were observed with Renshaw cells; in addition, the sensitivity of these cells to acetylcholine was depressed (Fig. 1). Although recovery after the ejection of noradrenaline was often rapid and at times was followed by enhancement of the sensitivity to excitants, the recovery was occasionally incomplete, with reduction in the amplitude of the spike potential. Depression produced by noradrenaline was not blocked by strychnine injected intravenously (strychnine hydrochloride, 0.05 to 0.1 mg/kg) or electrophoretically (20 to 40 na from a 0.01*M* solution of strychnine hydrochloride in 0.165*M* NaCl). Care was necessary that the currents used to eject strychnine were not of sufficient magnitude to produce extracellular concentrations of this alkaloid which alone reduced the sensitivity of Renshaw cells to excitants.

In marked contrast, the inhibition of Renshaw cells produced either by squeezing the ipsilateral hind paw (*6*) or by tetanic electrical stimulation of the contralateral medullary reticular formation (*7*) was readily blocked by strychnine. Such inhibition was measured by the reduction in the number of spikes evoked by stimulation of the ventral root and was accompanied by a diminution in the sensitivity of Renshaw cells to acetylcholine. Some difficulty was experienced in demonstrating a consistent blocking action of intravenously administered strychnine because of the marked increase in the "background" activity of both the observed neuron and other nearby cells (see also *6*). Inconsistency would also be expected from the polysynaptic nature of the pathways involved in these inhibitions (*6*, *7*). However, electrophoretically administered strychnine, which would affect structures only in the immediate vicinity of the tip of the micropipette, readily and reversibly reduced the inhibition of Renshaw cells (Fig. 2). No difficulty was experienced in controlling strychnine efflux from micropipettes used in this study, presumably because of the small size (1 to 2 μ) of the individual barrels of the electrode (*9*).

Thus these postsynaptic inhibitions of Renshaw cells were clearly blocked by strychnine, whereas the depression induced by noradrenaline was not. If the action of strychnine is one of antagonism with inhibitory transmitters at postsynaptic receptor sites, or one of interference with the increase in conductance which is associated with inhibition, these results suggest that noradrenaline is not an inhibitory transmitter acting upon Renshaw cells. As a consequence the noradrenaline "receptors" on these cells, and possibly also upon interneurons, may have no functional significance in synaptic processes. On the other hand, if strychnine blocks these inhibitions by presynaptic action, or if there are other inhibitory pathways converging on Renshaw cells, the effects of which are resistant to strychnine, then noradrenaline may indeed be a spinal inhibitory transmitter.

T. J. Biscoe
D. R. Curtis
Department of Physiology, Australian National University, Canberra

References

1. A. Fuxe, A. Dahlström, N.-Å. Hillarp, *Int. Cong. Physiol. Sci. 23rd Cong.* (1965), p. 419.
2. A. Lundberg, in *Studies in Physiology Presented to J. C. Eccles*, D. R. Curtis and A. K. McIntyre, Eds. (Springer, Berlin, 1965).
3. D. R. Curtis, J. W. Phillis, J. C. Watkins, *J. Physiol. London* **158**, 296 (1961).
4. K. Krnjević and J. W. Phillis, *Brit. J. Pharmacol.* **20**, 471 (1963); P. Andersen and D. R. Curtis, *Acta Physiol. Scand.* **61**, 100 (1964).
5. I. Engberg and R. W. Ryall, *Life Sci.* **4**, 2223 (1965).
6. V. J. Wilson and W. H. Talbot, *Nature* **200**, 1325 (1963); ——— and M. Kato, *J. Neurophysiol.* **27**, 1063 (1964).
7. R. Granit, J. Haase, L. T. Rutledge, *J. Physiol. London* **154**, 308 (1960); J. Haase and J. P. Van der Meulen, *J. Neurophysiol.* **24**, 510 (1961).
8. D. R. Curtis, in *Physical Techniques in Biological Research*, W. L. Nastuk, Ed. (Academic Press, New York, 1964), vol. 5.
9. D. R. Curtis, *Int. J. Neuropharmacol.* **1**, 239 (1962).

21 December 1965

Metachromatic Leucodystrophy: Isolation and Chemical Analysis of Metachromatic Granules

Abstract. *The abnormal, cytoplasmic metachromatic granules found in the brain of metachromatic leucodystrophy were isolated in high purity. They show metachromasia by von Hirsch-Peiffer's cresyl violet method. Electron microscopy revealed very slight contaminations by other components, such as mitochondria, myelin, and ribosomes. Chemical analysis of two separately obtained collections has shown that the molar ratio of cholesterol to galactolipids to phosphatides is 1 : 1 : 1. Most of the galactolipids are sulfatides.*

Metachromatic leucodystrophy (MLD) is a genetically determined metabolic disorder primarily involving the nervous system. It is characterized by a large number of abnormal, cytoplasmic granules which show brown-yellow metachromasia when stained by von Hirsch-Peiffer's cresyl violet method (*1*). Abnormally high sulfatide content in white matter of MLD brain was demonstrated by Austin (*2*) and Jatzkewitz (*3*) and subsequently confirmed by many investigators.

The membranous cytoplasmic bodies (MCB) characteristic of Tay-Sachs disease have been isolated in high purity and contain large amounts of gangliosides, the major lipid accumulated in this disease (*4*). The metachromatic granules in brain in MLD differ from Tay-Sachs cytoplasmic bodies in morphology and in their characteristic metachromatic staining property. It would be reasonable to assume that MLD granules might contain large amounts of sulfatides resulting in metachromasia.

The MLD granules were isolated by procedures similar to those devised for isolation of MCB. The procedures were carried out at 4°C. Tissue obtained at autopsy was frozen (1-g samples) and homogenized in 10 ml of 0.05*M* sodium ethylenediamine tetraacetate (EDTA), *p*H 7.5. The homogenate was centrifuged at 1500*g* for 4 minutes, and the

Table 1. Chemical analysis of MLD granules.

Components	Collection I			Collection II		
	Weight		Molar ratio	Weight		Molar ratio
	Total (mg)	Dry (%)		Total (mg)	Dry (%)	
Residue	7.3	47.1		4.9	46.7	
Total lipids	8.2	52.9		5.6	53.3	
Cholesterol	1.50		1.00	1.06		1.00
Galactolipids	3.88		1.07	2.48		0.97
Phosphatides	2.77		0.93	2.03		.96
Gangliosides			< .06			< .05

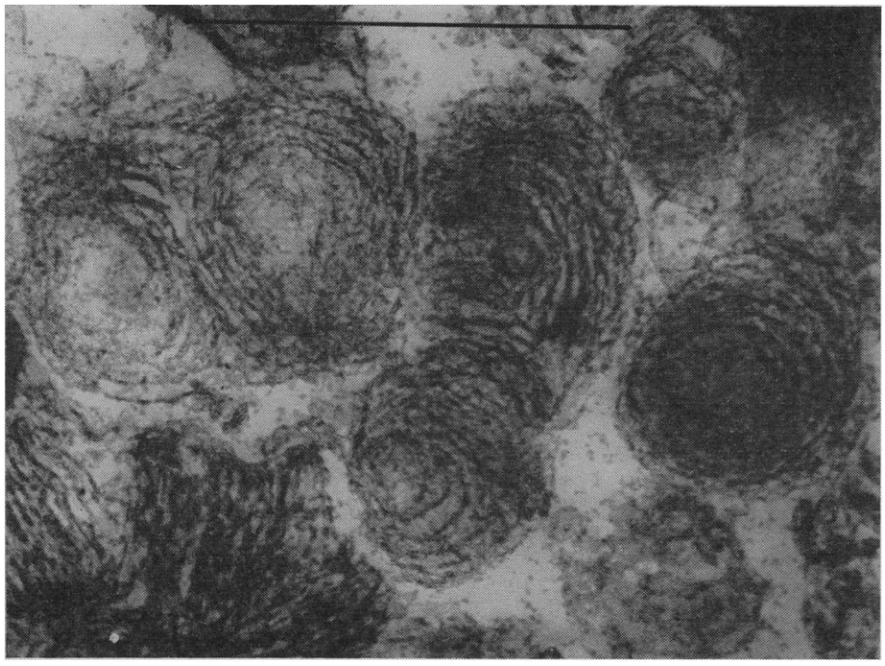

Fig. 1. Electron micrograph of the isolated MLD granules. Scale indicates 1 μm.

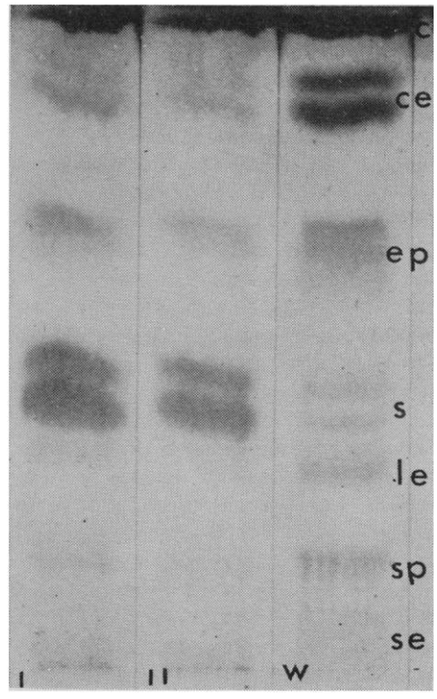

Fig. 2. Thin-layer chromatogram of the lipids of the isolated MLD granules. Comparison of collection I, collection II, and normal white matter (w). Approximately 180 μg of total lipids in each sample. Solvent system: a mixture of chloroform, methanol, and water (70:30:4, by volume), ascending. *c*, Cholesterol; *ce*, cerebrosides (2 bands); *ep*, ethanolamine phosphatides; *s*, sulphatides (two bands); *le*, lecithin; *sp*, sphingomyelin; *se*, serine phosphatides.

supernatant was then centrifuged at 7500*g* for 5 minutes. The precipitate was again washed, resuspended in 0.05*M* sodium EDTA, and centrifuged at 25,000 rev/min (Spinco SW-25.1) for 60 minutes on a discontinuous sucrose density gradient of 1.2*M*, 0.75*M*, and 0.25*M*. The layer floating at the interface of 1.2*M* and 0.75*M* sucrose was washed with 0.05*M* sodium EDTA and centrifuged. The pellet was resuspended in 0.05*M* sodium EDTA and again centrifuged (SW-25.1) at 25,000 rev/min for 60 minutes, but this time on a continuous gradient of 1.2*M* to 0.75*M* sucrose. Three bands were observed at the end of the centrifugation, the narrow and sharply defined top and bottom bands and the broad and somewhat ill-defined middle band. The middle band was the MLD fraction of high purity. This fraction was washed a few times with 0.05*M* sodium EDTA.

When stained by von Hirsch-Peiffer's cresyl violet method, the smear of this fraction showed uniform yellow-brown metachromasia. Light microscopic observation revealed little contamination by nonmetachromatic, purple-staining particles. Under the electron microscope, the major portion of the fraction consisted of bodies of lamellar structure, as expected from the observations by light microscopy (Fig. 1). As compared to the granules *in situ*, the lamellar structures are ruffled, very loosely arranged, and not membrane-bound. There are many fragments of these lamellar bodies in addition to well-formed ones. At high magnification, the individual lamellae appear to consist of a double membrane with a dense line, a clear space, and another dense line. There is scant contamination by myelin fragments and distorted mitochondria. Ribosome-like particles are also diffusely scattered among these bodies.

For chemical analysis, two collections were separately accumulated, each from a total of approximately 10 g of tissue. The smears of both collections showed uniform metachromasia; the concentration of pure granules was (estimated) 90 percent or more. The final pellet was suspended in 1 ml of water and it was then extracted with 19 ml of a mixture of chloroform and methanol (2:1, by volume). The extract was partitioned with the addition of 4 ml of water and washed once with solvent equivalent to the upper phase in composition but containing no salt. The amount of proteolipid protein was judged negligible after several complete dryings from the chloroform-methanol-water system, and the analysis of lipids was carried out directly on the total lower phase. The qualitative thin-layer chromatography of the lower phase lipids of both collections appear identical (Fig. 2), and, when compared to normal white matter lipids, they show reduced cerebrosides, greatly increased sulfatides, uniformly reduced phosphatides, and relatively normal cholesterol. Quantitative determinations were carried out for cholesterol (*5*), lipid phosphorus (*6*), and lipid hexose (*7*). Gangliosides were estimated on the dialyzed upper-phase fraction, by its total sialic acid content (*8*). The molar ratio was calculated on the basis that each phosphatide molecule contains one phosphorus atom and each galactolipid molecule contains one galactose moiety. Weights of lipids were tentatively calculated on the basis of the following molecular weights; galactolipids (100 percent sulfatides) 933, and phosphatide, 775 (Table 1).

The analytical data are in excellent agreement on both collections. The amount of chloroform-methanol insoluble residue is relatively high, in contrast to Tay-Sachs MCB (0 and 8 percent). The most striking feature, however, is the molar ratio of cholesterol:galactolipids:phosphatides, which

is almost precisely 1:1:1. However, this does not necessarily mean that the lamellar structure of the MLD granules consists of only one kind of abnormal membrane with equimolar amounts of cholesterol, galactolipids, and phosphatides, because the same analytical data could be obtained in various situations, for example, a mixture of equal amounts of two membrane structures, one with a ratio for cholesterol to galactolipids to phosphatides being 1:2:3, and the other 3 : 2 : 1. An extensive electron microscopic examination of biopsy specimens from three cases of MLD (*9*) has shown that the dimensions of these membranous structures appear to be constant, although the MLD granules vary in overall size or in the arrangements of the lamellae within the granules. This evidence supports the idea that the MLD granules contain only one kind of abnormal membrane with a cholesterol: galactolipids: phosphatides ratio of 1: 1: 1. The amount of gangliosides appears to be too small to be an integral part of the MLD granules and is probably derived from contaminating structures such as microsomes which are rich in gangliosides. Whether the considerable amount of protein in the MLD granules is an integral part of the abnormal membrane or whether it contains any enzyme activity is now unknown. The MLD granules appear to carry strong acid phosphatase activity as judged by light and electron microscopic histochemistry (*9*).

KUNIHIKO SUZUKI
KINUKO SUZUKI, GLORIA CHEN
Saul R. Korey Department of Neurology, Department of Pathology (Neuropathology), Albert Einstein College of Medicine, New York

References and Notes

1. T. von Hirsch and J. Peiffer, *Arch. Psychiat. Nerv.* **194**, 88 (1955).
2. J. H. Austin, *Neurology* **7**, 415 (1957).
3. H. Jatzkewitz, *Z. Physiol. Chem.* **311**, 279 (1958).
4. S. Samuels, S. R. Korey, J. Gonatas, R. D. Terry, M. Weiss, *J. Neuropathol. Exp. Neurol.* **22**, 81 (1963).
5. R. L. Searcy and L. M. Bergquist, *Clin. Chim. Acta* **5**, 192 (1960).
6. G. V. Marinetti, J. Erbland, E. Stotz, *Biochim. Biophys. Acta* **31**, 251 (1959); W. T. Norton and L. A. Autilio, *J. Neurochem.*, in press.
7. L. Svennerholm, *J. Neurochem.* **1**, 42 (1956).
8. ———, *Biochim. Biophys. Acta* **24**, 604 (1957); T. Miettinen and I-T. Takki-Luukkainen, *Acta Chem. Scand.* **13**, 856 (1959).
9. R. D. Terry, K. Suzuki, M. Weiss, in preparation.
10. We thank Dr. J. French who secured the brain tissue for this study. Supported by grant R-160-65C from the United Cerebral Palsy Foundation, and grants NB-03356 and NB-02255 from NIH.

15 October 1965

Protein Synthesis in Micromeres of the Sea Urchin Egg

Abstract. *A method was developed for isolating large quantities of micromeres from the 16-cell stage of the sea urchin, and measurements were made of their ability to incorporate C^{14}-L-valine into protein as compared with that of a mixed suspension of micromeres, mesomeres, and macromeres. Per cell, the rate of incorporation was considerably less for the micromeres than for the suspension of mixed cells. Per unit volume, however, the two types of suspensions showed no significant differences.*

In sea urchins the first three cleavages divide the fertilized egg into eight equal-sized, and similar-appearing, cells. At the fourth cleavage the "lower" (vegetal) four cells divide unequally and across the polar axis, forming four micromeres (vegetally) and four macromeres, while the upper four cells divide equally and meridionally to form a layer of eight mesomeres. Experiments (*1*) in which these and derivative cell types have been removed and regrouped have shown that they possess special properties and that they interact in a gradient system. Previous investigators (*2*), using cytochemical and autoradiographic methods, have reported that protein content and rate of incorporation of labeled amino acid into protein are approximately the same in the different cell types. In order to obtain more quantitative information on this subject we have developed a method for isolating micromeres in large quantity and have measured their ability to incorporate labeled amino acid into protein.

Gametes of the sea urchin *Lytechinus pictus* were obtained by injection of the animals with isotonic potassium chloride. Artificial sea water (*3*) was used throughout. Typically, 25 ml of a suspension of washed eggs were inseminated with 25 ml of a dilute sperm suspension containing $10^{-4}M$ ethylenediaminetetraacetate (EDTA) at *p*H 8.1.

The fertilization membrane was removed 4 minutes (20°C) later by a single rapid squirting of the suspension against the side of a beaker through a 50-ml pipette. The eggs were then washed, suspended in 250 ml of sea water, cultured to the 16-cell stage in a large (500 cm^2 area) pyrex dish, and transferred, with one washing, to $0.55M$ KCl containing EDTA at $2 \times 10^{-3}M$, *p*H 8.1. After 10 minutes of gentle agitation in this medium, the component cells of the eggs were generally completely separated from each other. The suspension was then centrifuged at about $1000g$ for 2 minutes in order to reduce its volume to 40 ml, and 10-ml portions were layered, in test tubes, over 10 ml of a mixture of isotonic sucrose and EDTA-KCl (3:7). The cells were allowed to settle for 10 to 15 minutes, and the upper 10-ml of the suspension were removed. This was composed predominantly (99 percent) of micromeres. This suspension was further concentrated by centrifugation at $1000g$ for 2 minutes. The entire procedure was carried out at 20°C. When returned to sea water, the micromeres exhibit normal reaggregation and continue with normal cell division. After such treatment, mixtures of micromeres, mesomeres, and macromeres will reaggregate and frequently gastrulate. Only a few of the aggregates develop into normal embryos, as expected in view of the random nature of the kinds and positions of the different cell types, which evidently retain their distinctive properties during the period of isolation (*1*).

For the measurements of incorporation of labeled amino acid into the proteins of micromeres, 0.05 ml of the suspension in sea water (containing 8 to 20 $\times$ 10^3 cells, depending on the experiment) were added to 0.25 ml of C^{14}-L-valine (specific activity 200 c/mole, 0.5 μc/ml) in sea water and incubated for 1 hour at 20°C with

Table 1. Incorporation of uniformly labeled C^{14}-L-valine into micromere and mixed-cell suspensions.

Cell suspension	Radioactivity (count/min)	
	Per 10^3 cells	Per unit volume
	Experiment 1	
Micromere	32	131
Mixed	183	183
	Experiment 2	
Micromere	99	406
Mixed	480	480
	Experiment 3	
Micromere	120	492
Mixed	370	370