parenchyma callose may have had was slight.

In spite of substantial increases in sieve-plate callose, the autoradiographs showed no indication of a restriction of longitudinal translocation. No unequivocal interpretation of this can be made at present. It may mean that sieve-plate callose, in the individual sieve tube, has no effect. This would imply that movement of assimilates across the sieve plate was not limited to connecting strands but was through the sieve plate as a whole. Alternatively, it may mean that the conductive capacity of the phloem exceeded the demands made upon it. There were always some sieve plates with small amounts of callose. It is conceivable that either removal of assimilates at sinks or some other process limited movement in these petioles with the result that the conducting capacity of the few functional sieve tubes was not exceeded. It is clear, at any rate, that sieve-plate callose was not limiting translocation in the untreated plants, since amounts far in excess of normal had no effect on longitudinal translocation.

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13. The term "sink" means tissue to which assimilates and other solutes move and are absorbed; sink is receiving tissue in contrast to source or supplying tissue.
14. Modified from a Ph.D. thesis presented to the Graduate Division, University of California, Davis. A more detailed report is in preparation. We thank Dr. S. Yamaguchi for advice on methods of autoradiography. This study was supported in part by NSF grant G-17825.

27 August 1965

Steroid Stimulation of Beating of Cultured Rat-Heart Cells

Abstract. *The addition of cortisol acetate and deoxycorticosterone acetate to cultured heart cells from rats, 4 to 6 days old, stimulated and prolonged beating. Protein synthesis was slowed down in the presence of steroids. Addition of steroids to cells that had stopped beating caused the reestablishment of the beat.*

Primary rat-heart cells in culture not only grow but continue to beat (*1*). This phenomenon was not expected since cells in culture usually lose their specific function as well as their distinguishing morphology. Recently (*2*) it was reported that serum lipids or certain fatty acids were essential for the maintenance of this beat. We now report on the relation between steroid hormones and the beat of heart cells in culture. Cortisol acetate and deoxycorticosterone (DOC) acetate stimulated the beat; cholestrol had no effect. While treatment with cortisol acetate was more effective than with DOC acetate, both caused prolonged beating and resulted in a higher percentage of beating areas. Cortisol acetate and DOC acetate also reestablished beating of cells which had stopped beating after 7 days in control growth medium. The reestablishment of beating was accompanied by morphological changes which, along with the beating, were maintained for as long as 2 months.

Heart cells were obtained from rats 4 to 5 days old. Thirty to forty rats were anesthetized with ether, and the hearts were removed aseptically. The techniques and medium for culturing the cells were those reported (*1*), with the following exceptions. The culture medium contained 20 percent bovine serum instead of 10 percent human serum and 10 percent fetal-calf serum. The washed and minced tissue was placed for treatment with trypsin in a 500-ml continuous-flow flask (Bellco Glass, Inc., Vineland, New Jersey) containing a magnetic stirring bar. The flask was surmounted by a separatory funnel containing 0.1 percent trypsin in Hanks saline solution. Both were attached to a ring stand and placed over a magnetic stirrer. Stirring was maintained to prevent the formation of bubbles. This whole assembly was kept sterile and was housed at 37°C. The trypsin-treated heart cells were removed from the bottom of the flask at 15-minute intervals. This cell suspension was allowed to collect in a roller tube contained in an ice bath. Fresh trypsin was added from the separatory funnel, and the treatment was continued for another 15-minute period. Five transfers of trypsin were usually sufficient to fragment the heart tissue into isolated heart cells.

The cell suspension was centrifuged at low speed for 4 minutes. Trypsin was then decanted, and 3 ml of fresh, cold, complete growth media (CGM) was added to the packed cells. The cell pellet was resuspended by mixing with a Vortex Junior Mixer and then placed on ice.

Cell suspensions were pooled, stirred slowly, and poured through two sterile stainless steel screens (1-mm² mesh) to remove clumps of undissociated heart tissue and to insure uniform distribution of cells. In most cases cells

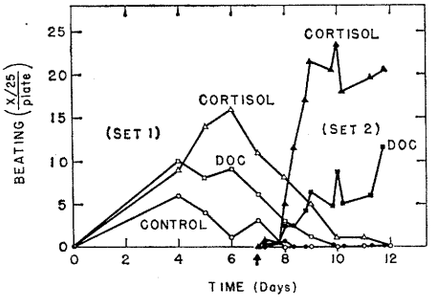

Fig. 1. Beating areas from 25 areas (per plate) randomly selected as a function of time after addition of cortisol acetate and DOC acetate. Zero represents 24 hours after culturing on control medium (CGM). Steroid-supplemented medium added at 0 days and 7 days (arrow) in set 1 and set 2, respectively.

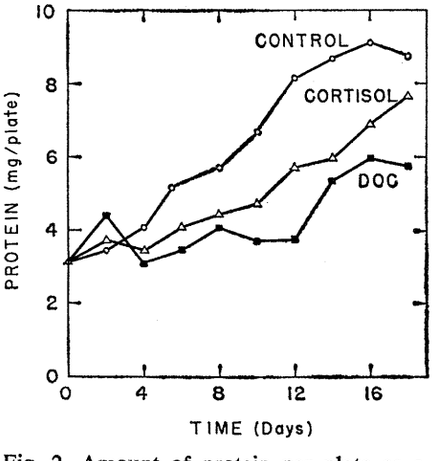

Fig. 2. Amount of protein per plate as a function of time after addition of cortisol acetate and DOC acetate. The zero time is 24 hours after culturing on control medium (CGM).

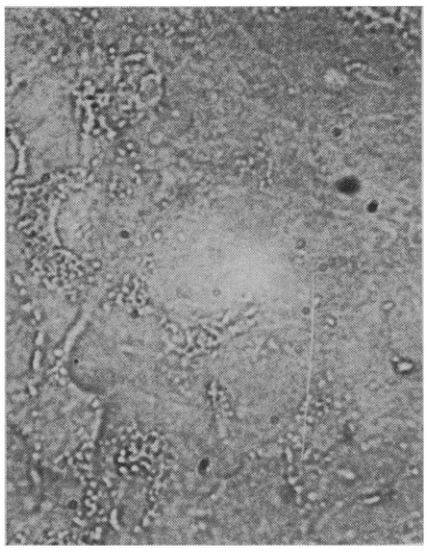

Fig. 3. Appearance of cells in control medium 10 to 12 days after culturing in control medium (CGM). Pictures taken with a polaroid camera back attached to a Unitron microscope (× 400).

equivalent to 0.75 heart were added to each polystyrene petri dish (60 by 15 mm), the total volumes per plate being 3.5 ml.

Beating was evaluated by counting the number of "beating areas" observed in 25 areas chosen at random on each plate. Any positive contraction in an area was considered as one. In most cases more than one center of contraction was observed (× 420) in one area.

Two types of experiments were performed with control medium and additions of steroid. In both cases protein and beating were measured as a function of time. The amount of protein per plate was determined (*3*). In all experiments the medium was changed 1 day after culturing and every 2 days thereafter. In the first set of experiments, steroids were added with the fresh media every 2 days, beginning 1 day after culturing. At this time cells had attached to the petri dishes, and beating could be observed. In the second set of experiments steroids were added with the fresh medium every 2 days after 7 days of incubation. This time was chosen because it was observed that the CGM-cultured cells had essentially stopped beating after 7 days under the conditions employed in our laboratory.

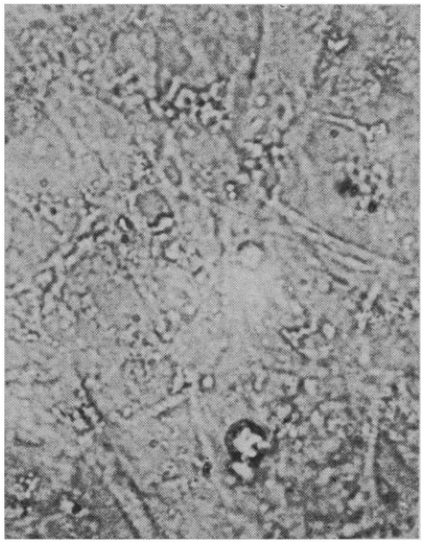

Fig. 4. Appearance of cells 3 to 5 days after addition of DOC acetate to cells that had stopped beating, that is, 10 to 12 days after culturing (× 400).

Typical curves for beating and protein synthesis in plates containing control medium and steroid-saturated control medium which was added 1 day after culturing are shown in Figs. 1 and 2. Cortisol and DOC increase the number of areas containing beating heart cells and prolong the time the cells beat in culture. In this medium, the cells did not beat synchronously but only as isolated cells even when in apparent contact with each other. Although the steroid-treated cells beat for an extended period of time they did eventually stop, and continued treatment had no apparent effect. On the other hand, cells which had been grown in CGM for 7 days and which had stopped beating did respond to the addition of the test steroids. These cells do not appear to become refractory when allowed to develop initially in control conditions.

Figure 1 (set 2) indicates the effects of adding steroids to cells which had stopped beating. The data for protein synthesis for set 2 are similar to those for set 1 (Fig. 2). At least 20 hours was required before any increase in the number of beating areas could be observed. This relative refractory period was the same for both compounds even though cortisol acetate had a greater effect on beating than DOC acetate. A change could also be observed in the appearance of the cells when beating was reestablished. The cells grown on medium enriched with cortisol acetate or DOC acetate showed fibrous centers from which the beat was apparently initiated, as in Figs. 3–5. Numerous centers were observed, all beating synchronously at a time which coincides with the period of 20 to 24 hours required for beating to begin in cells that had stopped beating and were then treated with the steroid.

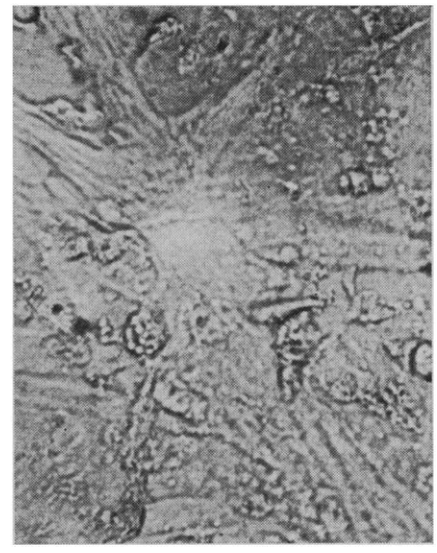

Fig. 5. Appearance of cells 3 to 5 days after addition of cortisol acetate to cells that had stopped beating, 10 to 12 days after culturing (× 400).

Replication of these experiments has substantiated the fact that cortisol acetate and DOC acetate stimulate and prolong heart beat in all trials; however, absolute variations as to the degree of stimulation or the length of prolongation were observed between various runs. These can be attributable to many factors, such as the number of hearts used, the initial amount of protein per plate, the age of the rat population, and the source of the rats. The significant point is that heart cells treated with natural corticoids exhibit a prolonged characteristic morphology and function. When these cells are allowed to adapt to the culture medium before treatment, they develop further and appear to undergo some natural differentiation necessary for synchronous beating.

The protein synthesis pattern was similar whether steroids were added 1 day or 7 days after culturing. The amount of protein per plate was less in medium containing cortisol acetate and DOC acetate than in controls, and the rate of increase of protein was slower. Therefore an inverse relationship exists between protein production and the presence of steroids.

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22 October 1965

Myelin Membrane: A Molecular Abnormality

Abstract. *Myelin was isolated from cerebral white matter from a patient who had died of metachromatic leukodystrophy, and its lipid composition was analyzed. Although the lipid content was nearly normal, the myelin contained a three- to fourfold excess of cerebroside sulfate and a threefold deficiency of cerebroside compared to normal myelin. The deficiency of cerebroside and the excess of cerebroside sulfate may account for defective myelination in this disease.*

Metachromatic leukodystrophy is an inborn error of metabolism characterized by faulty myelin formation. Cerebroside sulfate accumulates in the nervous system and in the viscera (*1*). Large round metachromatically stained bodies comprised of this lipid are present within glial cells in the central nervous system and within Schwann cells in the peripheral nervous system. In addition, a marked deficiency of cerebrosides has been found in white matter (*2, 3*), especially of those cerebrosides which contain very-long-chain fatty acids (19 to 26 carbon atoms) (*3*).

The relation of the cerebroside sulfate accumulation or the cerebroside deficiency, or both, to defective myelination in metachromatic leukodystrophy (MLD) is obscure. To shed more light upon this relation, the question whether the accumulation of cerebroside sulfate and the deficiency of cerebroside are also present in myelin in this disease needs to be answered. In partial answer we now report the analyses of myelin isolated from cerebral white matter from a boy, age 6 years, who expired from late infantile MLD.

White matter was dissected from frontal and occipital lobes and myelin was isolated from the white matter (*4*). The yield of myelin (4 mg) was 1/200 of that from the brain of a normal deceased child of a similar age. Examination by light microscopy showed that the myelin fraction was comprised of elongated tubules which were doughnut shaped on cross section. Electron microscopic examination showed that the myelin preparation was comprised of smooth-surfaced membranes, layered in lamellar fashion, many of which were wound in spiral fashion like native myelin. Granular material was also seen in the myelin preparation, but this was present in small proportions compared to the membranous material.

The myelin preparation was extracted with a mixture of chloroform and methanol (2 : 1) to obtain its constituent lipids. Similar to normal myelin (*4, 5*) the MLD myelin fraction was completely soluble in this solvent. The lipids could be obtained free of nonlipid residue by evaporating the myelin extract to dryness, drying the extract for a 24 hour period, and extracting again the dried residue with the chloroform-methanol (2 : 1) mixture (*5*). With this procedure, 76 percent of the dried extract became soluble in the chloroform-methanol. The lipids obtained from MLD myelin were then analyzed by chromatography on paper impregnated with silicic acid (*6*) for a comparison with normal myelin. The MLD myelin contained an excess of cerebroside sulfate and a deficiency of cerebroside compared to normal myelin.

The amounts of individual classes of lipids in the MLD myelin extract were then determined by x-ray fluorescence spectroscopy for analysis of sulfur and phosphorus (*7*), cholesterol determination (*8*), and colorimetric analysis by the anthrone method (*9*) for the determination of total galacto-lipids (predominantly cerebroside plus cerebroside sulfate). The lipids obtained from whole white matter from the patient's brain were also studied by column chromatographic procedures (*5, 6*).

The MLD myelin contained nearly the same lipid content as normal myelin but its composition was abnormal (Table 1). There was a three- to fourfold excess of cerebroside sulfate and a threefold deficiency of cerebroside compared to normal; in fact, the myelin values and the white matter values were nearly identical. The other lipids were present in proportions that were somewhat closer to normal.

These analyses indicate that central nervous system myelin is chemically abnormal in MLD. It is not possible to state that all the myelin in the central nervous system is abnormally constituted, since it is not known what fraction of the total myelin was isolated. However, the fact that the MLD myelin we obtained had a lipid composition very close to that of the white matter from which it was isolated suggests that there was little tendency toward the preservation of a normal-myelin lipid composition in this patient. It is also not possible to state whether myelin in MLD is abnormally constituted at the time of its initial synthesis. The patient studied here was in the late stages of his disease, and it is necessary to learn more about myelin at earlier ages to decide this point.

Mention should also be made of the deficiency of long-chain sphingolipids (those containing fatty acids with more than 18 carbon atoms) in MLD myelin. It was postulated (*3*) that a deficiency of long-chain sphingolipids may lead to the formation of unstable myelin in MLD since these molecules are thought to act in the stabilization of the myelin bimolecular lipid leaflet (*10, 11*). Unfortunately, it was not possible to determine the fatty acid composition of the sphingolipids in the MLD myelin we isolated because of insufficient quantity. The fatty acid compositions of cerebroside and sphingomyelin from white matter of the present patient, but not that of cerebroside sulfate, were shifted toward short-

Table 1. Analysis of myelin and white matter from metachromatic leucodystrophy. GP, glycerophosphatides. All values except total lipid are expressed as percent of the total lipid.

Component	Normal myelin*	MLD white matter	MLD myelin
Total lipid (% of dry weight)	78–81	47.7	76.0
Cholesterol	24.4	17.8	17.0
Total phospholipids	47.6	53.3	55.0
Ethanolamine GP	16.2	19.3	
Serine GP	6.0	7.5	
Choline GP	13.3	15.7	
Sphingomyelin	5.6	4.0	
Cerebroside	19.5	6.5	6.7
Cerebroside sulfate	5.6	20.4	20.2
Ceramide	1.3	2.0	
Uncharacterized†	6.5	6.8	

* Average of four humans aged 10 months, 6 years, 9 years, and 55 years (*5*). † Includes inositol glycerophosphatides as major components and smaller proportions of free fatty acids, gangliosides, and other phosphatides.