSON, 1970) it appears that the catalytic site has been strongly conserved and that functional change has been mediated through changes in the specificity of binding sites.

Another significant change is in residues 59 and 60 (Trp-Trp or Tyr-Trp in the lysozymes) which show considerable variability in the α-lactalbumins (e.g., Asp-Phe in guinea pig) and appear unlikely to play a role in the specifier activity of the protein.

An analysis of the invariant residues in the whole α-lactalbumin-lysozyme group (23 in number) produces some interesting results (see Table 3). Many of the invariant positions are of very obvious structural significance, for example, the two invariant glycines, the cystinyl residues, several residues present centrally in hydrophobic cores, and two residues which form internal H-bonds in lysozyme, and in the α-lactalbumin model. The invariance of such residues is further evidence for the conservation of conformation within the group. A number of invariant residues are exposed to the surface of the protein and are thus less likely to be

Table 3. Invariant residues of whole α-Lactalbumin-lysozyme group

Residue	Number	Positions	Structural role[a]
Cystine	7 or 8	6, 30, 65, 77, 81, 95, 116, 128	Obvious structural significance, exact number depends on C-terminal alignment
Glycine	2	55, 105	Both in positions where a non-glycine residue cannot be accomodated in conformation
Cleft region residues			Concerned directly in substrate binding in lysozyme
GLN		58	
ILE		59	
ASP		102	
TRP		109	
ALA		111	
ALA		42	
ASN		46	Not obvious
TYR		55	
SER		36	Internal residues forming internal H-bond. Probably structurally important
THR		40	
Other residues			
TYR		20	All central in hydrophobic core
LEU		25	
LEU		85	
LYS		97	Adjacent in 3-D structure to Tyr 20

[a] Suggested from studies of BROWN et al. (1969) or WARME et al. (1974).

of structural importance. These residues are all situated in the cleft region of the molecule and might be thought to indicate the conservation of some function, such as an ability to provide interactions for monosaccharides within the cleft region of the α-lactalbumin molecule, but evidence for this is only presumptive.

Within the α-lactalbumins as a group it is found that the conserved regions consisting of more than 3 consecutive residues are all clustered around the cleft region of the molecule. A particularly interesting group of such residues stretches from positions 101–109. Of these, many which correspond to lysozyme residues involved in substrate binding vary consistently in all the α-lactalbumins (for example Tyr 103, Leu 105) and could conceivably be associated with the functional change between α-lactalbumin and lysozyme. The effects of tyrosine chemical modification on the activity of α-lactalbumin are not surprising on this basis, as 2 tyrosines of bovine α-lactalbumin (Tyr 50 and Tyr 103) are in the cleft region and are conserved in all other α-lactalbumins of known sequence.

Again, the weight of evidence from the nature of the sequence variations in α-lactalbumin reinforces the view that the overall conformations of α-lactalbumin and lysozyme are probably closely similar, and that some conservation of function in the cleft region of α-lactalbumin has occurred. This function is evidently

different from the type of catalytic activity found in lysozyme, as one of the essential catalytic carboxyl groups of lysozyme is absent from α-lactalbumin. Possibly the provision of specific monosaccharide binding sites in the α-lactalbumin cleft may be part of its functional characteristics in lactose synthetase.

A claim by McKenzie (1971) that an α-lactalbumin isolated from the milk of the echidna (a monotreme) possessed both lactose synthetase specifier activity and lysozyme activity has not yet been supported by the publication of any data. Such a finding would be contrary to biological expectations, and to our present understanding of the structure and function of both α-lactalbumin and lysozyme, thus it is urgent that it soon be clarified.

D. Activity Differences among α-Lactalbumins

It is evident from many studies that α-lactalbumins from a range of different species are active with human and bovine milk galactosyl transferases for lactose synthetase (Khatra et al., 1974), and that N-acetyllactosamine synthetases from various origins are active with bovine α-lactalbumin. Furthermore quantitative differences with respect to lactose synthetase specifier protein activity are found with α-lactalbumins from various sources with respect to lactose synthesis, which do not reflect specific mutual adaptation between galactosyl transferase and α-lactalbumin in the individual species. A major basis of the functional differences between the species variants lies in varying association constants for a galactosyl transferase-substrate complex (see Section VII B Khatra et al., 1974) but it is not possible at present to relate these interspecies differences to differences in structure of α-lactalbumin, although it is to be hoped that this will be possible in the future. It also appears possible that differences in activity of α-lactalbumins may be related to the control of the rates of lactose synthesis in different mammalian species (Khatra et al., 1974).

E. Biosynthesis and Secretion of α-Lactalbumin

It is clear that milk α-lactalbumin is synthesized and secreted by the epithelial cells of the lactating mammary gland. Slices of mammary tissue from lactating guinea-pig mammary gland and an endoplasmic reticulum-derived subcellular fraction from homogenates of this tissue have been shown to carry out the biosynthesis of α-lactalbumin (Brew and Campbell, 1967). Similar observations have been made with a fraction from lactating bovine mammary gland (Beitz et al., 1969). In an extension of the studies of α-lactalbumin synthesis in lactating guinea-pig mammary gland, Fairhurst and coworkers (1972) obtained a polysome fraction from subcellular fractions of this tissue and demonstrated its ability to direct the incorporation of labelled amino acids into well-characterized guinea-pig α-lactalbumin. It appears reasonable to conclude that α-lactalbumin is synthesized on polysomes attached to intracellular membranes in the lactating mammary gland. On incubation of slices of lactating mammary gland with labelled

amino acids, a 15 to 20 minute delay was observed before labelled α-lactalbumin appeared in the incubation medium (BREW and CAMPBELL, 1967b). This delay, which is far greater than the time required for the biosynthesis of protein molecules, has been observed with other specifically secreted proteins and is attributed to the time required for newly synthesized proteins to pass through the lumina of the intracellular membranes during their passage from the cell. A wide range of evidence suggests that after biosynthesis, proteins pass through a well defined secretory pathway (rough endoplasmic reticulum to smooth endoplasmic reticulum to Golgi region) before being packaged into secretory vacuoles in the Golgi region and being exported from the cell by reverse pinocytosis [see BREW (1969) for references].

F. Glyco-α-Lactalbumin and other Components

It is generally recognized that during their passage through this secretory pathway of the cell, the carbohydrate moieties of exported glycoproteins become attached to the apoprotein moieties as a result of the serial addition of monosaccharides. This process is catalyzed by various glycosyl transferases which transfer monosaccharides from nucleotide sugars to a growing carbohydrate side chain attached to the protein. As a result of the association of such enzymes with the secretory pathway of the mammalian cell, it is not surprising that some secreted proteins which are not normally classified as glycoproteins, contain a proportion of glycosylated forms produced by the action of these enzymes (e.g., ribonuclease). Similarly, minor components of bovine α-lactalbumin preparations have been isolated by various workers which appear identical in amino acid composition to the major protein, but differ in possessing attached carbohydrates. GORDON and coworkers (1968) obtained a component which contained hexosamine. BARMAN (1970) isolated a minor component by ion exchange chromatography which contained mannose, fucose, galactose, galactosamine, glucosamine and N-acetylneuraminic acid.

HINDLE and WHEELOCK (1971) examined several preparations of bovine α-lactalbumin prepared by various procedures. By ion-exchange chromatography the preparations were found to contain, besides a major component devoid of carbohydrate, at least 2 minor components each containing varying amounts of the previously mentioned monosaccharides. The exact point of attachment of the carbohydrate side chain in glyco-α-lactalbumin is as yet uncertain, but BARMAN has found that the presence of carbohydrate has little or no effect on the biological activity of the protein (BARMAN, 1970). The attachment of the first and following monosaccharides to α-lactalbumins must therefore be a hit or miss process and it is possible that the rapid rate of synthesis and secretion of milk proteins in the mammary gland results is a substrate or enzyme-limited situation with respect to the attachment of carbohydrate. As yet there is no evidence to suggest that the glyco-α-lactalbumins have any separate biological function.

BARMAN (1973) has reported an isolation of an additional active component of bovine milk α-lactalbumin that appears to differ considerably in amino acid composition from the major components. It is claimed that this protein possesses

only 6 half-cystinyl residues and must therefore contain only 3 disulfide bonds. Unfortunately, the criteria used in ascertaining the purity of this component are uncertain and the evaluation of this finding must await further studies.

V. Hormonal Control

A. Levels of α-Lactalbumin and Galactosyl Transferase during Pregnancy and Lactation

It has been recognized for many years that the development of the mammary gland and the initiation and maintenance of lactation are under hormonal control (for a review of earlier studies see COWIE, 1969). The mammary epithelium of mature, nulliparous animals contains few secretory cells, but after pregnancy, differentiation begins and by mid-pregnancy many cells have secretory structural characteristics (MILLS and TOPPER, 1970), including a rough endoplasmic reticulum, Golgi apparatus, and secretory granules. The undifferentiated cells are metabolically quiescent compared to the secretory cells of lactating tissue, which contain a full complement of the enzymatic activities required to maintain themselves and produce the constituents of milk.

Examination of the hormonal control of the components of lactose synthetase was not possible until the discovery that α-lactalbumin (EBNER et al., 1966 and BRODBECK et al., 1967) and the galactosyl transferase (BREW et al., 1968) were required for lactose biosynthesis. Prior to this time, however, the changes in activity of several enzymes in the mammary gland at various stages of pregnancy and lactation in rats were examined. In general, the apparent activity of most of the enzymes involved in carbohydrate, fat, protein, and nucleic acid metabolism were at low levels throughout the first half of pregnancy (10 days) but increased markedly thereafter, reaching higher levels at parturition (20 days) and maximal levels after a few days of lactation (BALDWIN and MULLIGAN, 1966; KUHN and LOWENSTEIN, 1967). KUHN (1968) was one of the first to measure lactose synthetase activity in rat mammary tissue. He found that it was absent or very low until parturition, when the activity rose rapidly to reach maximal values within the first 6 days of lactation. Similarly, the lactose content of the glands during the last three days of pregnancy was also very low (0.091 μmole/g fresh weight tissue) but rose rapidly during the first 6 days after parturition (8–14 μmoles/g fresh weight tissue), and persisted at high levels during lactation Bovine α-lactalbumin stimulated the apparent lactose synthetase activity to a low but significant level when added to tissue homogenates of glands that were removed and assayed before and after parturition.

After recognition that lactose synthetase was composed of α-lactalbumin and the galactosyl transferase, TURKINGTON et al. (1968) measured the levels of both components by means of enzymic assays in homogenates of glands from pregnant and lactating mice. The transferase was measured in two ways: first, by its N-acetyllactosamine synthetase activity with N-acetylglucosamine as the acceptor

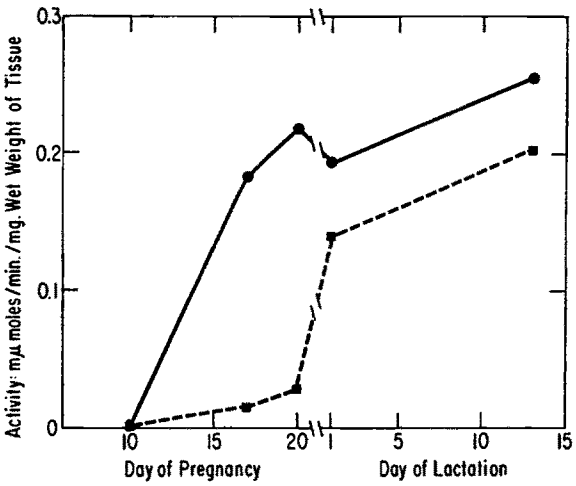


Fig. 6. The levels of galactosyl transferase (●) and α-lactalbumin (■) in mouse (C3H/HeN) mammary tissue homogenates at different stages of pregnancy and lactation. From TURKINGTON et al., 1968

substrate in the absence of added α-lactalbumin; and secondly, by its lactose synthetase activity in the presence of added bovine α-lactalbumin. Both types of assay gave virtually identical results under the specific conditions used. α-Lactalbumin levels were estimated from the lactose synthetase activity of homogenates after addition of a standard amount of bovine transferase. Under the conditions employed, it was assumed that there was a linear relationship between lactose synthetase activity and α-lactalbumin. By these methods it was found that both proteins were at very low levels during the first half of pregnancy but thereafter, the transferase levels increased considerably so that at parturition, they were at about the same level as observed during lactation (Fig. 6). In contrast, α-lactalbumin was at very low levels in mid-pregnancy, rose somewhat in late pregnancy, but never reached the high levels found in lactating tissue. There was, however, sufficient α-lactalbumin 1–2 days prepartum to support a low level of lactose synthetase activity. Thus, it appeared that the galactosyl transferase and α-lactalbumin were synthesized asynchronously during the last half of pregnancy and at parturition α-lactalbumin synthesis by the gland increased dramatically.

PALMITER (1969) and JONES (1972) in subsequent studies obtained results that were not completely consistent with those reported by TURKINGTON et al. (1968). Palmiter confirmed that the lactose synthetase activity was low in midpregnant mice and that the activity rose before parturition to about 20–30% the activity observed in lactating glands. However, synthesis of the galactosyl transferase and α-lactalbumin was not asynchronous and the galactosyl transferase as well as α-lactalbumin levels increased after parturition. It is difficult to assess the discrepancies between results from different laboratories but the somewhat different assay systems used by different workers may complicate comparison of results

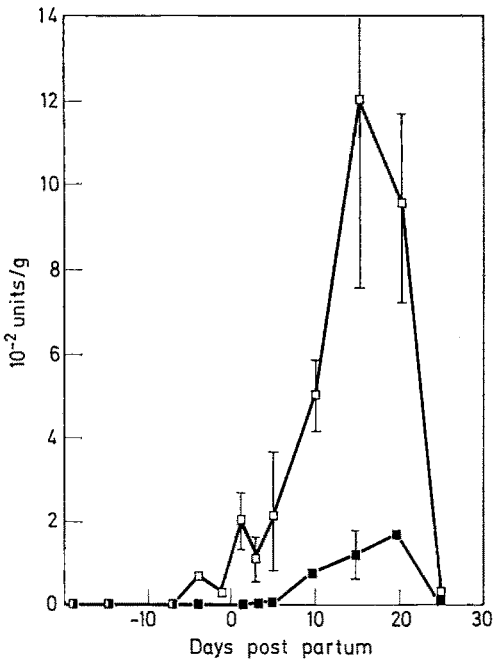


Fig. 7. The lactose synthetase activity of rat mammary tissue homogenates measured in the absence (■) and presence (□) of added exogenous α-lactalbumin as a function of time of pregnancy, lactation and involution. From McKenzie et al., 1971

of these studies. The studies by Jones (1972) emphasize this point. In accord with Turkington et al. (1968), Jones found that transferase levels increased after midpregnancy and did not change markedly before and after parturition. In contrast, however, he also found that the α-lactalbumin levels did not change significantly before and after parturition. The enzymatic assays of Jones (1972) for α-lactalbumin were somewhat different than those used by either Turkington et al. (1968) or Palmiter (1969), so it is difficult to compare exactly the results from the different studies. Jones (1972), however, properly points out that on homogenization of mammary tissue, the smooth endoplasmic reticulum to which the galactosyl transferase is bound, forms vesicles which may contain included α-lactalbumin and transferase so as to render them inaccessible to substrates (Coffey and Reithel, 1968a, b). Thus, estimates of α-lactalbumin and transferase levels may be in error unless the vesicles are disrupted. Digitoxin proved an effective agent for this purpose. Nevertheless, it is difficult to explain why the levels of α-lactalbumin estimated by Jones (1972) in lactating glands were about 2–6 μmolar as compared with concentrations of α-lactalbumin in milk of 0.1–1.0 millimolar. McKenzie et al. (1971) have also pointed out that there are species differences between the rat and mouse (Figs. 7 and 8). Thus, in the rat they detected neither lactose synthetase, galactosyl transferase nor α-lactalbumin until 25 hours prior to parturition but all activities increased markedly thereafter and

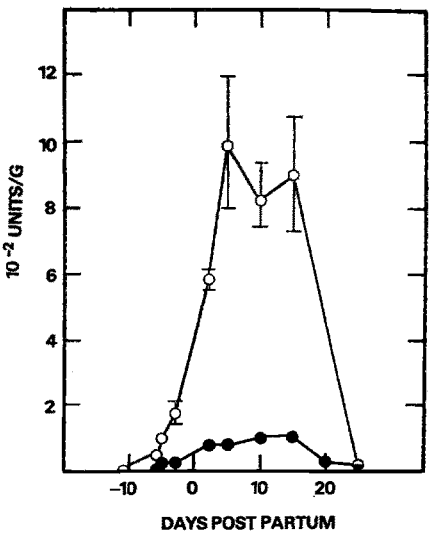


Fig. 8. The lactose synthetase activity of mouse (CD-1) mammary tissue homogenates in the absence (±) and presence (○) of added exogenous α-lactalbumin as a function of time of pregnancy, lactation and involution. From MCKENZIE et al., 1971

during lactation. In contrast, all activities were found in mice after 15 days of pregnancy, in accord with the results of TURKINGTON et al. (1968), and continued to rise prior to parturition and in early lactation. Because different strains of mice have been used by all workers who have attempted to measure the levels of lactose synthetase and its components by enzymatic methods, it is possible that the discrepancies among different studies could have resulted from differences among the strains examined. It is unfortunate that each study to date give somewhat different results concerning the exact levels of lactose synthetase, the transferase and α-lactalbumin during late pregnancy and lactation. Comparison of the results from rats and mice may be impossible but the discrepancies among studies with mice cannot be explained readily. It is likely that further work with mice will be required to settle these important issues, and methods in addition to enzymatic assays should be developed to measure the total amounts of transferase and α-lactalbumin in mammary tissue. Immunochemical methods may be useful in this respect.

MELLENBERGER et al. (1973) recently examined the lactose synthetase activity of multiparous cow mammary tissue at 30 and 7 days prepartum and 7 and 40 days postpartum. Mammary tissue had no lactose synthetase activity at 30 days prepartum but significant activity 7 days prepartum. There was a further increase in activity of about 3- and 5-fold at 7 and 40 days postpartum, respectively. These levels of activity were proportional to the α-lactalbumin content of the glands at the same periods of pregnancy and lactation, suggesting that lactose synthetase activity is regulated by the α-lactalbumin content of the mammary gland, as originally suggested (TURKINGTON et al., 1968).

B. Hormonal Requirements for Lactose Synthetase

TURKINGTON et al. (1968) also examined the hormonal requirements for induction of lactose synthetase and its components in mouse mammary glands *in vitro*, using the explant system of Topper and coworkers (JUERGENS et al., 1965; LOCKWOOD et al., 1966). Earlier studies with explants showed that differentiation of mammary epithelial cells into secretory alveolar cells required the presence of insulin, hydrocortisone and prolactin (JUERGENS et al., 1965). These same hormones were also required for synthesis of specific milk proteins, such as casein (LOCKWOOD et al., 1966). It was found that synthesis of the transferase and α-lactalbumin was stimulated by the same hormones (Fig. 9). Insulin alone, or combinations of insulin and hydrocortisone, or insulin and prolactin, did not induce either the transferase or α-lactalbumin in explants from midpregnant mice. Prolactin, however, induced the synthesis of each protein when explants were pretreated with insulin and hydrocortisone. Under the conditions used, the galactosyl transferase and α-lactalbumin were increased ten-fold and two-fold, respectively, by prolactin. Placental lactogen (human) was equally as effective as prolactin (ovine). In addition, inhibitors of protein synthesis prevented hormonal induction of the transferase and α-lactalbumin. The relative levels of the transferase and α-lactalbumin in the explants were similar to those observed in late pregnant mice, and the rate of synthesis of the transferase in explants appeared to be greater than that of α-lactalbumin, in accord with the asynchronous synthesis these workers observed for the two proteins *in vivo*. In subsequent studies PALMITER

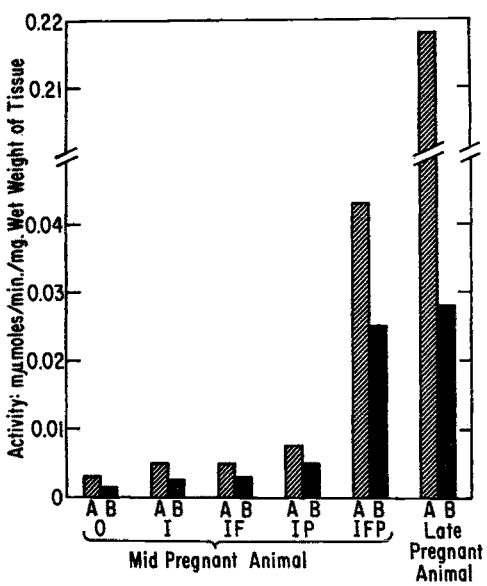


Fig. 9A and B. The effects of different concentrations of hormones on the levels of α-lactalbumin (B) and galactosyl transferase (A) in mouse mammary gland explants. Explants from mice (10–13 days pregnant) were incubated for 48 hours with the hormone combinations and then homogenized and assayed. *I* insulin, *F* hydrocortisone and *P* prolactin. From TURKINGTON et al., 1968

(1969) confirmed the hormonal requirements for lactose synthetase in mouse mammary explants. JONES and COWIE (1972) also found that prolactin stimulated lactose synthetase activity in hypophysectomized, lactating rabbits, which supports the important role of this hormone in regulating lactation.

VONDERHAAR et al. (1973) have examined in further detail the hormonal control of lactose synthetase in mouse mammary gland explants. They demonstrated that the induction of the galactosyl transferase and α-lactalbumin by insulin, hydrocortisone and prolactin differs in mammary tissue from pregnant and virgin mice. Mammary epithelium from mature virgin mice has very few cells with secretory characteristics and was found to contain little or no α-lactalbumin, very low levels of transferase and to synthesize only minute amounts of casein. Explants of mammary gland from virgin mice were cultured in the presence of the three hormones, and were found to develop the capacity to produce casein, α-lactalbumin and the transferase. Although maximal amounts of transferase and casein were obtained after 72 hours in culture, α-lactalbumin remained at low levels during this same period and reached maximal levels 24–48 hours later. In contrast, mammary gland explants from mice in midpregnancy synthesized casein at maximal rates and contained maximally inducible amounts of α-lactalbumin and galactosyl transferase at the same time (48 hours) after exposure to insulin, prolactin and hydrocortisone. Mammary explants from virgin mice pretreated with either 17 β-estradiol or prolactin, responded to prolactin, insulin and hydrocortisone in much the same way as explants from midpregnant animals. From these studies, prolactin was recognized to exert distinct effects on the components of lactose synthetase during pregnancy. First, prolactin is required for induction of maximal levels of α-lactalbumin at the same time that casein synthesis and galactosyl transferase levels are also maximal. Secondly, prolactin in synergy with insulin, is required for synthesis of casein and α-lactalbumin by cells which have previously developed secretory characteristics under the influence of insulin and hydrocortisone. This effect was also noted earlier for casein (OKA and TOPPER, 1971) and lactose synthetase (TURKINGTON et al., 1968). Finally, prolactin appears to be required for mammary cells to acquire a sensitivity to insulin and serum factors. Without this sensitivity, further growth and development of the gland is not obtained.

OWENS et al. (1972) have also demonstrated that the capacity to synthesize α-lactalbumin and casein is coupled to DNA synthesis, cellular development and cellular proliferation in mammary explants from virgin mice. In contrast, explants from midpregnant mice have the ability to synthesize these proteins under influence of hormones, but synthesis is not dependent on DNA synthesis and cell proliferation.

TURKINGTON and HILL (1969) proposed that progesterone may also be involved in regulating the synthesis of α-lactalbumin. Mammary gland explants from midpregnant mice induced synthesis of both the galactosyl transferase and α-lactalbumin in the presence of prolactin, insulin and hydrocortisone, as noted earlier (TURKINGTON et al., 1968), but in the presence of very low amounts of progesterone, α-lactalbumin production by the explants was repressed. Progesterone was without effect on the induction of synthesis of the galactosyl transferase under the same conditions. It is proposed that blood progesterone,

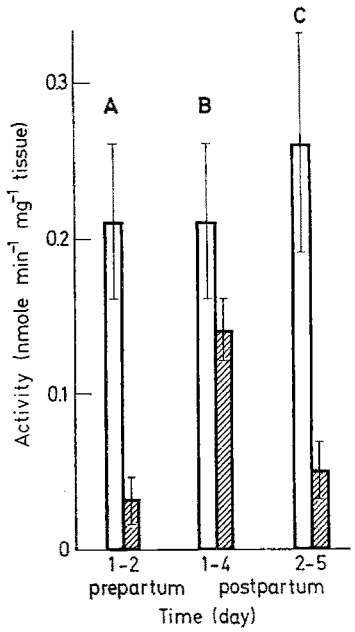


Fig. 10. The effect of progesterone on the levels of galactosyl transferase [open bars] and α-lactalbumin [hatched bars] in mammary glands from pre- and postpartum mice (C3H/HeJ). A and B. Levels without progesterone administration. C. Levels in mice injected daily with progesterone (1 mg) in lipid emulsion from 1–2 days prepartum to 2–5 days postpartum. From TURKINGTON and HILL, 1969

which is present in relatively high levels during pregnancy, inhibits synthesis of α-lactalbumin and that when progesterone levels fall at parturition, the rate of α-lactalbumin synthesis increases. In support of this proposal, it was also found that mammary glands from mice treated with progesterone from the final 1–2 days of pregnancy through 5 days of lactation, had much lower levels of α-lactalbumin than untreated animals (Fig. 10).

Related effects concerning the role of progesterone were noted by KUHN (1972) who found that the lactose content of rat mammary glands fell sharply after administration of gonadotropin. Ovariectomy of treated rats resulted in an increase in lactose concentration, suggesting that gonadotropin stimulates the ovaries to produce progesterone, which in turn, diminished lactose synthesis. Progesterone administration also appeared to lower lactose content of mammary glands of gonadotropin treated animals.

MURPHY et al. (1973) have extended the observations of KUHN (1972) and also showed that bilateral ovariectomy of pregnant rats resulted in an increased lactose content of mammary tissue. Lactose levels increased within 13 hours after ovariectomy and reached maximal levels after 30 hours. It is noteworthy that significant galactosyl transferase activity was detected in the late pregnant rat and increased 3.8 fold after ovariectomy. This increase was largely prevented by administration of progesterone. In addition, α-lactalbumin levels were very low at the time of ovariectomy but increased about 4-fold 30 hours later. Administration of progesterone prevented the rise in α-lactalbumin levels during this same period. These workers also measured other enzyme levels involved in biosynthesis of lactose and concluded that lactose synthetase activity is rate limiting in lactose biosynthesis from glucose, and that α-lactalbumin in particular determines the rate of lactose biosynthesis.

Although the exact mechanism of action of insulin, hydrocortisone and prolactin in lactogenesis and the control of lactose synthetase remains unknown, it is of interest that the requirement for hydrocortisone can be partially replaced by spermidine in mouse mammary explants (OKA, 1974). The α-lactalbumin levels of explants from midpregnant mice cultured in insulin, prolactin and spermidine was about two-thirds that found in explants cultured for the same period with the three hormones in the absence of spermidine. Spermidine is present in lactating mouse mammary glands, but further studies will be required to understand its action as well as its relationship to hydrocortisone.

VI. Organization of Lactose Synthetase and Its Control at the Subcellular Level

Lactose biosynthesis is regulated not only by molecular and hormonal means but also as the result of the subcellular organization of lactose synthetase in lactating secretory cells. Control of lactose synthesis by this means was first suggested by BREW (1969, 1970) who proposed a model for the compartmentation of the components of lactose synthetase and its substrates, which is consistent with present knowledge of the molecular and hormonal control mechanisms, the subcellular distribution of the galactosyl transferase and α-lactalbumin, as well as the biosynthesis and secretion of α-lactalbumin and other constituents of milk.

The fundamental features of the model for cellular control of lactose biosynthesis are shown diagrammatically in Fig. 11. Basically, the process of lactose

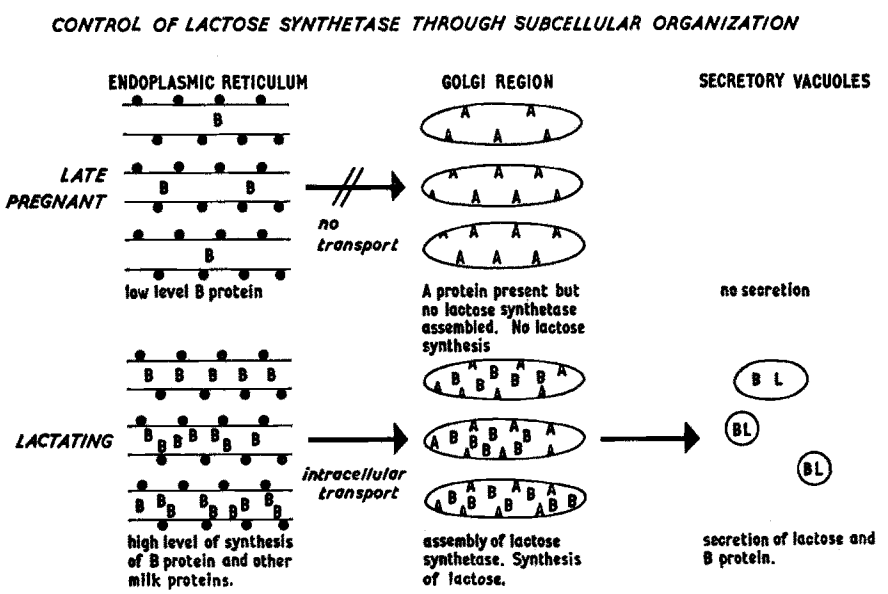


Fig. 11. A model for the cellular control of lactose biosynthesis by lactose synthetase in the mammary gland during pregnancy and lactation. *A* galactosyl transferase, *B* α-lactalbumin, *L* lactose

biosynthesis according to the model, is proposed to proceed as follows. α-Lactalbumin is continuously synthesized along with other specific milk proteins (principally casein) on ribosomes of the rough endoplasmic reticulum. After synthesis it and other milk proteins pass through the intracisternal space from the rough endoplasmic reticulum through the smooth endoplasmic reticulum into the Golgi apparatus. In the Golgi apparatus α-lactalbumin is available for reaction with the galactosyl transferase and lactose synthetase substrates, so as to promote lactose synthesis. Once lactose has been synthesized, α-lactalbumin, lactose, casein and other protein components are then secreted in membrane enclosed vacuoles by a process of exocytosis. The experimental basis for this model has been obtained from several observations including, the nature of particulate lactose synthetase, the biosynthesis of α-lactalbumin, the structure and enzymatic activities of Golgi membranes, hormonal control of the synthetase, as well as knowledge of the structure and function of the soluble transferase and α-lactalbumin. In the remainder of this section, the essential correctness of the model will be assumed, and the several diverse observations in its support will be discussed.

A. Particulate Lactose Synthetase

The subcellular control of lactose synthesis as proposed in Fig. 11 is consistent with studies of lactose synthetase in particles derived from homogenates of lactating tissue. Before the demonstration of the presence of two protein components in (milk) lactose synthetase, WATKINS and HASSID (1962) discovered that lactose synthetase activity in homogenates of lactating mammary tissue from guinea-pigs and cows is associated with a particulate fraction. This particulate fraction must therefore contain both the galactosyl transferase and α-lactalbumin maintained in close physical association. In homogenates of mammary tissue produced by a highly disruptive technique (Virtis overhead homogenizer) BRODBECK and EBNER (1966b) found that galactosyl transferase is bound to the microsomal fraction whereas α-lactalbumin is distributed between the microsomal and soluble cell fractions. Such a distribution for α-lactalbumin may result from the rather violent disruptive method used or from the presence of considerable quantities of milk in the tissue used. The association of newly-synthesized α-lactalbumin with particulate fractions of the mammary gland was indicated by studies of the biosynthesis of the protein by a cell-free system derived from the lactating guinea-pig mammary gland (BREW and CAMPBELL, 1967b). α-Lactalbumin synthesized in the system was presumably enclosed in microsomal vesicles as it was only released by disrupting the membranes by treatment with ultrasonic vibrations. Studies of lactose synthetase particles by COFFEY and REITHEL (1968a, b) have confirmed that both components are associated with a single particulate fraction. In this work, homogenization was accomplished by the more gentle procedure of pulverizing frozen mammary tissue followed by hand homogenization. After purification by differential centrifugation, lactose synthetase particles were investigated by electron microscopy and for sedimentation properties and enzymic activities. The particles were found to be vesicular

in structure, and their properties were closely similar to those of the membranes of the Golgi apparatus. One significant feature of these "lactose synthetase particles" was their response to treatment with ultrasonic vibrations or to homogenization with a Virtis Homogenizer. Under these treatments, lactose synthetase activity was lost, apparently as a result of a disruption of the close association of the two protein components, as it was restored by the addition of high concentrations of α-lactalbumin. Similar observations were made with particles obtained from homogenates of mouse mammary gland by PALMITER (1969), who concluded that intracellular lactose synthetase is an enzyme with very different properties from the soluble milk enzyme. It was assumed that the galactosyl transferase and α-lactalbumin are closely bound together, possibly through the mediation of a third component and that on sonication, the third component is destroyed with the production of a form of the enzyme more similar to that found in milk where the galactosyl transferase-α-lactalbumin association is loose and mediated by substrates. Subsequent studies have not supported this conclusion and no evidence for a third component has been found.

Lactose synthetase particles studied by COFFEY and REITHEL (1968a, b) and others can, on the basis of the model shown in Fig. 11, be interpreted as vesicular fragments pinched off from the Golgi complex during the process of homogenization. α-Lactalbumin is contained within the intravesicular contents, and can associate with the galactosyl transferase attached to the inner membrane surface. Treatment of these structures with ultrasonic vibrations or by vigorous homogenization will lead to a disruption of the membranes with a loss of the vesicular contents into the medium. The effective dilution of α-lactalbumin thus produced will lead, on the basis of the properties of the milk enzyme, to a decreased lactose synthetase activity which will only be restored by adding back high concentrations of α-lactalbumin, just as observed by COFFEE and REITHEL (1968a, b).

Experimental support for the model in Fig. 11 has also been obtained by KEENAN et al. (1969) who isolated highly purified lactose synthetase particles from homogenates of lactating rat mammary gland. Electron microscopic examination showed that the particles contained a large proportion of recognizable Golgi elements. The presence of the galactosyl transferase in the Golgi apparatus of other tissues has also been noted for bovine liver (FLEISCHER and FLEISCHER, 1970; FLEISCHER et al., 1969) and rat testis (CUNNINGHAM and MOLLENHAUER, 1970). The galactosyl transferase as well as a sialyl and an N-acetylglucosaminyl transferase, which are concerned in the synthesis of a terminal trisaccharide group of many exported glycoproteins were also found to be present in a single Golgi-derived particulate fraction in homogenates of rat liver (SCHACHTER et al., 1970).

B. Control through α-Lactalbumin Synthesis and Secretion

Unless α-lactalbumin plays a major nutritional role in milk, which seems improbable, as casein accounts for the overwhelming amount of the protein in milk, the secretion of large quantities of this protein can only be advantageous to the

animal if the process plays some role in the regulation of lactose biosynthesis. On the basis of the properties of lactose synthetase it would be expected that the flow of α-lactalbumin through the Golgi apparatus of the lactating mammary cell can potentially act as a controlling link between milk protein synthesis and lactose synthesis in the lactating mammary gland. Regulation of the rate of biosynthesis and secretion of α-lactalbumin (and hence its local concentration at the site of lactose synthesis) could therefore be an effective means of regulating lactose production in the mammary gland. The regulation of α-lactalbumin production could also potentially serve as an on-off switch for controlling the production of lactose, because its presence in mammary tissue is essential for lactose synthesis and cessation of α-lactalbumin synthesis would ultimately stop lactose production. That α-lactalbumin synthesis serves as an on-off switch for lactose production does not have firm experimental support. As noted in Section IV (hormonal control), somewhat conflicting results have been reported concerning the exact amounts of α-lactalbumin present in mammary tissue from rats, mice and cows during late pregnancy and early lactation, thus it is difficult to assess whether production of α-lactalbumin is the unique event controlling lactose synthesis. From the data available it appears that amounts of the transferase and α-lactalbumin required to support some lactose synthesis may be present a few days prior to parturition although the lactose content of the gland is not as high as that found in lactating tissue. It is possible that just prior to parturition, the gland is almost fully primed to assume full lactose synthesis but that its ultimate capacity is not realized until milk secretion commences. The fact that lactose can be detected in the blood and urine of ruminants just prior to parturition indicates that prelactational mammary cells have the capacity to synthesize lactose (WHEELOCK and ROOK, 1966). The fact that lactation has not commenced until parturition, however, may indicate that not only α-lactalbumin synthesis *but also its movement to the Golgi region during secretory activity* are required for maximal lactose synthesis. Whether other factors, including those suggested by PALMITER (1969) also play a role in regulating the lactose content of milk, remains unclear. Nevertheless in view of its unique requirement for lactose production at physiological levels, it is likely that α-lactalbumin synthesis and secretion may together be the major essential requirements for regulation of the lactose content of milk.

C. Control through Substrate Compartmentation and Transport

Within the cell, the site of lactose synthesis is segregated within the Golgi apparatus, whereas, the enzyme concerned in the final step in the synthesis of UDP-galactose (UDP-galactose 4-epimerase) appears to be present in the soluble cell fraction (COHN and SEGAL, 1969). Both substrates of lactose synthetase must therefore pass across the Golgi membrane to reach the enzyme. Although the properties of the Golgi membranes have not been subjected to investigation, it might be expected that glucose would pass freely across the membrane although some direct mechanism of transport for glucose from the outside of the cell to the site of lactose synthesis cannot be excluded. UDP-galactose would not be

expected to pass freely across the membrane and it seems quite likely that some transport system may operate for this sugar. It has been shown that the activity of lactose synthetase particles is stimulated by UTP, in contrast to the soluble enzyme which is inhibited by UTP (Coffey and Reithel, 1968b). An energy-driven transport process would explain this observation, and would constitute a possible point for the control of lactose biosynthesis.

D. Origin of Milk Galactosyl Transferase

Electron microscopic studies of lactating mammary cells (Helminen and Ericsson, 1968) and other tissues (e.g. Caro and Palade, 1964) suggest that the secretory vacuoles in which protein and lactose are secreted, are formed by budding off from the Golgi complex and are presumably composed from the membranes of the Golgi region. The mechanism by which the contents of these vacuoles are discharged into the lumen of the gland is thought to be one of reverse pinocytosis in which the vacuole membranes fuse with the cell surface and the vacuole emptied by being effectively turned inside out. During this process, the inner surface of the vacuole (formerly the intracisternal surface of the Golgi membranes) goes to form a section of the external surface of the cell. Thus the galactosyl transferase and other glycosyl transferases would be exposed on the cell surface and can dissociate into the milk. This is the most reasonable explanation for the presence of the transferase in soluble form in milk. The intracellular galactosyl transferase is presumably replenished simultaneously with the production of new Golgi membranes. As yet, the origin of these membranes in the animal cell is uncertain (see Beans and Kessel, 1968) and the site of synthesis of galactosyl transferase in the mammary cell and its mode of assembly into Golgi membranes is at present unknown.

E. Evolutionary Origins of the Organization of Lactose Synthetase

N-acetyllactosamine synthetase is widely distributed in different animal tissues and has also been found in non-mammalian species. In all cases where it has been tested (see Hill et al., 1968 and Schachter, 1970) the enzyme is inactive for lactose synthesis, but becomes active on the addition of α-lactalbumin. The most remarkable observation in this respect is the observation that Golgi membranes from onion tips synthesize lactose when bovine α-lactalbumin is present (Powell and Brew, 1974a). The distribution of this enzyme in birds as well as mammals and plants suggests that its activity in glycoprotein synthesis preceded the evolutionary development of lactose synthetase. As the evolutionary ancestor of the α-lactalbumins was probably a lysozyme and was certainly a secreted protein (as it contained disulphide linkage), it can be seen how the unique organization of lactose synthetase arose when mutational changes in the α-lactalbumin ancestor gave rise to lactose synthetase specified activity. The passage of this protein through the secretory pathway of the cell would automatically bring it into contact with the galactosyl transferase in the Golgi region of the cell and the full potential of the functional change exploited. The synthesis and secretion of lactose would then inevitably occur, assuming that the substrates were available.

The improved nutritional properties of the secretion so formed must have increased the chances of survival of the offspring of the animal, and the new biochemical system for lactose synthesis eventually became fixed in the population.

VII. Mechanism of Lactose Synthetase

A. Complex Formation between Galactosyl Transferase and α-Lactalbumin

For α-lactalbumin to exert its regulatory effects on galactosyl transferase, the formation of a complex between the two proteins is necessary. Thus, BREW et al. (1968) found that when α-lactalbumin and galactosyl transferase are separated by a dialysis membrane in the presence of UDP-galactose, Mn^{2+} and glucose, negligible synthesis of lactose occurred. The same authors deduced from the kinetic effects of α-lactalbumin that its attachment to galactosyl transferase or to a galactosyl transferase substrate complex is reversible in nature.

The first direct evidence pertaining to the nature of complexes formed between the two proteins was the observation by HILL, TRAYER and co-workers (TRAYER et al., 1970; TRAYER and HILL, 1971) and by ANDREWS (1970) that columns containing α-lactalbumin covalently bound to Sepharose, reversibly absorbed or retarded galactosyl transferase specifically in the presence of monosaccharides. N-acetylglucosamine was considerably more effective than glucose in promoting complex formation. Mawal and co-workers (MAWAL et al., 1971) subsequently found that attachment of galactosyl transferase to α-lactalbumin-Sepharose was promoted by several combinations of substrates and inhibitors, a combination of Mn^{2+} and UDP-galactose being particularly favorable for complex formation.

Recently ANDREWS et al. (1973) investigated the effects of varying concentrations of N-acetylglucosamine and glucose on the retardation of human galactosyl transferase on columns of human α-lactalbumin bound to Sepharose. The results were interpreted in terms of the following equilibrium reactions (3 and 4).

$$\text{Enzyme} + \text{MS} \rightleftharpoons \text{Enzyme} \cdot \text{MS} \tag{3}$$

$$\text{Enzyme} \cdot \text{MS} + \alpha\text{-lactalbumin} \rightleftharpoons \text{Enzyme MS} \cdot \alpha\text{-lactalbumin} \tag{4}$$

and values determined for the association constants (K_a) of galactosyl transferase with the monosaccharides. With N-acetylglucosamine, a value for K_a of 200 M^{-1} was obtained, and with glucose a value of 0.57 M^{-1}. These values are similar to the Michaelis constants for these monosaccharides determined, in the absence of α-lactalbumin, from kinetic studies. These quantitative studies have not yet been extended to investigate the binding of Mn^{2+} and UDP-galactose by the enzyme.

Using a different technical approach, KLEE and KLEE (1972) followed the sedimentation of bovine α-lactalbumin (labelled in the visible spectrum by nitration) in the presence of bovine milk galactosyl transferase and combinations of the substrates of lactose synthetase. A marked increase in the rate of sedimentation was observed only in the presence of a combination of Mn^{2+} and UDP-galactose. The interaction of the proteins in the presence of Mn^{2+} (30 mM) and N-acetylglucosamine (27 mM) was analyzed quantitatively by observing the

sedimentation of a band of galactosyl transferase through solutions containing varying concentrations of α-lactalbumin. Interaction was measured by the increase in sedimentation rate, and also as an increased area under the sedimenting band. With the implicit assumption that galactosyl transferase is saturated with N-acetylglucosamine under these conditions, the data were analyzed by a Scatchard plot, which indicated that complexes containing 1:1 molar ratios of the proteins were formed with an association constant (for E. GlcNAc + α-lactalbumin $\rightleftharpoons$ E. GlcNAc · α-lactalbumin) of about $10^5 M^{-1}$. Unfortunately, the experiments were not repeated in the presence of Mn^{2+} and UDP-galactose, even though these substrates had been shown to promote the formation of a complex. That 1:1 complexes of α-lactalbumin and galactosyl transferase are formed in the presence of N-acetylglucosamine has also been shown by ANDREWS (1970) using gel filtration and by IVATT and ROSENMEYER (1973) using sedimentation equilibrium ultracentrifugation.

It is evident that several types of complex can be formed between the two components of lactose synthetase. Under equilibrium conditions, the association can be interpreted in terms of reactions 5 and 6.

$$E + A \rightleftharpoons EA \tag{5}$$

$$EA + \alpha\text{-lactalbumin} \rightleftharpoons EA \cdot \alpha\text{-lactalbumin} \tag{6}$$

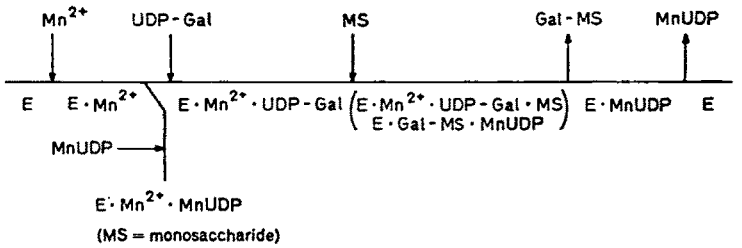
where A can be N-acetylglucosamine, glucose or a combination of Mn^{2+} and UDP-galactose. The ordered attachment of a substrate and α-lactalbumin may reflect that a conformational change in the galactosyl transferase must occur before α-lactalbumin can attach. Although the association constants for galactosyl transferase with monosaccharides and for galactosyl transferase substrate complex with α-lactalbumin determined from the binding studies are of some interest, they do not indicate which types of complex are significant in the catalytic functioning of lactose synthetase. The only presently available information on this has been obtained from steady-state kinetic studies with the enzyme system.

B. Kinetic Mechanism of Lactose Synthetase

A satisfactory kinetic mechanism for lactose synthetase must encompass the ability of the galactosyl transferase to catalyze disaccharide synthesis in the absence of α-lactalbumin, as well as the effects of α-lactalbumin in the system. Major effects of α-lactalbumin are threefold; (1) promotion of lactose synthesis, involving the reduction in the K_m for glucose [BREW et al., 1968; ANDREWS, 1970; KLEE and KLEE (1970)]; (2) inhibition of N-acetyllactosamine synthesis at concentrations of N-acetylglucosamine above 3 mM, and enhancement of synthesis at concentrations below 3 mM (BREW et al., 1968; KLEE and KLEE, 1970), and (3) a lack of inhibition by α-lactalbumin with oligomers of N-acetylglucosamine or glycoproteins as substrates (HILL et al., 1968; SCHANBACHER and EBNER, 1970).

The obvious initial consideration in a kinetic analysis of the enzyme system is the mechanism of the galactosyl transferase in the absence of α-lactalbumin. In separate studies, MORRISON and EBNER (1971 a), using partially purified bovine milk galactosyl transferase and KHATRA et al. (1974) using pure human milk galactosyl transferase (50000 mol. wt.) came to similar conclusions regarding the mechanism of disaccharide synthesis in the absence of α-lactalbumin. Mn^{2+},

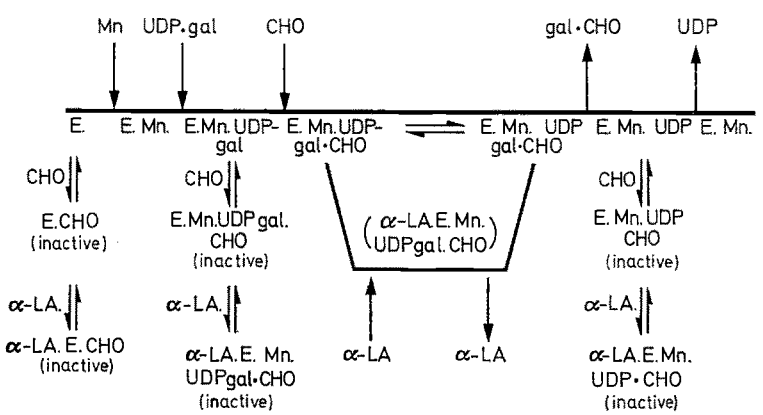
UDP-galactose and N-acetylglucosamine add to the enzyme in an ordered sequential manner, and the products are released in an ordered manner, disaccharide followed by UDP and Mn^{2+}. MORRISON and EBNER (1971a) were unable to investigate inhibition of the enzyme by UDP, while KHATRA et al. (1974) concluded from studies of the inhibition by the Mn^{2+} complex of UDP, that this complex is the most probable final product released by human galactosyl transferase under their assay conditions. Their proposed mechanism is shown in Scheme I.



Scheme I. Kinetic mechanism of the galactosyl transferase. From MORRISON and EBNER (1971a)

Additional features of the mechanism deduced from the study with the human enzyme were that the Mn^{2+} complex of UDP-galactose can act as a substrate and that the binding of UDP-galactose to EMn^{2+} is virtually irreversible.

For reactions catalyzed in the presence of α-lactalbumin, MORRISON and EBNER (1971b, c) deduced that a similar ordered sequential addition of substrates (Mn^{2+}, UDP-galactose and then glucose) to the galactosyl transferase occurs. They suggest that α-lactalbumin attaches to the enzyme after the addition of all three substrates, forming an enzyme Mn^{2+}· UDP-galactose · glucose · α-lactalbumin complex, and exerts its activating effects by increasing the concentration of complexes containing all three substrates (i.e. central complexes) through mass action. The inhibition by α-lactalbumin of N-acetyllactosamine synthesis, at higher concentrations of N-acetylglucosamine, which is uncompetitive in nature with respect to Mn^{2+} and UDP-galactose is not easily explained by this mechanism (Scheme II).



Scheme II. A proposed kinetic mechanism for lactose synthetase. From MORRISON and EBNER (1971c)

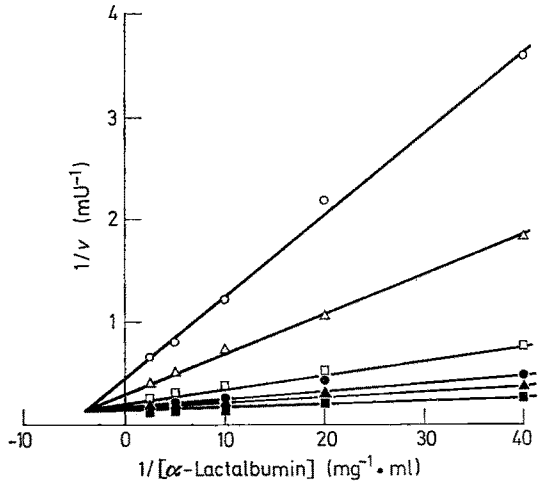


Fig. 12. A double reciprocal plot showing the effect of different concentrations of human α-lactalbumin on the rate of lactose synthesis by human milk galactosyl transferase at different fixed concentrations of glucose. The concentrations of Mn^{2+} and UDP-galactose were fixed at 10 mM and 0.63 mM respectively and the concentrations of glucose were: ○, 1 mM; △, 2 mM; □, 5 mM; ●, 10 mM; ▲, 15 mM; ■, 20 mM. Taken from KHATRA, HERRIES and BREW (1974)

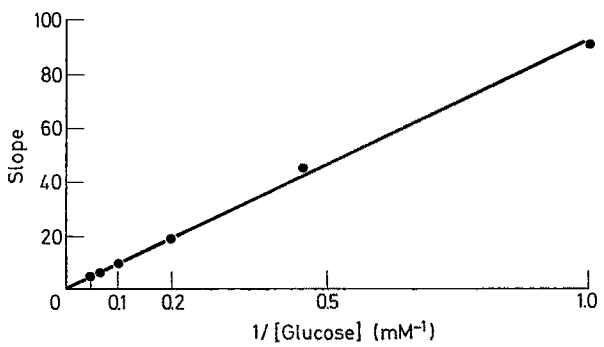


Fig. 13. A replot of the slopes of the lines from Fig. 12 against 1/glucose

In their kinetic studies with human galactosyltransferase, in the presence of human or bovine α-lactalbumins, KHATRA et al. (1974) observed a characteristic kinetic interconnection between α-lactalbumin and glucose. As shown in Figs. 12–14 for lactose synthesis in the presence of human α-lactalbumin, double reciprocal plots for initial velocity and α-lactalbumin concentration at a series of fixed concentrations of glucose consist of a series of lines intersecting to the left of the vertical axis. Replotting the slopes of these lines against the reciprocal of the glucose concentration generates a straight line that passes through the origin. Alternatively, the same data may be visualized as double reciprocal plots for initial velocity and glucose concentration at a series of fixed concentrations of α-lactalbumin, which consist of a series of lines intersecting on the 1/velocity

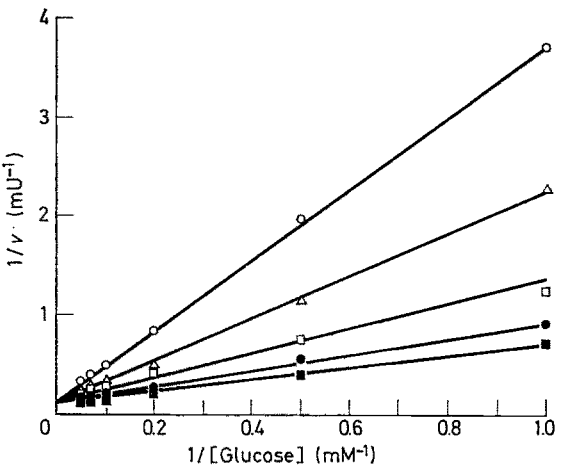


Fig. 14. A double reciprocal plot obtained using the same data from Fig. 12 with glucose as the variable substrate. The concentrations of human α-lactalbumin were: ○, 0.025 mg/ml; △, 0.05 mg/ml; □, 0.10 mg/ml; ●, 0.20 mg/ml; ■, 0.40 mg/ml

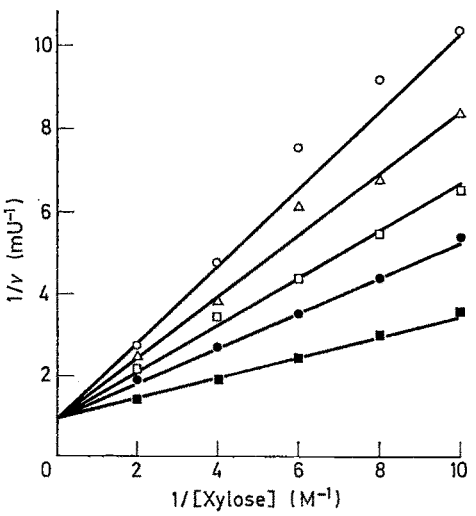


Fig. 15. A double reciprocal plot showing the effect of xylose concentrations on the rate of disaccharide (gal β1–4 xylose) synthesis by human milk galactosyl transferase at different fixed concentrations of human α-lactalbumin. The concentrations of Mn^{2+} and UDP-galactose were held constant at 10 mM and 0.63 mM respectively and the concentrations of α-lactalbumin were: ○, 0.05 mg/ml; △, 0.063 mg/ml; □, 0.083 mg/ml; ●, 0.125 mg/ml; ■, 0.25 mg/ml. Taken from KHATRA, HERRIES and BREW (1974)

axis. The kinetic patterns are observed when bovine α-lactalbumin is substituted for the human protein, and when alternative monosaccharide acceptors are substituted for glucose. For example D-xylose is a virtually unacceptable substrate for the human galactosyl transferase in the absence of α-lactalbumin (K_m of

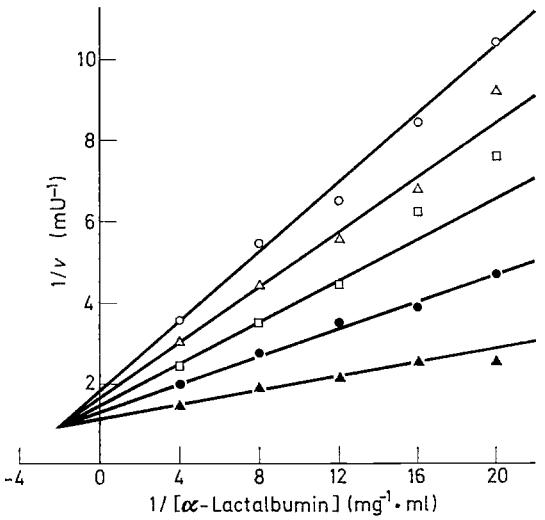


Fig. 16. A replot of the data from Fig. 14 with α-lactalbumin as the variable substrate. The concentrations of xylose were: ○, 0.10 M; △, 0.125 M; □, 0.17 M; ●, 0.25 M; ▲, 0.50 M

about 30 M) but becomes a progressively better substrate at increasing concentrations of human α-lactalbumin with kinetic patterns exactly similar to those observed with glucose (see Figs. 15–16). Extrapolation to conditions of saturation with respect to α-lactalbumin gives a K_m for xylose of 81 mM. This kinetic behavior in the enzyme system can only be described by rate equations in which the Michaelis constant for α-lactalbumin is zero, which indicates that the binding of α-lactalbumin is a rapid-equilibrium step that precedes the binding of monosaccharides within an ordered steady-state mechanism. As inhibition patterns indicate that UDP-galactose binds to the galactosyl transferase prior to α-lactalbumin and glucose, reaction 7 and 8 must occur in the reaction mechanism.

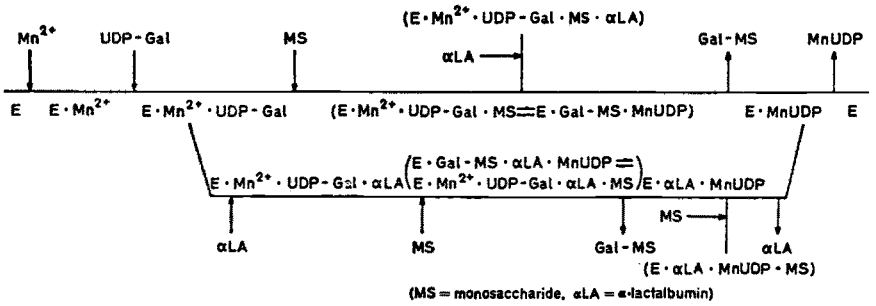
$$E \cdot Mn^{2+} \cdot \text{UDP-galactose} + \alpha\text{-lactalbumin} \rightleftharpoons E \cdot Mn^{2+} \cdot \text{UDP-galactose} \cdot \alpha\text{-lactalbumin} \quad (7)$$

$$E \cdot Mn^{2+} \cdot \text{UDP-galactose} \cdot \alpha\text{-lactalbumin} + \text{glucose} \rightleftharpoons E \cdot Mn^{2+} \cdot \text{UDP-galactose} \cdot \alpha\text{-lactalbumin} \cdot \text{glucose} \quad (8)$$

As $E \cdot Mn^{2+} \cdot$ UDP-galactose is the first complex in the ordered reaction mechanism, to which, according to binding studies, α-lactalbumin can attach (see section A), reaction (1) is therefore consistent with the results of binding studies.

If reaction (1) is considered to be at thermodynamic equilibrium, then at infinitely high concentrations of glucose, the reaction rate will be effectively independent of the concentration of α-lactalbumin, because only one mole of α-lactalbumin will be required per mole of enzyme to obtain a maximum velocity at saturating glucose concentrations. As this amount of α-lactalbumin is negligable compared with the total amount present, the Michaelis constant for α-lactalbumin

appears to be zero, and double reciprocal plots for glucose (or other monosaccharides) as variable substrate at different concentrations of α-lactalbumin will intersect on the 1/velocity axis, whereas the corresponding plots with α-lactalbumin as variable substrate at fixed concentrations of glucose will intersect to the left of the vertical axis, as observed. KHATRA et al. (1974) therefore suggest that the kinetic effects observed with galactosyl transferase in the presence and absence of α-lactalbumin can be explained in terms of the general mechanism shown in Scheme III.



Scheme III. The kinetic mechanism for lactose synthetase. From KHATRA et al. (1974)

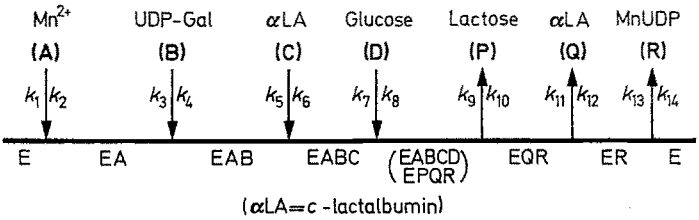
The upper line in this scheme is the same as the mechanism deduced for the galactosyl transferase in the absence of α-lactalbumin. The lower pathway incorporates the steps in the mechanism deduced from the kinetic effects of α-lactalbumin. It is proposed that in this pathway α-lactalbumin attaches to an enzyme · Mn^{2+} · UDP-galactose complex, prior to monosaccharide, to produce a new enzyme form which has a greatly increased affinity for monosaccharide, thus generating the characteristic activation effects observed.

The activation of galactosyl transferase reaction with N-acetylglucosamine as substrate (i.e. N-acetyllactosamine synthesis) by α-lactalbumin at N-acetylglucosamine concentrations below 3 mM is suggested to occur because under these conditions the rate of formation of E · Mn^{2+} · UDP-gal · GlcNAc is slow. Most reaction flux then occurs through the lower pathway in which α-lactalbumin exerts its activating effects. At higher concentrations of N-acetylglucosamine, the rate of formation of E · Mn^{2+} · UDP-galactose · GlcNAc is higher, diverting flow into the upper pathway. It is proposed that α-lactalbumin inhibits this pathway by attaching to a central complex (E · Mn^{2+} · UDP-gal · GlcNAc or E · MnUDP · Gal-GlcNAc) to form a dead-end inhibitory complex.

Such inhibition should be uncompetitive with respect to Mn^{2+} and UDP-galactose, as observed. α-Lactalbumin is not an effective inhibitor of galactose transfer to N-acetylglucosamine oligomers or to ovalbumin (SCHANBACHER and EBNER, 1970) which can be suggested to result from the steric prevention of α-lactalbumin from binding to a central complex containing high molecular weight substrates. It is also interesting to note that ANDREWS (1972) has observed that activation and inhibition of N-acetyllactosamine synthesis by α-lactalbumin is sensitive to temperature. At 25°, inhibition, but not activation, is observed. The inhibition, which can be observed at varying N-acetylglucosamine concen-

trations under these conditions was found to be uncompetitive with respect to N-acetylglucosamine, as well as to Mn^{2+} and UDP-galactose. This can only be attributed to the binding of α-lactalbumin to a complex containing all three substrates. The loss of activation at the lower temperature can be explained as a result of a large decrease in the rate of attachment of α-lactalbumin to E · Mn^{2+} · UDP-galactose, thus eliminating the lower pathway in Scheme III.

For glucose at millimolar concentrations and xylose at sub-molar concentrations, the upper pathway is insignificant, because the galactosyl transferase alone has a low affinity for these substrates. The mechanism (lower pathway only) then becomes the ordered sequential mechanism shown in Scheme IV.



Scheme IV. The ordered-sequential mechanism for lactose synthetase. From KHATRA et al. (1974)

In the absence of products (apart from α-lactalbumin which is both substrate and product), and assuming that k_6 is overwhelmingly greater than the maximum velocity in the reaction scheme, i.e. the binding of α-lactalbumin is at thermodynamic equilibrium, the following rate equation (9) was derived.

$$\frac{1}{V} = \frac{K_{ia}K_{ib}K_{ic}K_d}{V_1ABCD} + \frac{K_{ib}K_{ic}K_d}{V_1BCD} + \frac{K_{ic}K_d}{V_1CD} + \frac{K_{ia}K_b}{V_1AB} + \frac{K_d}{V_1D} + \frac{K_b}{V_1B} + \frac{K_1}{V_1A} + \frac{1}{V_1}. \quad (9)$$

A, B, C and D are the concentrations of Mn^{2+}, UDP-galactose, α-lactalbumin and glucose, respectively, K_a, K_b and K_d are Michaelis constants for Mn^{2+}, UDP-galactose and glucose, respectively, and K_{ia}, K_{ib} and K_{ic} are the dissociation constants for Mn^{2+} from E · Mn^{2+}, for UDP-galactose from E · Mn^{2+} · UDP-galactose and for α-lactalbumin from E · Mn^{2+} · UDP-galactose · α-lactalbumin, respectively.

The values of some of these parameters for the human galactosyl transferase—human α-lactalbumin system are given Table 4. Different α-lactalbumins can be characterized in terms of the dissociation constant K_{ic} (for α-lactalbumin from E · Mn · UDP-galactose · α-lactalbumin), and K_d, the Michaelis constant for glucose (at saturating α-lactalbumin concentration) and a maximum velocity (V_{max}). Of four α-lactalbumins examined (human, bovine, pig and guinea-pig), two showed the rapid equilibrium binding patterns discussed above (human and bovine), whereas the other two showed different kinetic patterns which indicate that the binding of those α-lactalbumins to E · Mn^{2+} · UDP-galactose is not at thermodynamic equilibrium during the reaction cycle. In the case of these proteins an additional term is present in the denominator of the rate equation, KcABD,

Table 4. Apparent and true values for kinetic constants associated with human lactose synthetase

Substrate	Kinetic constant	Pair of substrates varied	Apparent value (mM)	True value (mM)	Mean (mM)
Mn^{2+}	K_{ia}				1.42
UDP-galactose	K_b	B and C	0.14	0.135	
		B and D	0.21	0.184	0.16
	K_{ib}	B and C	0	0	
		B and D	0	0	0
α-lactalbumin	K_c	C and D (glucose or xylose)	0	0	0
	K_{ic}	C and D (glucose)	0.029	0.029	
		C and D (xylose)	0.036	0.086	
Glucose	K_d	B and D	14.9	2.31	
		C and D	1.4	1.87	2.1
Xylose	K_d	C and D	81		
GlcNAc	K_d	C and D	2.7		

From KHATRA et al., 1974. B UDP-galactose; C α-lactalbumin; D glucose or xylose.

Table 5. A comparison of the kinetic constants associated with different α-lactalbumins for lactose synthesis with human galactosyl transferase

α-Lactalbumin	K_c (mM)	K_{ic} (mM)	K_d (mM)	V_1 (milliunits)	% Lactose in milk
Human	0	0.029 0.036	1.4	6.29	7.0
Bovine	0	0.028	0.89	4.49	4.4
Pig	0.0004	0.034	0.87	4.23	3.8
Guinea-pig	0.0026	0.076	0.71	3.32	2.8

From KHATRA et al., 1974.

where Kc is a Michaelis constant for the α-lactalbumin. A comparison of the kinetic parameters associated with the 4 α-lactalbumins is given in Table 5 can be seen that considerable differences exist between the α-lactalbumins with respect to their association constants for the human galactosyl transferase · Mn · UDP-galactose complex and also in their relative V_{max} values. The latter parameter shows an interesting relationship with the lactose content of the milk of the species from which the α-lactalbumin was obtained, suggesting that species variation in the structure and activity of α-lactalbumin may to some degree control species differences in milk lactose levels. The same differences between the activities of

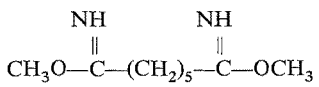
the α-lactalbumin seem to persist irrespective of the source of galactosyl transferase.

The role played by α-lactalbumin in the mechanism proposed by KHATRA et al. (1974) is clearly that of an activator of monosaccharide binding to the galactosyl transferase. It is closely similar both in kinetic effects (rapid equilibrium ordered binding of activator, followed by substrates) and in functional nature to the role played by metals in certain enzymes [for a recent example with pig liver pyruvic carboxylase, see WARREN and TIPTON (1974)]. It was suggested on this basis that α-lactalbumin may contribute directly to a binding site for monosaccharides in the enzyme Mn^{2+}· UDP-galactose · α-lactalbumin complex.

Taking into account the structural similarity of α-lactalbumin to lysozyme, and the ability of the cleft region of lysozyme to bind monosaccharides, it was speculated that the attachment of α-lactalbumin to the enzyme Mn^{2+}· UDP-galactose complex may bring the surface region of α-lactalbumin which corresponds to the binding site in lysozyme, into proximity with the monosaccharide binding site of the galactosyl transferase. If this region of α-lactalbumin can provide additional favorable interactions for monosaccharides, all the observed kinetic effects would follow.

C. Crosslinked Complexes of α-Lactalbumin and Galactosyl Transferase

As an alternative approach to the mechanism of lactose synthetase, BREW et al. (1974) have investigated the crosslinking of bovine α-lactalbumin and bovine milk galactosyl transferase with the bifunctional reagent dimethylpimelimidate.



At pH 8.1, α-lactalbumin and galactosyl transferase are crosslinked to form 1:1 complexes only in the presence of substrates. N-acetylglucosamine or a combination of Mn^{2+} and UDP-galactose were particularly effective in promoting crosslinking, but glucose or MnUDP were less effective (Fig. 17). The crosslinked complexes were separated from uncrosslinked materials by gel filtration and affinity chromatography with α-lactalbumin-Sepharose 4B. They were found to have a high activity for lactose synthesis at low concentrations of glucose which was not affected by the addition of exogenous α-lactalbumin (see Fig. 18). The activity of the complexes for N-acetyllactosamine synthesis was relatively lower and they showed no activity for transfer to the oligosaccharide moiety of ovalbumin (a substrate for uncrosslinked galactosyl transferase). More detailed kinetic studies indicated that while the kinetic parameters associated with Mn^{2+} and UDP-galactose are little different from those of the normal galactosyl-transferase, the Michaelis constants for monosaccharides (glucose, xylose and N-acetylglucosamine) were reduced by several orders of magnitude by the attachment of α-lactalbumin (see Tables 6 and 7). In addition, the turnover number of the enzyme is reduced by 100. The properties of the complex were interpreted as showing the maximum activation effects by α-lactalbumin (i.e., lowered K_m values for

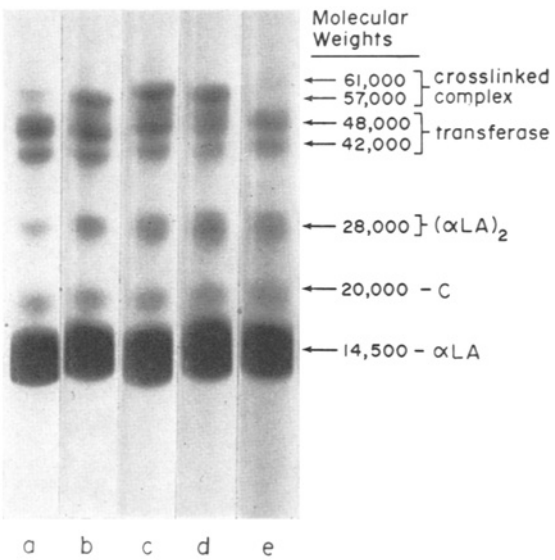


Fig. 17a—e. Analysis of the crosslinking of α-lactalbumin and galactosyl transferase by dimethylpimelimidate in the presence of N-acetyl-glucosamine by gel electrophoresis in sodium dodecyl sulfate. α-Lactalbumin (1 mg/ml), galactosyl transferase (0.1 mg/ml), N-acetylglucosamine (50 mM) and dimethylpimelimidate (1 mg/ml) in 0.2 M triethanolamine, pH 8.1, were incubated at 23°. Samples were removed at intervals and mixed with an equal volume of 1 M glycine, pH 8.5, exhaustively dialyzed against water, lyophilized and analyzed on the gels. The gel patterns shown were after reaction for 3 minutes (a), 30 minutes (b), 60 minutes (c) and 120 minutes (d). Gel (e) is the pattern after crosslinking for 120 minutes exactly as described above except that N-acetylglucosamine was not present in the reaction mixture. From BREW et al. (1974)

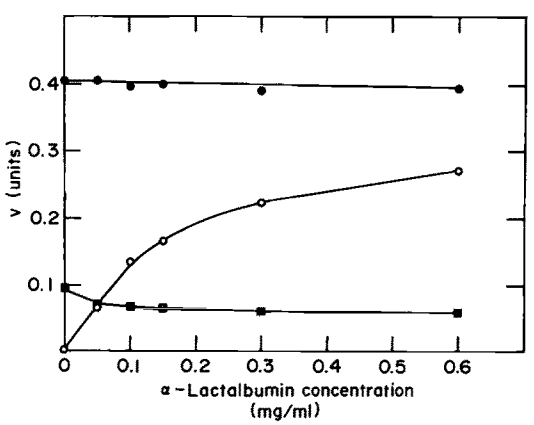


Fig. 18. Lactose synthetase activity (●) and N-acetyllactosamine synthetase activity (■) of a crosslinked α-lactalbumin-galactosyl transferase complex and lactose synthetase activity of uncrosslinked galactosyl transferase (○) as a function of α-lactalbumin concentration

monosaccharides) in combination with maximum product inhibition by α-lactalbumin (low V_{max}). A 1:1 complex of galactosyl transferase and α-lactalbumin is therefore necessary and sufficient for the exertion of the maximum effects by

Table 6. Comparison of the apparent kinetic parameters associated with different monosaccharides as substrates of galactosyl transferase and the crosslinked complex

Monosaccharide	Enzyme			
	Galactosyl transferase		Complex	
	app. K_m (mM)	V_m (mU/mg)	app. K_m (mM)	V_m (mU/mg)
N-acetylglucosamine	7.35	15000	0.036	42
Glucose	1000	—	0.35	137
Xylose	∞	—	24	66

From BREW et al. (1974).

Table 7. A comparison of the kinetic constants associated with substrates for lactose synthesis by the crosslinked complex and galactosyl transferase

Substrate	Complex			Human galactosyl transferase (Ref.: 6)
	Kinetic constant	Apparent value	True value	
Mn^{2+} (A)	K_a	0.15 mM	0.16 mM	0.083 mM[a]
	K_{ia}	1.64 mM	1.64 mM	1.42 mM
UDP-galactose	K_{ib}	0	0	0
	K_b	0.025 mM	0.027 mM	0.024 mM
Glucose	K_c	0.33 mM	0.33 mM	2260 mM

[a] Not determined for lactose synthesis by galactosyl transferase. The value given is for N-acetyllactosamine synthesis. From BREW et al. (1974).

α-lactalbumin. When crosslinked with galactosyl transferase, α-lactalbumin cannot be exerting mass action effects in the system. The kinetic properties of the complex therefore strongly indicate that α-lactalbumin acts at the level of monosaccharide binding in the enzyme system. The results of the crosslinking studies, including the conditions which are most favorable for the production of crosslinked complexes (i.e. presence of Mn^{2+} and UDP-galactose, or N-acetylglucosamine) are entirely consistent with the mechanism proposed by KHATRA (1974), but are not easily reconciled with the role proposed for α-lactalbumin by MORRISON and EBNER (1971c).

VIII. Future Studies on Lactose Synthetase

Although our present understanding of lactose biosynthesis and lactose synthetase has increased considerably over the past decade, several problems remain unanswered and much experimental work will be required to obtain vital information that is needed to understand lactose synthetase in detail.

Further information about the structure-function relationships of the galactosyl transferase and α-lactalbumin is essential. The successful elucidation of the covalent structures of α-lactalbumins from several animal species has clearly established its structural similarity to certain animal lysozymes and provides a reasonably clear view of its evolutionary origins. Nevertheless, the remarkable structural similarities among α-lactalbumin and lysozymes has shed very little light on the exact role of α-lactalbumin in lactose synthetase. α-Lactalbumin itself is not an enzyme but acts only when interacting with the transferase. In addition, lysozyme cannot replace α-lactalbumin in lactose synthetase. Thus, it is essential to determine whether α-lactalbumin, in accord with earlier predictions, does indeed have a conformation similar to that of the lysozymes. X-ray crystallographic analysis of α-lactalbumin for determination of its three-dimensional structure is perhaps one of the most important studies remaining incomplete. If the conformation of α-lactalbumin is similar to that of egg-white lysozyme, then perhaps the considerable body of knowledge about the structure-function relationships of lysozyme (IMOTO et al., 1972) could prove useful in deducing the exact role α-lactalbumin plays in lactose synthetase. Whether or not its conformation is similar, however, this information should prove valuable in designing further experiments concerning the function of α-lactalbumin.

The detailed structure of the galactosyl transferase is virtually unknown. Its covalent structure has not been established, its active site has not been probed either structurally or functionally, and very little is known of the nature of its interaction in structural terms with α-lactalbumin. This information may be very valuable but it may also be inadequate to provide a complete understanding of lactose synthetase in molecular terms. Such understanding is likely to be obtained only after crystallographic analysis of the transferase and α-lactalbumin as well as the transferase-α-lactalbumin complex.

Further understanding of lactose synthetase in a more biological sense is also clearly needed. The hormonal regulation of the two components of the synthetase remains unclear in view of the somewhat conflicting reports at present. Some of the more descriptive aspects of hormonal regulation should be easily clarified by additional, well controlled studies, although understanding hormonal regulation in molecular terms will likely emerge only as the molecular basis of hormone action in general also advances. In addition, the fundamental aspects of the subcellular organization of the synthetase are apparently known but further knowledge of the properties of the synthetase bound to Golgi membranes must be assessed in order to ascertain whether studies with the soluble synthetase reflect its behavior intracellularly.

Finally, lactose synthetase appears unique in many respects and another completely analogous enzyme system has not been identified at present. Other enzymes which require two quite dissimilar subunits, each of which is synthesized separately and interact only during cellular secretion within a short period of time required for a special physiological function (lactation) are unknown. Because of the close structural and evolutionary relatedness among α-lactalbumin and lysozymes, it is possible that lysozyme serves a regulatory role as yet unidentified. Evidence for such an enzymic function for lysozyme is totally lacking but perhaps should be kept in mind in the future studies.

References

ACHTER, E. K., SWAN, I. D. A.: On the conformation of lysozyme and α-lactalbumin in solution. Biochemistry **10**, 2976—2978 (1971).

ANDREWS, P.: Lactose synthetase A protein from human milk. Biochem. J. **111**, 14P–15P (1968).

ANDREWS, P.: Purification of lactose synthetase A protein from human milk and demonstration of its interaction with α-lactalbumin. FEBS Lett., **9**, 297–300 (1970).

ANDREWS, P.: The effect of temperature on a reaction catalyzed by lactose synthetase A protein. FEBS Lett. **26**, 333–335 (1972).

ANDREWS, P., KITCHEN, B. J., WINZOR, D. J.: Use of affinity chromatography for the quantitative study of acceptor-ligand interactions: the lactose synthetase system. Biochem. J. **135**, 897–900 (1973).

ATASSI, M. Z., HABEEB, A. F. S. A., RYDSTEDT, L.: Lack of immunochemical cross-reaction between lysozyme and α-lactalbumin and comparison of their conformations. Biochim. Biophys. Acta **200**, 184–187 (1970).

AUNE, K.: The thermodynamics of the denaturation of lysozyme. Ph. D. Dissertation, Duke University 1968.

BABAD, H., HASSID, W. Z.: A soluble lactose-synthesizing enzyme from bovine milk. J. Biol. Chem. **239**, PC-946-948 (1964).

BABAD, H., HASSID, W. Z.: Soluble uridine diphosphate: D-glucose β-4-0-galactosyl transferase from bovine milk. J. Biol. Chem. **241**, 2672–2678 (1966).

BALDWIN, R. L., MULLIGAN, L. P.: Enzymatic changes associated with the initiation and maintenance of lactation in the rat. J. Biol. Chem. **241**, 2058–2066 (1966).

BAREL, A. O., PRIEELS, J. P., MAES, E., LOOZE, Y., LÉONIS, J.: Comparative physicochemical studies of human α-lactalbumin and human lysozyme. Biochim. Biophys. Acta **257**, 288–296 (1972a).

BAREL, A. O., TURNEER, M., DOLMANS, M.: Spectral studies of the interaction of bovine α-lactalbumin and egg-white lysozyme with 2-p-toluidinyl naphthalene-6-sulfonate. Eur. J. Biochem. **30**, 26–32 (1972b).

BARMAN, T. E.: Purification and properties of bovine milk glyco-α-lactalbumin. Biochim. Biophys. Acta **214**, 242–244 (1970).

BARMAN, T. E.: The modification of the tryptophan residues of bovine α-lactalbumin with 2-hydroxy-5-nitrobenzyl bromide and with dimethyl (2-hydroxy-5-nitrobenzyl) sulphonium bromide. I. Characterization of the modified protein. Biochim. Biophys. Acta **258**, 297–313 (1972).

BARMAN, T. E., BAGSHAW, W.: The modification of the tryptophan residues of bovine α-lactalbumin with 2-hydroxyl-5-nitrobenzyl bromide and with dimethyl-(2-hydroxy-5-nitrobenzyl)-sulphonium bromide. II. Effect on the specifier protein activity. Biochim. Biophys. Acta **278**, 491–500 (1972).

BARKER, R., OLSEN, K., SHAPER, J. H., HILL, R. L.: Agarose derivatives of uridine diphosphate and N-acetylglucosamine for the purification of a galactosyl transferase. J. Biol. Chem. **247**, 7135–7147 (1972).

BARKER, R., TRAYER, I. P., HILL, R. L.: Nucleoside phosphates attached to agarose. Methods in Enzymol., in press (1974a).

BARKER, R., TRAYER, I. P., HILL, R. L.: Monosaccharides attached to agarose. Methods in Enzymol., in press (1974b).

BAXTER, C. F., KLEIBER, M., BLACK, A. L.: The bood precursors of lactose as studied with ^{14}C-labeled metabolites in intact dairy cows. Biochim. Biophys. Acta **21**, 277–285 (1956).

BEANS, H. W., KESSEL, R. G.: The Golgi apparatus, structure and function. In "International Review of Cytology" **23**, 209–276. Ed. BOURNE, G. H., and DANIELLI, J. F., NewYork and London: Academic Press 1968.

BEITZ, D. C., MOHRENWEISER, H. W., THOMAS, J. W., WOOD, W. A.: Synthesis of milk proteins in a cell-free system isolated from lactating bovine mammary tissue. Arch. Biochem. Biophys. **132**, 210–222 (1969).

BLAKE, C. C. R., MAIR, G. A., NORTH, A. C. T., PHILLIPS, D. C., SARMA, V. R.: On the conformation of the hen egg-white lysozyme molecule. Proc. Roy. Soc. B **167**, 365–377 (1967).

BREW, K., CAMPBELL, P. N.: The characterization of the whey proteins of guinea pig milk. The isolation and characterization of α-lactalbumin. Biochem. J. **102**, 258–264 (1967a).

BREW, K., CAMPBELL, P. N.: Studies on the biosynthesis of protein by lactating guinea pig mammary gland. Characteristics of the synthesis of α-lactalbumin and total protein by slices and cell-free systems. Biochem. J. **102**, 265–274 (1967b).

BREW, K., VANAMAN, T. C., HILL, R. L.: Structural studies on α-lactalbumin, the B protein of lactose synthetase. Fed. Proc. **26**, 724 (1967a).

BREW, K., VANAMAN, T. C., HILL, R. L.: Comparison of the amino acid sequence of bovine α-lactalbumin and hens egg-white lysozyme. J. Biol. Chem. **242**, 3747–3749 (1967b).

BREW, K., VANAMAN, T. C., HILL, R. L.: The role of α-lactalbumin and the A protein in lactose synthetase: a unique mechanism for the control of a biological reaction. Proc. Natl. Acad. Sci. (U.S.) **59**, 491–497 (1968).

BREW, K.: Secretion of α-lactalbumin into milk and its relevance to the organization and control of lactose synthetase. Nature **223**, 671–672 (1969).

BREW, K.: Lactose synthetase: evolutionary origins, structure and control. "Essays in Biochemistry" P. N. CAMPBELL and F. DICKENS, eds., **6**, 93–118 (1970).

BREW, K., CASTELLINO, F. J., VANAMAN, T. C., HILL, R. L.: The complete amino acid sequence of bovine α-lactalbumin. J. Biol. Chem. **245**, 4570–4582 (1970).

BREW, K., HILL, R. L.: The isolation and characterization of the tryptic, chymotryptic, peptic and cyanogen bromide peptides from bovine α-lactalbumin. J. Biol. Chem. **245**, 4559–4569 (1970).

BREW, K.: The complete amino acid sequence of guinea pig α-lactalbumin. Eur. J. Biochem. **27**, 341–353 (1972).

BREW, K., FINDLAY, J. B. C., KHATRA, B. S.: Structure and regulation of lactose synthetase. In "Behavior of Regulatory Enzymes". Biochemical Society Monograph (Ed. by THORNE, C. J. O. R., TIPTON, K. F.) pp. 19–32. London and New York: Academic Press (1973).

BREW, K., STEINMAN, H. M.: HILL, R. L.: A partial amino acid sequence of α-lactalbumin-I of the grey kangaroo (*Macropus giganteus*). J. Biol. Chem. **248**, 4739–4742 (1973).

BREW, K., SHAPER, J. H., OLSEN, K. W., TRAYER, I. P., HILL, R. L.: Crosslinking of the components of lactose synthetase by dimethylpimelimidate. J. Biol. Chem., in press, 1975.

BRODBECK, U., EBNER, K. E.: Resolution of a soluble lactose synthetase into two protein components and solubilization of microsomal lactose synthetase. J. Biol. Chem. **241**, 762–764 (1966a).

BRODBECK, U., EBNER, K. E.: The sucellular distribution of the A and B proteins of lactose synthetase in bovine and rat mammary tissue. J. Biol. Chem. **241**, 5526–5532 (1966b).

BRODBECK, U., DENTON, W. L., TANAHASHI, N., EBNER, K. E.: The isolation and identification of the B protein of lactose synthetase as α-lactalbumin. J. Biol. Chem. **242**, 1391–1397 (1967).

BROWN, W. J., NORTH, A. C. T., PHILLIPS, D. C., BREW, K., VANAMAN, T. C., HILL, R. L.: A possible three-dimensional structure of bovine α-lactalbumin based on that of hen's egg-white lysozyme. J. Mol. Biol. **42**, 65–86 (1969).

CANFIELD, R. E.: The amino acid sequence of egg-white lysozyme. J. Biol. Chem. **238**, 2698–2707 (1943).

CANFIELD, R. E., LIU, A. K.: The disulfide bonds of egg-white lysozyme (Muramidase). J. Biol. Chem. **240**, 1997–2002 (1965).

CANFIELD, R. E., KAMMERMAN, G., SOBEL, J. H., MORGAN, F. T.: Primary structure of lysozymes from man and goose. Nature. New Biology **232**, 16–17 (1971).

CAPUTTO, R., LELOIR, L. F., CARDINI, C. E., PALLADINI, J. R.: Isolation of the coenzyme of the galactose-glucose phosphate transformation. J. Biol. Chem. **184**, 333–350 (1950).

CARO, L. G., PALADE, G. E.: Protein synthesis, storage and discharge in the pancreatic cell. An autoradiographic study. J. Cell. Biol. **20**, 473–495 (1964).

CASTELLINO, F. J., HILL, R. L.: The carboxymethylation of bovine α-lactalbumin. J. Biol. Chem. **245**, 417–424 (1970).

COFFEY, R. G., REITHEL, F. J.: The lactose synthetase particles of lactating bovine mammary gland. Preparation of particles with intact lactose synthetase. Biochem. J. **109**, 169–176 (1968a).

COFFEY, R. G., REITHEL, F. J.: The lactose synthetase particles of lactating bovine mammary gland. Characteristics of the particles. Biochem. J. **109**, 177–183 (1968b).

COHN, R., SEGAL, S.: Some characteristics and developmental aspects of rat uridine diphosphogalactose 4-epimerase. Biochim. Biophys. Acta **171**, 333–341 (1969).

COWBURN, D. A., BRADBURY, E. M., CRANE-ROBINSON, C., GRATZER, W. B.: An investigation of the conformation of bovine α-lactalbumin by proton magnetic resonance and optical spectroscopy. Eur. J. Biochem. **14**, 83 (1970).

COWBURN, D. A., BREW, K., GRATZER, W. B.: An analysis of the circular dichroism of the lysozyme-α-lactalbumin group of proteins. Biochemistry **11**, 1128–1234 (1972).

COWIE, A. T.: The initiation of milk secretion at parturition (REYNOLDS, M., and FOLLIE, S. J., eds.) p. 157. Philadelphia: University of Pennsylvania Press (1969).

CUNNINGHAM, W. P., MOLLEHAUER, H. H.: Isolation and purification of Golgi apparatus from rat testis. J. Cell. Biol. **47**, 449 (Abstracts 10th Ann. Meeting Am. Soc. Cell. Biol.) (1970).

DEN, H., KAUFMAN, B., ROSEMAN, S.: Properties of some glycosyltransferases in embryonic chicken brain. J. Biol. Chem. **245**, 6607–6615 (1970).

DENTON, W. L., EBNER, K. E.: The effect of tyrosyl modification on the activity of α-lactalbumin in the lactose synthetase reaction. J. Biol. Chem. **246**, 4053–4059 (1971).

DIMANT, E., SMITH, V. R., LARDY, H. A.: Lactose synthesis in the mammary gland perfused with 1-^{14}C-glucose. J. Biol. Chem. **201**, 85–91 (1953).

EBNER, K. E., DENTON, W. L., BRODBECK, U.: The substitution of α-lactalbumin for the B protein of lactose synthetase. Biochim. Biophys. Res. Commun. **24**, 232–236 (1966).

EBNER, K. E.: A biological role for α-lactalbumin as a component of an enzyme requiring two proteins. Acc. Chem. Res. **3**, 41–47 (1970).

EBNER, K. E.: Lactose synthetase in "The Enzymes", P. BOYER, ed., Vol. IX, Part B, 363–377, Academic Press, New York, 1973.

FAIRHURST, F., MCILREAVY, D., CAMPBELL, P. N.: The protein-synthesizing activity of ribosomes isolated from the mammary gland of lactating and pregnant guinea pigs. Biochem. J. **123**, 865–874 (1971).

FINDLAY, J. B. C., BREW, K.: The complete amino acid sequence of human α-lactalbumin. Eur. J. Biochem. **27**, 65–86 (1972).

FITZGERALD, D. K., BRODBECK, U., KIYOSAWA, I., MAWAL, R., COLVIN, B., EBNER, K. E.: α-Lactalbumin and the lactose synthetase reaction. J. Biol. Chem. **245**, 2103–2108 (1970).

FLEISCHER, B., FLEISCHER, S., OZAWA, H.: Isolation and characterization of Golgi membranes from bovine liver. J. Cell. Biol. **43**, 59–79 (1969).

FLEISCHER, B., FLEISCHER, S.: Preparation and characterization of Golgi membranes from rat liver. Biochim. Biophys. Acta **219**, 301–319 (1970).

GANDER, J. E., PETERSON, W. E., BOYER, P. O.: On the mechanism of the enzymatic synthesis of lactose. Arch. Biochem. Biophys. **60**, 259–261 (1956).

GORBUNOFF, M. J.: Exposure of tyrosine residues in protein. Reaction of cyanuric fluoride with ribonuclease, α-lactalbumin and β-lactoglobulin. Biochemistry **6**, 1606–1615 (1967).

GORDON, W. G., SEMMETT, W. F.: The isolation of crystalline α-lactalbumin from milk. J. Am. Chem. Soc. **75**, 328–330 (1953).

GORDON, W. G., ASCHAFFENBURG, R., SEN, A., GHOSH, S. K.: Amino acid composition of several α-lactalbumins. J. Dairy Sci. **51**, 947 Abs. M103 (1968).

GORDON, W. G.: α-Lactalbumin. In "Milk Proteins" (H. A. MCKENZIE, ed.) II, pp. 331–365. New York and London: Academic Press 1971.

GOTTSCHALK, A.: Biosynthesis of glycoproteins and its relationship to heterogeneity. Nature **222**, 452–454 (1969).

GRANT, G. A.: The metabolism of galactose. 1. Lactose synthesis from (a) glucose-galactose mixture, (b) phosphoric esters, by slices of the active mammary gland *in vitro*. 2. The effect of prolactin on lactose synthesis in the mammary gland. Biochem. J. **30**, 2027–2035 (1936).

GUIRE, P.: Differences in tryptophan exposure between chicken egg-white lysozyme and bovine α-lactalbumin. Biochim. Biophys. Acta **221**, 383–386 (1970).

HABEEB, A. F. S. A., ATASSI, M. Z.: Enzymic and immunochemical properties of lysozyme. IV. Demonstration of conformational differences between α-lactalbumin and lysozyme. Biochem. Biophys. Acta **236**, 131–141 (1971).

HELMINEN, H. J., ERICSSON, J. L. E.: Studies on mammary gland involution. 1. On the ultra structure of the lactating mammary gland. J. Ultrastruct. Res. **25**, 193–213 (1968).

HILL, R. L., BREW, K., VANAMAN, T. C., TRAYER, I. P., MATTOCK, P.: The structure, function and evolution of α-lactalbumin. Brookhaven Symposium in Biology No. 21, 139–154 (1968).

HINDLE, E. J., WHELLOCK, J. V.: Carbohydrates of bovine α-lactalbumin preparations. Chimia **25**, 188–190 (1971).

IMOTO, T., JOHNSON, L. N., NORTH, A. C. T., PHILLIPS, D. C., RUPLEY, J. A.,: Vertebrate lysozymes, in "The Enzymes", P. BOYER, ed., pp. 666–868, Vol. VII. New York: Academic Press 1972.

IVATT, R. J., ROSENMEYER, M. S.: The complex formed between the A and B proteins of lactose synthetase. FEBS Letts. **28**, 195–197 (1972).

IYER, K. S., KLEE, W. A.: Direct spectrophotometric measurement of the rate of reaction of disulfide bonds. The reactivity of the disulfide bonds of bovine α-lactalbumin. J. Biol. Chem. **248**, 707–710 (1973).

JENNESS, R.: Protein composition of milk in milk proteins, Chemistry and Molecular Biology (H. A. MCKENZIE, ed.) pp. 17–43. New York: Academic Press Inc. 1970.

JOLLÉS, P.: Relationship between chemical structure and biological activity of hen egg-white lysozyme and lysozymes of different species. Proc. Roy. Soc. (London) Ser. B **167**, 350–364 (1967).

JONES, E. A.: Studies on the particulate lactose synthetase of mouse mammary gland and the role of α-lactalbumin in the initiation of lactose synthesis. Biochem. J. **126**, 67–78 (1972).

JONES, E. A., COWIE, A. T.: The effect of hypophysectomy and subsequent replacement therapy with sheep prolactin or bovine growth hormone on the lactose synthetase activity of rabbit mammary gland. Biochem. J. **130**, 997–1002 (1972).

JUERGENS, W. G., STOCKDALE, F. E., TOPPER, Y. J., ELIAS, J. J.: Hormone-dependent differentiation of mammary gland *in vitro*. Proc. Natl. Acad. Sci. (U.S.A.) **54**, 629–634 (1965).

KEENAN, T. W., MORRE, D. J., CHEETHAM, R. D.: Lactose synthetase by a Golgi apparatus fraction from rat mammary gland. Nature **228**, 1105–1106 (1970).

KHATRA, B. S., HERRIES, D. G., BREW, K.: Some kinetic properties of human milk galactosyl transferase. Eur. J. Biochem. **44**, 537–560 (1974).

KLEE, W. A., KLEE, C. B.: The role of α-lactalbumin and the A protein of lactose synthetase. Biochem. Biophys. Res. Commun. **39**, 833–841 (1970).

KLEE, W. A., KLEE, C. B.: The interaction of α-lactalbumin and the A protein of lactose synthetase. J. Biol. Chem. **247**, 2336–2344 (1972).

KLEIBER, M., BLACK, A. L., BROWN, M. A., BAXTER, C. R., LUICK, J. R., STADTMAN, F. H.: Glucose as a precursor of milk constituents in the intact dairy cow. Biochem. Biophys. Acta **17**, 252–260 (1955).

KON, S. K., COWIE, A. T., eds.: "Milk: The Mammary Gland and Its Secretion." 515 pp. Vol. I. New York: Academic Press 1961 a.

KON, S. K., COWIE, A. T., eds.: "Milk: The Mammary Gland and Its Secretion." 423 pp. Vol. II. New York: Academic Press 1961 b.

KRIGBAUM, W. R., KUGLER, F. R.: Molecular conformation of egg-white lysozyme and bovine α-lactalbumin in solution. Biochemistry **9**, 1216–1223 (1970).

KRONMAN, M. J., HOLMES, L. G.: Inter- and intramolecular interactions of α-lactalbumin. IV. Location of tryptophan groups. Biochemistry **4**, 526–532 (1965).

KRONMAN, M. J.: Similarity in backbone conformation of egg-white lysozyme and bovine α-lactalbumin. Biochem. Biophys. Res. Commun. **33**, 535–541 (1968).

KRONMAN, M. J., HOLMES, L. G., ROBBINS, F. M.: Inter- and intramolecular interactions of α-lactalbumin X. Effect of acylation of tyrosyl and lysyl side chains on molecular conformations. J. Biol. Chem. **246**, 1909–1921 (1971).

KUHN, N. J., LOWENSTEIN, J. M.: Lactogenesis in the rat. Changes in metabolic parameters at parturition. Biochem. J. **105**, 995–1002 (1967).

KUHN, N. J.: Lactogenesis in the rat. Metabolism of uridine diphosphate galactose by mammary gland. Biochem. J. **106**, 743–748 (1968).

KUHN, N. J.: The lactogenetic action of human chorionic gonadotropin in the rat. Biochem. J. **129**, 495–496 (1972).

KUHN, R., LÖW, I.: Über ein Vorkommen von Milchzucker im Pflanzenreich. Chem. Ber. **82**, 479–481 (1949).

LAWFORD, G. R., SCHACHTER, H.: Biosynthesis of glycoprotein by liver. The incorporation *in vivo* of ^{14}C-glucosamine into protein-bound hexosamine and sialic acid of rat liver subcellular fractions. J. Biol. Chem. **241**, 5409–5418 (1966).

LELOIR, L. R., CARDINI, L. E.: The biosynthesis of lactose. In "Milk: The Mammary Gland and its Secretion", KON, S. K., and COWIE, A. T., eds., Vol. I, 421–440. New York: Academic Press 1961.

LIN, T.: Lactose synthetase. Modification of carboxyl groups in α-lactalbumin. Biochemistry **9**, 984–995 (1970).

LING, E. R., KON, S. K., PORTER, J. V. R.: The composition of milk and the nutritive value of its components. In "Milk: The Mammary Gland and its Secretion", KON, S. K., and COWIE, A. T., eds., Vol. II, 195–263 (1961).

LOCKWOOD, D. H., TURKINGTON, R. W., TOPPER, Y. J.: Hormone-dependent development of milk protein synthesis in mammary gland *in vitro*. Biochim. Biophys. Acta **130**, 493–501 (1966).

LYSTER, R. L. J.: Reviews of the progress of dairy science. Sect. C. Chemistry of milk proteins. J. Dairy Res. **39**, 279–318 (1972).

MAGEE, S. C., MAWAL, R., EBNER, K. E.: Proteolytic conversion of the molecular forms of bovine milk transferase. J. Biol. Chem. **248**, 7565–7569 (1973).

MAWAL, R., MORRISON, J. F., EBNER, K. E.: Studies on galactosyl transferase. Detection of enzyme-reactant complexes by means of affinity chromatography. J. Biol. Chem. **246**, 7106–7109 (1971).

MCGUIRE, E. J., JORDIAN, G. W., CARLSON, D. M., ROSEMAN, S.: Incorporation of D-galactose into glycoproteins. J. Biol. Chem. **240**, PC4113–PC4115 (1965).

MCKENZIE, H. A.: Milk proteins. Adv. Protein Chemistry **22**, 56–234 (1967).

MCKENZIE, H. A., ed.: "Milk Proteins: Chemistry and Molecular Biology", 519 pp., Vol. I. New York: Academic Press 1970a.

MCKENZIE, H. A., ed.: "Milk Proteins: Chemistry and Molecular Biology", 552 pp., Vol. II. New York: Academic Press 1970b.

MCKENZIE, L., FITZGERALD, D. K., EBNER, K. E.: Lactose synthetase activities in rat and mouse mammary glands. Biochim. Biophys. Acta **230**, 526 (1971).

MELLENBERGER, R. W., BAUMAN, D. E., NELSON, D. R.: Metabolic adaptations during lactogenesis. Fatty acid and lactose synthesis in cow mammary tissue. Biochem. J. **136**, 741–748 (1973).

MILLS, E. S., TOPPER, Y. J.: Some ultrastructural effects of insulin, hydrocortisone and prolactin on mammary gland explants. J. Cell Biol. **44**, 310–328 (1970).

MORRÉ, J. D.: Isolation of Golgi apparatus. Methods in Enzymology **22**, 130–148 (1971).

MORRÉ, J. D., CHEETHAM, R. D., NYQUIST, S. E., OVTRACHT, L.: A Simplified procedure for the isolation of Golgi apparatus from rat liver. Prep. Biochem. **2**, 61–69 (1972).

MORRISON, J. F., EBNER, K. E.: Studies on galactosyl transferase. Kinetic investigation with N-acetylglucosamine as the galactosyl group acceptor. J. Biol. Chem. **246**, 3977–3984 (1971a).

MORRISON, J. F., EBNER, K. E.: Studies on galactosyl transferase. Kinetic investigation with glucose as the galactosyl acceptor. J. Biol. Chem. **246**, 3985–3991 (1971b).

MORRISON, J. F., EBNER, K. E.: Studies on galactosyl transferase. Kinetic effects of α-lactalbumin with N-acetylglucosamine and glucose as galactosyl group acceptors. J. Biol. Chem. **246**, 3992–3998 (1971c).

MURPHY, G., ARIYANAYAGAM, A. D., KUHN, N. J.: Progesterone and the metabolic control of the lactose biosynthetic pathway during lactogenesis in the rat. Biochem. J. **136**, 1105–1116 (1973).

OKA, T., TOPPER, Y. J.: Is prolactin mitogenic for mammary epithelium? Proc. Natl. Acad. Sci. (U.S.A.) **69**, 1693–1696 (1972).

OKA, T.: Spermidine in hormone dependent differentiation of mammary gland *in vitro*. Science **184**, 78–80 (1974).

OWENS, I. S., VONDERHAAR, B. K., TOPPER, Y. J.: Concerning the necessary coupling of development to proliferation of mouse mammary epithelial cells. J. Biol. Chem. **248**, 472–477 (1973).

PALMITER, R. D.: Hormonal induction and regulation of lactose synthetase in mouse mammary gland. Biochem. J. **113**, 409 (1969a).

PALMITER, R. D.: Properties of lactose synthetase from mouse mammary gland: role of a proposed third component. Biochim. Biophys. Acta **178**, 35–46 (1969b).

PALMITER, R. D.: What regulates lactose content in milk? Nature **221**, 912–914 (1969c).

PATTON, S., FOWKES, F.M.: The role of the plasma membrane in the secretion of milk fat. J. Theoret. Biol. **15**, 274–281 (1967).

PEDERSEN, K. O.: Ultracentrifugal studies and electrophoretic studies on the milkp roteins. I. Introduction and preliminary results with fractions from skim milk. Biochem. J. **30**, 948–970 (1936).

PERUTZ, M. F., MUIRHEAD, H., COX, J. M., GOAMAN, L. C. G.: Threedimensional fourier synthesis of horse oxyhaemoglobin at 2.8 Å resolution: the atomic model. Nature **219**, 131–139 (1968).

PESSEN, H., KUMOSINSKI, T. F., TIMASHEFF, S. W.: The use of small-angle X-ray scattering to determine protein conformation. J. Agric. Food. Chem. **19**, 698–702 (1971).

PILSON, M. E. Q., KELLY, A. L.: Composition of milk from *Zalophus californianus*, the California sea lion. Science **135**, 104–105 (1962).

POWELL, J. T., BREW, K.: Glycosyl transferases of onion stem Golgi membranes. Biochem. J. **142**, 203–210 (1974a).

POWELL, J. T., BREW, K.: Isolation and characterization of two forms of bovine galactosyl transferase. Eur. J. Biochem. **48**, 217–228 (1974b).

RAWITCH, A. B.: The rational diffusion of bovine α-lactalbumin: a comparison with egg-white lysozyme. Arch. Biochem. Biophys. **151**, 22–27 (1972).

REISS, O. K., BARRY, J. M.: The synthesis of lactose from glucose in the mammary gland. Biochem. J. **55**, 783–785 (1953).

REITHEL, F. J., HAROWITZ, M. G., DAVIDSON, H. M., KITTINGER, G. N.: Formation of lactose in homogenates of mammary gland. J. Biol. Chem. **194**, 839–848 (1952).

ROBBINS, F. M., HOLMES, L. G.: Binding of N-methylnicotinamide chloride by tryptophan residues of α-lactalbumin. J. Biol. Chem. **247**, 3062–3065 (1972).

SCHACHTER, H., JABBAL, I., HUDGIN, R. L., PINTERIC, L., MCGUIRE, E. J., ROSEMAN, S.: Intracellular location of liver sugar nucleotide glycoprotein glycosyl transferases in a Golgi-Rich fraction. J. Biol. Chem. **245**, 1090–1100 (1970).

SCHANBACHER, F. L., EBNER, K. E.: Galactosyl transferase acceptor specificity of the lactose synthetase A protein. J. Biol. Chem. **245**, 5057–5061 (1970).

SCHMIDT, D. V., WALKER, L. E., EBNER, K. E.: Lactose synthetase activity in Northern fur seal milk. Biochem. Biophys. Acta **252**, 439–442 (1971).

SHAPER, J. H., BARKER, R., HILL, R. L.: Purification of wheat germ agglutinin by affinity chromatography. Anal. Biochem. **53**, 564–570 (1973).

SHOTTON, D. M., WATSON, H. C.: Three-dimensional structure of tosyl-elastase. Nature **225**, 811–816 (1970).

SOMMERS, P. B., KRONMAN, M. J., BREW, K.: Molecular conformation and fluorescence properties of α-lactalbumin from four animal species. Biochem. Biophys. Res. Commun. **52**, 98–105 (1973).

SØRENSEN, M., SØRENSEN, S. P. L.: The proteins in whey. C. r. Trav. Lab. Carlsberg, Ser. Chim. **23**, 55–99 (1939).

SPATZ, L., STRITTMATTER, P.: A form of reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. J. Biol. Chem. **248**, 793–799 (1973).

SVEDBERG, T., PEDERSEN, K. O.: "The Ultracentrifuge" p. 379, Oxford Univ. Press 1940.

SVEDBERG, T.: The ultracentrifuge and the study of high-molecular compounds. Nature **139**, 1051 (1957).

TAMBURRO, A. M., JORI, G., VADILI, G., SCATTURIN, A., SACCOMANI, G.: Studies on the structure in solution of α-lactalbumin. Biochim. Biophys. Acta **263**, 704–713 (1972).

TRAYER, I. P., MATTOCK, P., HILL, R. L.: Purification and properties of the A protein from lactose synthetase. Fed. Proc. **29**, 597 (1970).

TRAYER, I. P., HILL, R. L.: The purification and properties of the A protein of lactose synthetase. J. Biol. Chem. **246**, 6666–6675 (1971).

TRAYER, I. P., BARKER, R., HILL, R. L.: α-Lactalbumin-Sepharose, a specific adsorbent for a galactosyl transferase. Methods in Enzymology, in press (1974).

TURKINGTON, R. W., BREW, K., VANAMAN, T. C., HILL, R. L.: The hormonal control of lactose synthetase in the developing mouse mammary gland. J. Biol. Chem. **243**, 3382–3387 (1968).

TURKINGTON, R. W., HILL, R. L.: Lactose synthetase: progesterone inhibition of the initiation of α-lactalbumin. Science **163**, 1458–1460 (1969).

VANAMAN, T. C., BREW, K., HILL, R. L.: The disulfide bonds of bovine α-lactalbumin. J. Biol. Chem. **245**, 4583–4590 (1970).

VENKATARAMAN, R., REITHEL, F. J.: Carbohydrates of the sapotaceae. I. The origin of lactose in *A. Sapota*. Arch. Biochem. Biophys. **75**, 443–452 (1958).

VONDERHAAR, B. K., WWENS, I. S., TOPPER, Y. J.: An early effect of prolactin on the formation of α-lactalbumin by mouse mammary epithelial cells. J. Biol. Chem. **248**, 467–471 (1973).

WARME, P. K., MOMANY, F. A., RUMBALL, S. V., TUTTLE, R. W., SCHERAGA, H. A.: Computation of structures of homologuous proteins. α-lactalbumin from lysozyme. Biochemistry **13**, 768–781 (1974).

WARREN, G. B., TIPTON, K. F.: Pig liver pyruvate carboxylase. The reaction pathway for the carboxylation of pyruvate. Biochem. J. **139**, 311–302 (1974).

WATKINS, W. M., HASSID, W. Z.: The synthesis of lactose by particulate enzyme preparations from guinea pig and bovine mammary glands. J. Biol. Chem. **237**, 1432–1440 (1962).

WHEELOCK, I. V., ROOK, J. A. R.: Reviews of the Progress of Dairy Science. C. Dairy Chemistry. The secretion of water and of water-soluble constituents in milk. J. Dairy Res. **34**, 273–287 (1967).

WOOD, H. G., SCHAMBYE, P., PEETERS, G. L.: Lactose synthesis: II. The distribution of C^{14} in lactose of milk from the perfused isolated cow udder. J. Biol. Chem. **226**, 1023–1034 (1957a).

WOOD, H. G., SIU, P., SCHAMBYE, P.: Lactose synthesis. III. The distribution of C^{14} in lactose of milk after inter-arterial injection of acetate-1-C^{14}. Arch. Biochem. Biophys. **69**, 390–404 (1957b).

WOOD, H. G., GILLESPIE, R., JAFFE, S., HANSEN, R. G., HARDENBROOK, H.: Lactose synthesis. V. C^{14} in lactose, glycerol and serine as indications of the triose phosphate isomerase reaction and pentose cycle. J. Biol. Chem. **233**, 1271–1278 (1958).

WOOD, H. G.: My life and carbon dioxide fixation. "Miami Winter Symposium", The Molecular Basis of Biological Transport, J. R. WOESSNER, JR., and F. HUIJING, eds., Vol. 3, pp. 1–54. New York: Academic Press, Inc. (1972).

Author Index

Page numbers in *italics* refer to the bibliography

Subject Index