EXPERIMENTALLY INDUCED CHANGES IN THE BASE COMPOSITION OF THE RIBONUCLEIC ACIDS OF ISOLATED NERVE CELLS AND THEIR OLIGODENDROGLIAL CELLS

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ABSTRACT

The effect of tricyano-amino-propene, a dimer of malononitrile, on the base composition of the RNA in isolated Deiters' nerve cells and their oligodendroglial cells has been studied using a microelectrophoretic method. Tri-a-p in a dose of 20 mg/kg has the effect of increasing the RNA and protein content per nerve cell by 25 per cent and decreasing the glia RNA by 45 per cent. The RNA base composition of the nerve cells from the control animals differs from that of their glial cells. The guanine of the nerve cell is significantly higher than that of the glia, but the content of cytosine is higher in the glia than in the RNA of nerve cell. The cytosine of nerve cells decreased significantly after tri-a-p administration. In the glial cells the cytosine showed a 20 per cent increase, and the guanine a 25 per cent decrease. Tri-a-p sharpened the difference in RNA composition already existing between the control nerve cells and their glial cells by almost 300 per cent for the guanine and by 400 per cent for the cytosine. The chemical and functional relationship between the nerve cell and its oligodendroglial cells is discussed.

Several years ago malononitrile given to animals was found to increase the concentration of ribonucleic acid and proteins in nerve cells (Hydén, 14; Hydén and Hartelius, 16). Mendelson *et al.* (19) ascribed the malononitrile action to a compound formed in the aged solution of malononitrile. This compound has now been identified as 1,1,3-tricyano-2-amino-1-propene (tri-a-p), a dimer of malononitrile (Eberts, 5).

Grenell and Hydén (12) have found that tri-a-p in small amounts in 1 hour caused a remarkable increase in the amount of RNA, proteins, and free lipids per nerve cell, and a concomitant decrease in RNA in the oligodendroglial cells surrounding the nerve cells. If administered for 6 days a successive increase of the cytochrome oxidase activity by more than 700 per cent was found. Table I summarizes the results.

In view of the rapid RNA changes occurring as a result of this treatment, it becomes a matter of interest to see whether there had occurred also qualitative changes in the RNA of the nerve and glial cells of such a nature that they are reflected in changes in base composition. In the present work the purine-pyrimidine composition of isolated nerve cells and glial cells has been measured after administration of tri-a-p to animals.

Preparation of Isolated, Fresh Nerve Cells and Samples of Oligodendroglial Cells of the Same Volume

In all, 23 rabbits were used for the present study, 11 for the controls and 12 for the tri-a-p studies. Tri-a-p was given intravenously, 20 mg/kg, and the animals were killed 1 hour later as described

TABLE I

A. Effect of Tricyano-Amino-Propene on the Total Amount of RNA and Proteins per Nerve Cell (Deiters'), and of RNA per Glia Cell Sample

Rabbits injected with 20 mg/kg and killed 1 hour later

Number of analyses: 320

	Nerve cell Protein	Nerve cell RNA	Glia cell RNA
	μμg/cell	*μμg/cell*	*μμg/cell*
Control	16,400	1,550	123
1 hour after injection	20,800 (27% increase)	2,120 (26% increase)	68 (45% decrease)

B. Effect of Tricyano-Amino-Propene on the Respiratory Enzyme Activities per Nerve Cell (Deiters') after Administration for Several Days

Number of analyses: 320

	Nerve Cell (Enzyme activities expressed as μl $O_2 \times 10^{-4}$/hr $\times$ cell)	
	Cytochrome oxidase	Succinic oxidase
Control	4.2	2.2
2 days	13.9	—
5 days	10.7	4.2
6 days	30.6	—

below. The only symptom observable was a moderately increased body temperature. The giant nerve cells of Deiters, situated within the lateral vestibular nucleus of Deiters, and the oligodendroglial cells immediately surrounding each nerve cell have constituted the material. For the description of this area, see Brodal and Pompeiano (2, 3).

The cell samples were isolated from the fresh tissue according to the following procedure. After being given an air embolus (20 ml of air injected in the ear vein), the animal became unconscious within a few seconds and its carotid arteries were cut to permit the remaining heartbeats to drain the blood from the body. The brain was rapidly removed and a slice was cut through the desired locus and placed in sterile 0.25 M sucrose solution. From the nucleus to be studied were then removed single nerve cells and the same weight (= volume) of oligodendroglial cells as that of the nerve cells.

The cells, with their surrounding glial cells, were lifted out by hand under a stereomicroscope (provided with hand supports) at a magnification of 64 or 100. The instrument used was a 15 μ thick, stainless steel thread, shaped like a spatula at its end with a point of a few microns. The thread was inserted in a glass handle. This spatula was inserted under the nerve cell, and the cell plus adhering glial cells was lifted out of the thick section submerged in sucrose solution and placed in a drop of sterile sucrose for dissection. By gentle manipulation the nerve cells were freed from the glial cells, which stuck together. It is to be noted that those glial cells immediately surrounding the cell body were taken and not the glial cells within the ramifications of the dendrites projecting from the poles of the nerve cell, the latter being more contaminated with the fine processes of nerve cells than the former. The structural composition of the glia samples was checked regularly in the phase and light microscopes after staining of the fresh material with Victoria blue solution. For some of the analyses larger samples of glial cells were taken, approximately 10^6 to 10^7 μ^3 in volume. These samples were then freed from nerve cells and processes but were not as pure oligodendroglial cell samples as those described above.

Staining has shown that nerve cells completely free from surrounding glia can be obtained by this technique. It is important to note that the larger parts of the dendrites remain intact until the processes reach a certain minimal diameter (Fig. 1). The enzyme activity in these parts of the cell is high. They also contain about 10 per cent of the RNA of the nerve cell.

The nerve cells are easily found if a drop of a highly diluted solution of methylene blue in isotonic sucrose is applied for several seconds to the desired locus. Note that the cells themselves must not be allowed to take up the stain. Only the synapses are stained, and they are seen as a finely dotted border around the area occupied by the unstained nerve cell. When experience has been gained in this type of work, no staining is needed for locating the nerve cells. In most cases, the appearance of the nerve cell in the incident light differs slightly from that of its surroundings. For most of the present work no staining was used.

The glial cells removed from the nerve cell stick together and are easily trimmed down to approximately the same volume as that of the nerve cell. The amount of nerve threads or parts of dendrites left in the trimmed glial cells, found by teasing out and staining such material, is small and can probably be overlooked as a source of error. The isolation and dissection of 10 nerve cells did not take more than 5 minutes.

Methods of Analysis

For the purine-pyrimidine analysis of RNA the procedure developed by Edström (6, 8–10) was applied.

The fresh nerve cell and glia cell samples were precipitated with ice cold 10 per cent perchloric acid

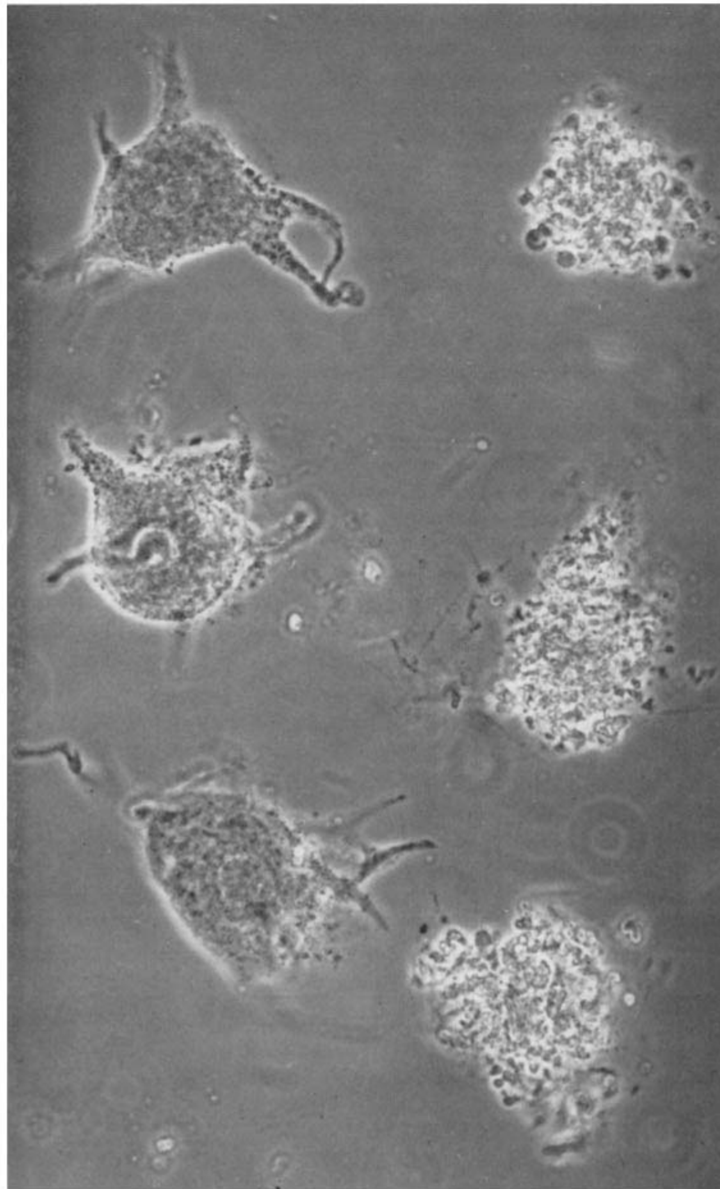

FIGURE 1

Three fresh, isolated Deiters' nerve cells photographed in the phase contrast microscope. To the right of each nerve cell is a cluster of glial cells which originally surrounded the body of each nerve cell and was of the same volume. × 230.

for 5 minutes, ethanol 5 minutes, and chloroform 5 minutes. RNA was extracted with a buffered solution of ribonuclease. The buffer contained 0.2 N ammonium bicarbonate–acetate solution of a pH of 7.6; the electrolytes are thus volatile. Each sample was extracted three times with approximately 0.5 mμl of this solution for a total of 90 minutes (3 × 30 minutes). These steps were performed in a paraffin oil chamber. After drying, the extracted and digested RNA was hydrolyzed with 4 N HCl in a micropipette at 100°C for 30 minutes.

The subsequent electrophoretic separation and

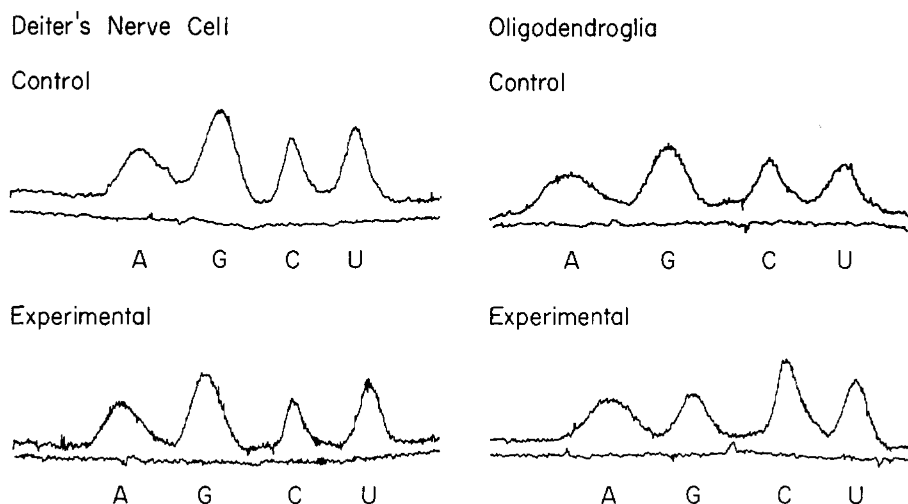

FIGURE 2

Microelectrophoretic separations of hydrolysates containing about 500 μμg of RNA from isolated Deiters' nerve cells and glial cells from control animals, and from experimental animals after administration of 20 mg/kg tricyano-amino-propene. *A*, *G*, *C*, *U* denote adenine, guanine, cytidylic acid, and uridylic acid.

TABLE II

The Composition of the RNA in Control Deiters' Nerve Cells and Their Oligodendroglial Cells in Rabbits. Microelectrophoretic Analysis of the RNA

Nerve cell: 1,550 μμg of RNA per cell. Average dry weight: 20,000 μμg
Glia: 125 μμg of RNA per sample. Average dry weight: 20,000 μμg
Purine and pyrimidine bases as molar proportions in percentages of the sum
Number of animals: 5
Number of analyses: 49

	Nerve cell		Glia Mean		
	Mean	*v*	Mean	*v*	*P*
Adenine	19.7 ± 0.37	4.2	20.8 ± 0.28	3.0	
Guanine	**33.5** ± 0.39	2.6	**28.8** ± 0.64	5.0	0.001
Cytosine	**28.8** ± 0.36	2.8	**31.8** ± 0.27	2.0	0.001
Uracil	18.0 ± 0.18	2.3	18.6 ± 0.55	6.7	0.001

v = the coefficient of variation, $\frac{S \times 100}{\text{Mean}}$.

P = probability after t-test

determination of the purines and the pyrimidine nucleotides were performed using a microscopic cellulose fiber. The diameter of these fibers after treatment with alkali is 25 to 35 μ. The fiber had been treated with a buffer of very high viscosity containing H_2SO_4, glucose, and glycerol. After the hydrolysis, the RNA samples, containing about 500 μμg of RNA, were placed on even parts of the fiber. The fiber was placed on a quartz slide and finally transferred to a constant humidity chamber of 42 per cent relative humidity. The fiber was then covered with liquid paraffin, and 2000 to 3000 volts per cm was applied for 10 minutes, after which time the separation had been completed. In the last steps the fiber was photographed at 2570 A and its optical density recorded with a microdensitometer according to Walker (20). From the curves recorded, the purine and pyrimidine were calculated as molar proportions in percentages of the sum (Fig. 2). In the present study, for 500 μμg of RNA the average values of the determinations showed a coefficient of variation of ±5 per cent.

RESULTS

The results presented in Table II show that the RNA base composition of the neuron differs significantly from that of the glia. The adenine and the uracil proportions agree very well. The guanine of the nerve cell predominates over that of the glial cells, but the cytosine content of the latter is higher than that in the RNA of the nerve cell. The variation coefficients of the glia values show that the samples constitute a sufficiently pure and uniform material for the experiments.

TABLE III

Microelectrophoretic Analyses of the Composition of the RNA in Deiters' Nerve Cells from Rabbits Treated with 20 mg/kg of Tricyano-amino propene and Killed 1 Hour Later

Purine and pyrimidine bases in molar proportions in percentages of the sum
Number of animals: 11
Number of analyses: 117

	Controls	Tri-a-p		
	Mean	Mean	v	P
Adenine	19.7	20.5 ± 0.31	3.8	
Guanine	**33.5**	**34.6** ± 0.28	2.0	
Cytosine	**28.8**	**26.7** ± 0.24	2.2	0.001
Uracil	18.0	18.2 ± 0.20	2.6	

Quotient	Controls	Tri-a-p
Adenine/cytosine	0.65	0.52
Guanine/uracil	1.55	1.13

TABLE IV

Microelectrophoretic Analyses of the Composition of the RNA in Oligodendroglial Cells Surrounding the Deiters' Nerve Cells from Rabbits Treated with 20 mg/kg of Tricyano-amino-propene and Killed 1 Hour Later

Purine and pyrimidine bases in molar proportions in percentages of the sum
Number of animals: 11
Number of analyses: 115

	Controls	Tri-a-p		
	Mean	Mean	v	P
Adenine	20.8	20.1 ± 0.74	9.1	
Guanine	**28.8**	**21.9** ± 2.15	24.0	0.01
Cytosine	**31.8**	**38.6** ± 2.40	15.2	0.01
Uracil	18.6	19.4 ± 0.65	8.2	

Quotient	Controls	Tri-a-p
Adenine/cytosine	0.68	0.77
Guanine/uracil	1.86	1.90

Tables III and IV show the results of treatment of Deiters' nerve cells and the surrounding oligodendroglial cells with tri-a-p. In general, it can be said that the differences in the RNA base composition already existing between the nerve cell and its oligodendroglial cells have become quite pronounced as a result of the action of the tri-a-p. The increase of the guanine of the experimental nerve cells and its concomitant decrease in the glial cells has intensified the difference in guanine by almost 300 per cent. Still more accentuated is the cytosine change. The decrease of the cytosine content in the nerve cells and its concomitant increase in the glial cells has sharpened the difference by 400 per cent.

If the changes in the RNA base composition of the control nerve cells are compared with the changes occurring in the experimental nerve cells, with the increased amount of RNA, it is apparent that there was a significant decrease in the cytosine from 28.8 per cent to 26.7 per cent. The glial cell changes after tri-a-p injection were even more drastic. The guanine decreased from 28.8 per cent to 21.9 per cent. The cytosine increased on an average from 31.8 per cent to 38.6 per cent. It is to be noted that the total amount of RNA in these glial cell samples had decreased by 45 per cent.

These were all average values. As was mentioned above, the glia cell samples used differed in the amount of glia. The purest were the smallest glia samples, which had the same volume as that of the nerve cell. Great pains were taken to collect only the oligodendroglial cells which surrounded the cell body and were of the same volume, and to avoid the glia situated within the ramification of the dendrites. In the latter case, too many nerve cell processes rendered the glia mass less pure.

In some instances, the neuroglia sample had a volume approximately 50 times greater than that of the unicell glia sample. These samples were cleaned of nerve cell bodies and processes.

In the total group of glial cells from the experimental animals, approximately one-third consisted of unicell samples and two-thirds of the large type samples. However carefully such large samples may be

cleaned, they are always contaminated by a certain amount of small nerve cell processes, and probably contain more than 10 per cent of astrocytes.

Table V is an example of the results from a "pure" glial cell sample. The effect of the tri-a-p was indeed considerable. Note that the purine bases have decreased and the pyrimidine bases increased, especially the cytosine.

In view of the considerable changes in the base composition in the microsamples, it was of interest to see whether such changes were discernible also in an analysis of a large piece of brain tissue. Four brains from control rabbits and four from rabbits injected with 20 mg/kg of tri-a-p and killed 1 hour later were used as material. RNA was prepared according to Davidson and Smellie's (4) modification of the Thannhauser method. The base composition was obtained by electrophoretic analysis.

The result is shown in Table VI. This negative finding is to be expected when the structural composition of the brain and the microchemical RNA analyses in the present study are considered. The neuroglia cells outnumber the neurons by a factor of 10 or more. The glia compartment takes up at least the same volume as that of the nerve cells. The relation between oligodendro- and astroglia varies very much in different areas. The microchemical analyses presented showed that the base changes in nerve and glial cells went in opposite directions. A bulk analysis of the central nervous tissue can therefore not be expected to detect qualitative changes in its components under such conditions.

TABLE V

Example of the Effect of Tricyano-amino-propene on the RNA Composition of Oligodendroglial Cells from a Rabbit Given 20 mg/kg of Tri-a-p and Killed 1 Hour Later

The microelectrophoretic analyses give the purine and pyrimidine bases in molar proportions in percentages of the sum.

	Controls	Tri-a-p
Adenine	20.8	17.2
Guanine	28.8	12.3
Cytosine	31.8	49.5
Uracil	18.6	21.0

TABLE VI

Macroelectrophoretic Analyses of the Composition of RNA in Brains from Rabbits Treated with 20 mg/kg of Tricyano-amino-propene and Killed 1 Hour Later

Purine and pyrimidine bases in molar proportions in percentages of the sum
Number of animals: 8
Number of analyses: 50

	Controls	Tri-a-p
Adenine	19.8	19.1
Guanine	31.8	31.9
Cytosine	28.2	27.5
Uracil	20.2	21.5

SIGNIFICANCE OF THE CHANGES OBSERVED

The Nerve Cell

The changes in the RNA base composition of the neurons can be discussed as such and in relation to the concomitant changes in the glial cells.

The Deiters' nerve cells have an average of 1,550 $\mu\mu$g of RNA per cell. These nerve cells are the richest in RNA of those hitherto analyzed in mammals. The content of proteins lies at 16.000 $\mu\mu$g per cell, which gives the expected RNA: protein ratio of approximately 1:10. Little can be said about the mechanism whereby tri-a-p has brought about such a high increase—more than 25 per cent of the total RNA and protein content per cell in 1 hour. The short time is, however, not surprising. Hammarsten (13) has *e.g.* recently found a protein which constitutes 0.15 per cent of the purified RNA and has the capacity to act as a primer enzyme.

One possibility is that tri-a-p acts by inhibiting a cellular control mechanism of RNA synthesis, resulting in an acceleration of those RNA syntheses recently initiated by the cell.

The base changes found may reflect the increased production within the neuron of one particular RNA fraction. The increase of such an RNA fraction and its special composition can account for the quantitative, as well as the qualitative, changes. Or, it may reflect the terminal attachment of bases to the RNA chains recently produced. It is true that the RNA extracted from one nerve cell represents a statistical average of all the RNA fractions of the cell, the nucleolar

and nuclear RNA, the transfer RNA, and the highly polymerized cytoplasmic RNA. The molar changes found here are, however, of such a magnitude that they may reflect a rapid increase of a particular RNA fraction, activating at an increased rate the amino acids for the increased protein synthesis. In this connection it may be noted that Edström (9), in his studies on egg cells, has found that the composition of the nucleolar and cytoplasmic RNA agreed, but the composition of the nuclear RNA differed from that of the RNA of the nucleolus and the cytoplasm. The nuclear RNA contained more adenine than the RNA of the two other cytological compartments. In sections through nerve cells from animals given tri-a-p, it was typically found that although the absorption at 2570 A was increased for the whole cell, the increase in density was especially striking in the nucleus. This observation may have a bearing on our results.

In other instances of experimentally induced increase of the RNA content in nerve cells, as, for example, during the maturation stage in nerve regeneration (Edström, 7; Brattgård *et al.*, 1), or after administration of dimethylamino-propyl-iminodibenzyl (Hydén and Løvtrup, 17), no changes could be found in the composition of the nerve cells.

On the other hand, in macrochemical studies on samples of brain cortex from electrically stimulated cats, Geiger *et al.* (11) found a marked increase of adenine and of cytidine in the stimulated areas. These changes were reversible within minutes and thus differ in time from the changes reported here. The findings of Geiger show how rapidly the nucleic acid components can change following increased brain activity, and are of great importance.

The Oligodendroglial Cells and Their Relation to the Nerve Cell

The RNA base changes in the oligodendroglial cells consistently went in a direction opposite to that of the RNA base changes observed in the nerve cells. The magnitude of the glial changes was much greater than that of the nerve cell changes. In the present experiment, in spite of the pronounced molar changes of the bases, the glial cell content of RNA decreased from 125 $\mu\mu$g to 70 $\mu\mu$g per sample, *i.e.* by 44 per cent. This suggests that the production of glial RNA under the experimental circumstances cannot keep up with the utilization. If the qualitative changes in both the neurons and the glia are considered, it is evident that tri-a-p had the effect of altering the chemical composition of a major part of the central nervous tissue.

These glial changes give further evidence of a functional relationship between the neuron and the oligodendroglial cells immediately surrounding the nerve cell body. In a recent study Hydén (15) and Hydén and Pigon (18) found that intermittent stimulation of the Deiters' nerve cells caused an *increase* of the RNA and protein content and the respiratory enzyme activities per nerve cell, but a concomitant *decrease* of the RNA and enzyme activities per the same volume of their glial cells.

The tentative working hypothesis was advanced that the nerve cells obtain priority for the energy of the respiratory chain at increased brain activity, the glial cells resorting to other sources for their energy supply. It was also suggested that the oligodendroglia supply the neuron with energy rich compounds such as nucleotides.

If the relationship between neuron and glia is through RNA synthesis, tri-a-p may influence the oligodendroglia to change the neuron they support. Whatever the mechanism, the RNA base changes reported here give additional evidence of a mutual relationship between neuron and glia. From a general biological point of view these cells represent only one example of a functional relationship between two intimately related structures, this relationship being a prerequisite enabling the most important cell to carry out its function.

With respect to the physiological effect on the nervous function of the induced changes, little can be said. Tri-a-p does not cause grave intoxication symptoms in the animals or long-lasting pathological changes. The substance uncouples the oxidative phosphorylation like many other substances (Eberts, 5). It strongly stimulates the formation of enzymes in the nerve cells as reported here.

If a speculation is made, it may be that tri-a-p stimulates the RNA and consequently the protein production and increases the energy utilization in the neurons. According to their function, the oligodendroglia then support the neuron at an increasing rate by furnishing energy to the neurons or by giving priority to the neurons in utilizing the energy of the respiratory chain.

In both types of cells the particular RNA fractions needed are produced at an increasing rate.

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REFERENCES

1. BRATTGÅRD, S.-O., EDSTRÖM, J.-E., and HYDÉN, H., *J. Neurochem.*, 1957, **1**, 316.
2. BRODAL, A., and POMPEIANO, O., *J. Anat.*, London, 1957, **91**, 438.
3. BRODAL, A., and POMPEIANO, O., *Acta Morphol. Neerl.-Scand.*, 1958, **1**, 306.
4. DAVIDSON, J. N., and SMELLIE, R. M. S., *Biochem. J.*, 1952, **52**, 594.
5. EBERTS, F. S., JR., *Biochem. Biophys. Res. Comm.*, 1960, **3**, 107.
6. EDSTRÖM, J.-E., *Biochim. et Biophysica Acta*, 1956, **22**, 378.
7. EDSTRÖM, J.-E., *J. Comp. Neurol.*, 1957, **117**, 295.
8. EDSTRÖM, J.-E., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 39.
9. EDSTRÖM, J.-E., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 47.
10. EDSTRÖM, J.-E., and HYDÉN, H., *Nature*, 1954, **174**, 128.
11. GEIGER, A., YAMASAKI, S., and LYONS, R., *Am. J. Physiol.*, 1956, **184**, 239.
12. GRENELL, R. G., and HYDÉN, H., to be published.
13. HAMMARSTEN, E., *Ark. Kemi*, 1960, in press.
14. HYDÉN, H., *Symp. Soc. Exp. Biol.*, 1947 **1** 152.
15. HYDÉN, H., *Nature*, 1959, **184**, 433.
16. HYDÉN, H., and HARTELIUS, H., *Acta Psychiat. et Neurol.* 1948, Suppl. 48.
17. HYDÉN, H., and LØVTRUP, S., to be published.
18. HYDÉN, H., and PIGON, A., *J. Neurochem.*, 1960, **6**, 57.
19. MENDELSON, J., MENDELSON, J. H., FAX, B. J., and GRENELL, R. G., *Science*, 1954, **120**, 266.
20. WALKER, P. M. B., *Exp. Cell Research*, 1955, **8**, 567.