New Light on How Certain Amines Act in the Brain

Chemical deficit in Parkinsonism, rates of brain amine synthesis, S100 and AChE changes in glial cells reported.

A new link between brain biochemistry and function was formed by four converging lines of evidence at a recent symposium on Parkinson's disease. According to work reported by researchers from three countries, a deficit of dopamine (3,4-dihydroxyphenylethylamine) in certain subcortical brain centers is associated with Parkinson's disease in humans and with certain induced symptoms in animals.

Giving added import to the dopamine findings and increasing hope for improved chemotherapy of the disease is the outcome of a search that has stretched over 27 years and a half-dozen countries. This is the discovery of the metabolic pathways by which the sympathetic nerve transmitter, norepinephrine, is made in nerve cells from a dietary amino acid. In the last step of this three-enzyme pathway, dopamine is oxidized to norepinephrine.

While one of the enzymes acting in the chain was discovered in 1939 by Holtz and his associates, not until 1964 was the rate-limiting enzyme operating at the first step, conversion of tyrosine to dopa [3-(3,4-dihydroxyphenyl)alanine, precursor of dopamine], found. This discovery was the result of work by neuropharmacologists of the National Institutes of Health, several of whom, as participants in a final panel session, evaluated the new vistas opened.

Among the 600 researchers attending the symposium, who represented all the many specialties now being focused on study of the brain, there was an excitement rare at such scientific events. This was set off when, as one panel member said afterward, "the four independent reports on dopamine convinced the audience that this chemical deficit is operative in Parkinson's disease."

The sense of progress increased as recent findings on brain metabolism were seen to fit together, suggesting that surgical removal of diseased body organs may ultimately be replaced by a sort of microchemectomy, perhaps targeted against specific enzymes. Beyond this is the even more remote possibility that the component parts of enzymes in short supply might eventually be supplied.

At the final session, Columbia's Elvin Kabat suggested how immunochemistry may open a path to recognition of specific molecular abnormalities. It may eventually be possible, Kabat said, to use antibody-forming mechanisms to track structural differences in enzymes, nucleic acids, and other chemical constituents operating in the central nervous system and to correlate small structural differences with differences in function and with the remarkable specificity of brain cells in retaining information. It is already possible, Kabat reminded, to produce antibodies specific to nucleic acids.

Arranged by the Parkinson's Disease Information and Research Center at Columbia University's College of Physicians and Surgeons, the program was an unusual demonstration of the advances that can be made through basic studies of cell function, as against narrower objectives keyed to clinical descriptions of disease.

The program was organized by Melvin Yahr, Columbia neurologist and executive director of the Parkinson's Disease Foundation, together with Erminio Costa, Lucien Coté, and David Nachmansohn, all of whom are Columbia faculty members and members of the Foundation's scientific staff. Reflecting the policy of basic research made possible for the Foundation by an unrestricted gift of $600,000 from William Black, New York philanthropist, the program was jointly sponsored by the Foundation and by the National Institute of Neurological Diseases and Blindness.

The four main findings pointing to dopamine as the compound with a primary role in biochemical abnormality related to Parkinson's disease were as follows:

► An almost complete lack of dopamine in Parkinson patients in forebrain motor centers where it is normally highly concentrated. This was reported by pharmacologist Oleh Hornykiewicz of the University of Vienna, who has been able to make post mortem analyses of 40 brains of persons who had had Parkinson's disease and to compare the chemical components with those extracted post mortem from brains of patients who had not had this disease.

► A high concentration of dopamine in the same forebrain motor nuclei in a number of species of animals. These findings, made some years ago, were discussed in a paper given by Arvid Carlsson, a pharmacologist and one of a group that has been studying brain catecholamines for 10 years at the universities of Lund and Göteborg in Sweden. Early in his work, Carlsson said, he was surprised to find that dopamine "occurred in the brains of normal mammals in about the same quantities as norepinephrine. This made us think that dopamine might have a function of its own apart from being a precursor of norepinephrine." Soon afterward, A. Bertler and E. Rosengren found that dopamine was highly concentrated in these forebrain motor centers—a finding which suggested that it has a role in Parkinson's disease.

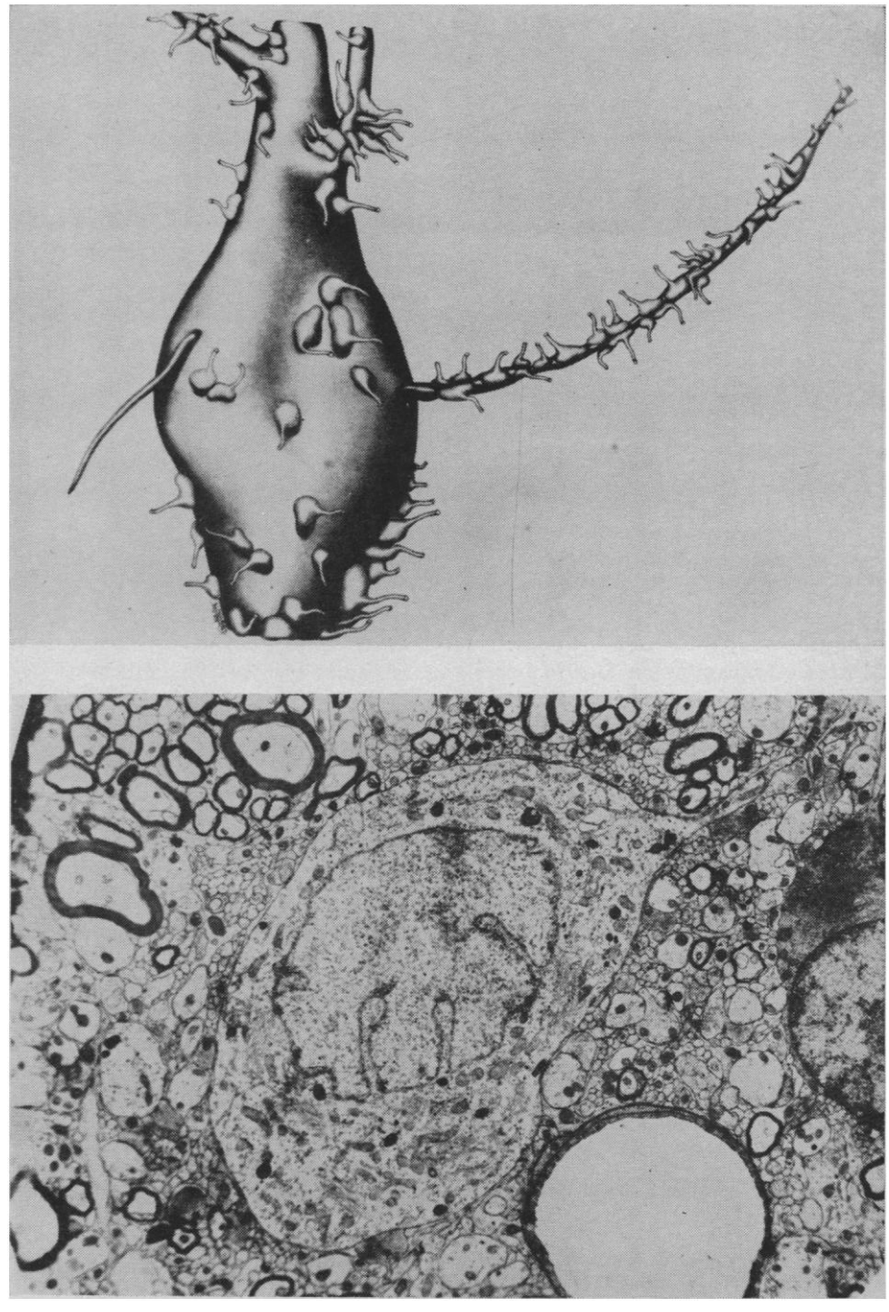
► A high concentration of dopamine in the nerve pathway connecting the same forebrain nuclei with the largest nucleus of the human midbrain, the dark, half-moon-shaped substantia nigra. This was reported by Kjell Fuxe, who, with Anden, was able to trace dopamine inside nerve fibers originating in cell bodies of the substantia nigra to their terminus, using a new fluorescence technique developed by Hillarp and Falck that makes it possible to detect monamines within a single nerve cell. Paraformaldehyde

This report was made possible by the assistance of all the participants whose work is summarized. Lucien Coté, a member of the Department of Neurology, Columbia University, and one of the symposium organizers, was general consultant.

was used to convert dopamine to a fluorescent compound. By cutting the nerve pathways at various levels, the researchers produced, above the lesions, a concentration of dopamine high enough for the compound to be clearly visible by fluorescence microscopy. Two smaller dopamine-containing pathways were also found; one of these terminates in a nerve network at the pituitary gland and is "probably involved in the control of anterior pituitary secretion," according to Carlsson.

► Reproduction, in more than 40 monkeys, of both Parkinsonism symptoms and the dopamine deficit in the forebrain. T. L. Sourkes of McGill University and Louis Poirier of Laval University did this by cutting the nerve pathway linking the substantia nigra to the forebrain nuclei, where dopamine concentration is normally high. Where loss of substantia nigra cells was incomplete, dopamine loss was only partial.

Degeneration and disappearance of melanin- and dopamine-containing cells in the substantia nigra is the only pathology that has been consistently demonstrated by post mortem studies of patients with Parkinson's, as Abner Wolf, Columbia neuropathologist, said at the symposium. Thus the painstaking work of these four research groups strongly suggests that degeneration of a nerve pathway from the substantia nigra to the forebrain motor centers causes Parkinson's disease. But certain complexities remain, and discussion of these is reported below.



Functional significance of neuron structure is studied by means of model made by Fritiof Sjöstrand, who showed these slides at the meeting. Model shows part of a neuron dissected from the lateral geniculate body of rat forebrain. Main fibers of the optic tract terminate in this center, from which fibers of visual perception lead to the visual cortex. Model was sized on the basis of 500 cross sections like the one shown above. Ulf Karlsson collaborated in this study at the University of California, Los Angeles.

Drugs Can Induce Symptoms

Since James Parkinson described paralysis agitans in 1817, many combinations of tremor, muscle rigidity, and loss of certain spontaneous associated movements have been called Parkinson's disease. The constellation of symptoms sometimes follows encephalitis. Among the mysteries surrounding the disease is the fact that symptoms sometimes disappear during sleep; a postencephalitic patient was described as being able to walk and talk only during sleep. In the United States, one million persons are estimated to have Parkinson's disease.

A number of drugs have been used for treatment (many with atropine-like action), each of which is of limited effectiveness. Reserpine and, to a lesser degree, the group of phenothiazine tranquilizers also produce Parkinson-like symptoms in experimental animals and, as a side effect, in some human patients.

More effective than either of these drugs in inducing tremor in animals is tremorine (1,4-dipyrrolidino-3-butyne). This compound was synthesized for this experimental purpose by Guy Everett of Abbott Laboratories, who told the symposium that oxytremorine has recently been recognized as the active metabolite of tremorine and has been synthesized. This compound also has possibilities for the study of neural temperature-control mechanisms, Everett said.

The forebrain centers in which these researchers showed a dopamine deficit are the gray nuclei (the caudate nucleus and the putamen) of the corpus striatum, a subcortical mass of gray and white matter lying above the thalamus in each cerebral hemisphere.

Control center of the old (extrapyramidal) motor system, the corpus striatum is the most important brain center in birds and other animals in which the cerebral cortex is absent or rudimentary. With the advent of the cerebral cortex, the pyramidal system appeared as the main motor pathway, linking cerebral controls directly to lower motor pathways. While the role of the extrapyramidal system in man is not entirely clear, there is evidence that this system acts to modulate the pyramidal pathways and to control posture and gross body movements. Cajal found collateral nerves linking both old and new motor systems.

In reporting the dopamine deficit found in extrapyramidal control centers of the human brain, Hornykiewicz said the dopamine deficit could not be made up in patients by prolonged treatment with monoamine oxidase inhibitors. Monoamine oxidase is the enzyme operative at one of two breakdown pathways known for dopamine. Treatment with L-dopa, precursor of dopamine, relieved symptoms but had to be discontinued because of side effects, one of the most severe being emetic action.

Suggesting that dopamine's normal role may be inhibition of neuron activity in the old motor centers, Hornykiewicz cited work of other groups who have found that dopamine inhibits the activity of certain brain cells in animal species ranging from the cat to the garden snail. He suggested that some symptoms of Parkinsonism are caused by degeneration of dopamine-containing neurons that normally inhibit the activity of the old motor centers.

Possible Storage Disturbance

The level of the main breakdown product of dopamine, homovanillic acid, was also found to be subnormal in the striatal nuclei, the substantia nigra, and the globus pallidus of patients with Parkinson's disease. But in the globus pallidus the level of homovanillic acid was found to be 20 times that of dopamine, a ratio much higher than that for normal brain. The higher relative concentration of the breakdown product, Hornykiewicz said, suggests a possible disturbance of the dopamine-storing mechanism.

A surgical technique introduced by Irving Cooper, by which lesions are made in the globus pallidus, has

Melvin Yahr organized basic research.

been effective in relieving rigidity in a large number of patients. Hornykiewicz suggested, as a working hypothesis, that rigidity may occur when neuronal activity from the substantia nigra to the striatal nuclei is interrupted and dopamine is released from the striatal outer structures to the inner globus pallidus. Rapid breakdown there would account for the high ratio of homovanillic acid to dopamine.

Building on the structure laid down by U. S. von Euler, the famed physiologist of the Karolinska Institute who established in 1946 that norepinephrine is the chemical transmitter of the sympathetic nervous system, and upon fundamental contributions by the late N. A. Hillarp, Swedish work in exploring the chemistry of the brain is especially lively. Thus Carlsson's report dealt both with his own pioneering findings on the distribution of dopamine in the brain in many animal species and with the findings of other workers at Göteborg and Lund.

Carlsson was able to show that reserpine, while inducing Parkinson-like symptoms in animals, also depletes brain concentrations of dopamine. Bjorn-Erik Roos, a member of the Göteborg group now working at Emory University, Atlanta, showed that dopa, precursor of dopamine, counteracts the motor effects of reserpine and also raises the blood concentration of dopamine to the normal level. Unlike dopamine, dopa can penetrate the blood-brain barrier.

Only fluorometric methods are sensitive enough to detect dopamine and other brain amines in microgram quantities. Working with a method introduced by Ehrlen in 1948 for norepinephrine detection, Carlsson developed it and obtained a dopamine spectrum markedly different from the spectrum of norepinephrine. By this method the monoamines are extracted from nerve tissue, purified by ion exchange in a resin column, separated by elution, and oxidized to hydroxyindoles. In alkali, the reaction products rearrange to form fluorescent compounds. With a change to acid *p*H, fluorescence of the dopamine fluorophore is increased in intensity and occurs at a wavelength lower than that of norepinephrine.

All of the workers who reported dopamine estimates at the symposium use this method or variations of it, and all of them said they read their results on a spectrophotofluorometer of the type designed by Robert Bowman of the National Heart Institute. The instrument was devised in collaboration with Bernard Brodie, Sidney Udenfriend, and other NHI pharmacologists exploring the function of brain amines.

Cutting Pathway Gives Symptoms

When Sourkes and Poirier placed lesions along the dorsomedial edge of the substantia nigra of monkeys, hypokinesia of contralateral limbs appeared. In these animals, marked depletion of dopamine was found in the corpus striatum on the same side. Striatal serotonin was at a normal level.

The substantia nigra, a relay station of the extrapyramidal pathways, does not appear in evolution below mammals, while the melanin-containing nerve cells that give the center its name are most highly pigmented in man. This may explain why Parkinson's disease is unknown in animals.

When Sourkes and Poirier cut dorsomedial fibers of the cerebral peduncle and the corresponding pathway connecting the nucleus ruber with spinal nerves, animals showed chorea-like movements of the contralateral limbs.

"Such lesions were associated with a low concentration of serotonin and a normal concentration of dopamine in the corresponding striatum," Sourkes said.

A combination of the lesions described above produced spontaneous tremor of the contralateral limbs, accompanied by a loss of both serotonin and dopamine from the striatum. Sourkes and Pourier consider that this work, which correlated neurochemi-

cal, histological, and functional studies, has demonstrated roles for both serotonin and dopamine in the central nervous system.

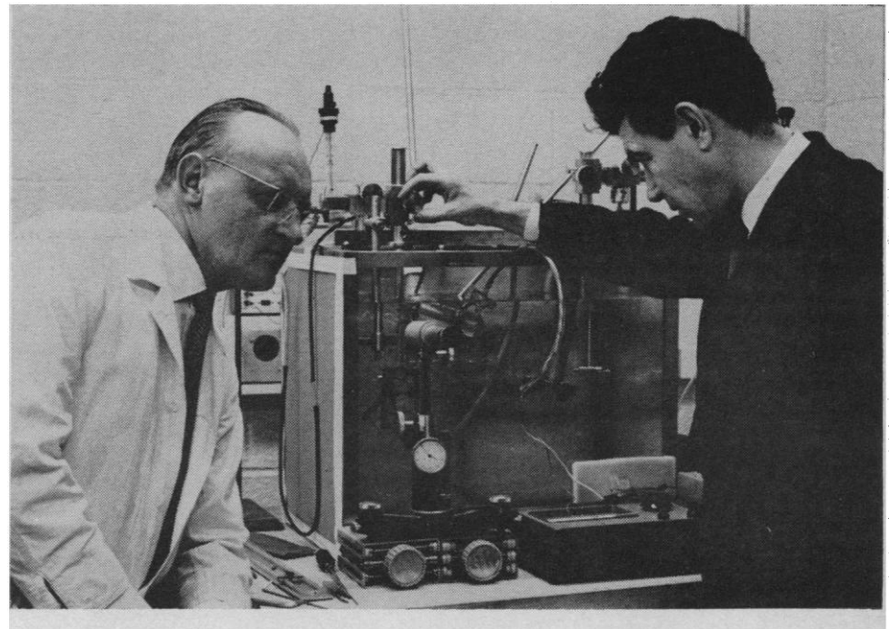
Discussing these findings, Columbia neuroanatomist Malcolm Carpenter said that "the anatomists have had a hard time demonstrating what the biochemists can demonstrate with ease. Most of the efferent fibers from the substantia nigra can be traced to the globus pallidus. Projection from the corpus striatum to the nigra can be demonstrated, but the reverse pathway cannot be shown by anatomists."

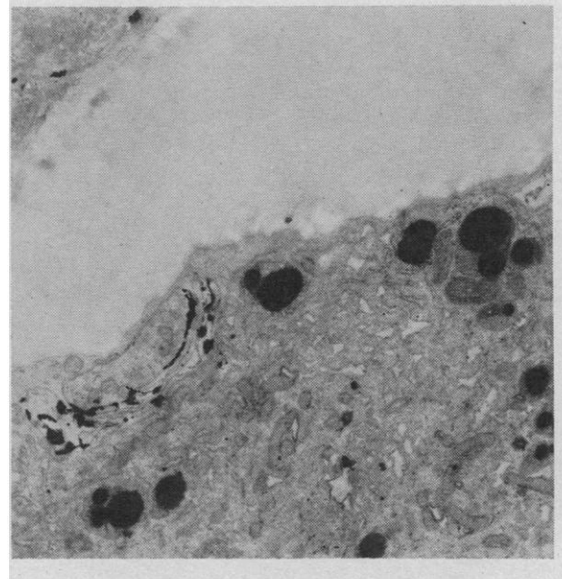
In the final panel, Dominick Purpura, Columbia neurophysiologist, said it is "difficult to demonstrate a monosynaptic pathway from the nigra to the striatum. If the pathway exists, it should be possible to stimulate at the nigra and record excitation in striatal centers."

Sourkes also said that α-methyl dopa, a synthetic analog of dopa, increases tremor in patients with Parkinson's disease and is thought to compete for the enzyme that decarboxylates dopa to form dopamine. This analog has been available for research since its synthesis by Merck and Company in the 1950's. Working collaboratively with the National Heart Institute research group, Merck made a number of other compounds that have been of great value in experimental study of neuroamines. Clinical use seemed unlikely at the time Merck invested in synthesis. Only recently has α-methyl dopa found wide clinical acceptance in the treatment of hypertension. But this effect, Albert Sjoerdsma of the National Heart Institute told the symposium, is not the result of enzyme inhibition. It probably occurs because the analog is metabolized to α-methylnoradrenaline. "The latter amine is thought to act as a weak or 'false' transmitter at sympathetic nerve endings."

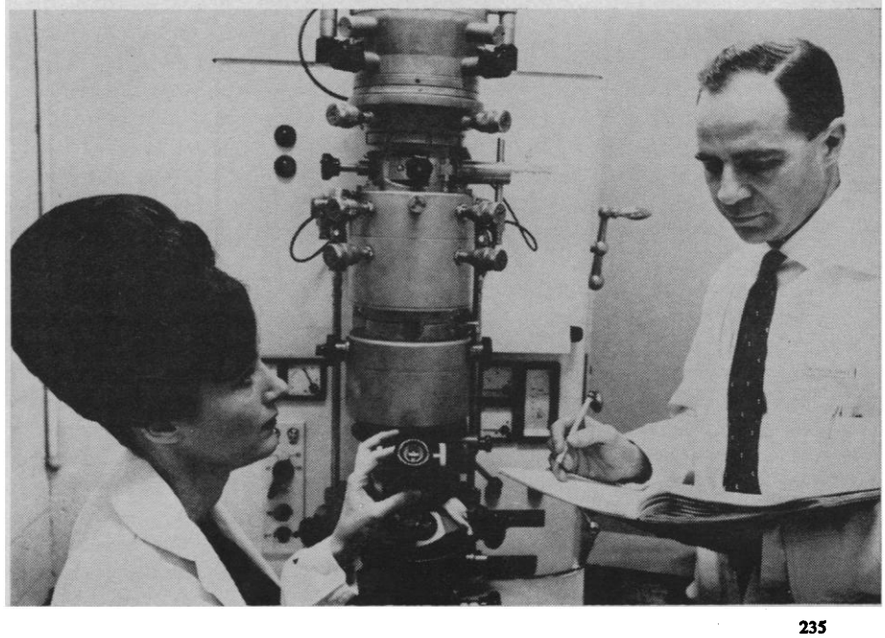
Sjoerdsma is now studying clinical use of another member of the analog group made a decade ago by Merck. This is α-methyl-*p*-tyrosine, a strong inhibitor of the enzyme that converts tyrosine to dopa. The analog produces up to 70-percent inhibition of neuroamine synthesis in humans. In patients with pheochromocytoma the drug reduces blood pressures. In other patients there has been little effect on blood pressure, and the drug produces a mild sedation with few toxic side effects.

While the dopamine findings meant





Enzyme activity in single nerve cell is measured in magnetic microdiver. Miro Brzin, University of Lubljana, Yugoslavia (right, above), is a co-inventor of the instrument. Brzin worked with Columbia electron microscopists Virginia Tennyson and Philip Duffy (below) to combine biochemical with structural study. Two frog sympathetic neurons shown in micrograph (× 12,600) were analyzed for 110 minutes gasometrically in the diver for acetylcholinesterase activity. Same cells, treated histochemically, show AChE as black lines and dots in electron micrograph. (Large black ovals may be lysosomes.) Rudi Pavlin (left, above) also reported AChE study by microdiver (see page 238).



progress, no one in this field underestimates the complexities that must be explored before it can be said whether dopamine deficit causes Parkinsonism or whether the deficit is only one of many results of a mechanism as yet unseen.

"In the life sciences, the situation is always more complicated than we think," Seymour Kety of the National Institute of Mental Health said at the final panel session. "How do the neurochemicals fit into the fantastically complicated, remarkably efficient structure of the nervous system? Despite decades of research, we still don't clearly understand the mechanism of acetylcholine action. ACh effects on the neuromuscular junction may be quite clear, but effects on the central synapses are less clear.

"Many newly discovered biogenic amines are being studied as possible transmitters in the brain. Are these compounds transmitters or modulators? Perhaps they may create a chemical field that modulates action of the neurons. There may be a complex interplay of amines at different sites rather than clear-cut and general actions throughout."

Sidney Udenfriend, the NHI pharmacologist who recently identified the rate-limiting enzyme in the tyrosine-to-norepinephrine chain, suggested that it may be more useful to map the distribution of enzymes than to map that of substrates. "The brain amines are small-molecular substances, particularly subject to distortion by staining and other analytical procedure."

Turnover Rates Now Known

While levels of brain amines can be measured, the results tell nothing about the rate of synthesis or breakdown. Udenfriend said that studies with labeled precursors show that amine turnover is higher in brain than in other tissues. In guinea pig brain, for example, norepinephrine turns over at a rate corresponding to a half-life of 2 to 3 hours as compared with 300 hours in guinea pig adrenal gland. Recently radioactive tyrosine and dopa with high biological activity became available, and Udenfriend used these in studies which indicate that turnover of dopa in brain is even faster than turnover of norepinephrine.

"Initial findings indicate that the total daily synthesis of catecholamine is higher in brain than in the adrenal gland," he told the symposium.

Columbia pharmacologist Erminio Costa has been using tritium-labeled dopamine and norepinephrine to calculate sizes of storage pools and rates of synthesis of stores in various peripheral tissues of animals.

Since these amines cannot pass the blood-brain barrier, this method is useless for study of the central nervous system. Costa told the symposium that he has been able to calculate the rates of synthesis of dopamine and norepinephrine in brain by using α-methyltyrosine to block synthesis. By blocking the enzyme acting at the tyrosine-to-dopa conversion, α-methyl-tyrosine causes an exponential decline in brain levels of the amines. By measuring the initial rate of decline, Costa and N. H. Neff have been able to calculate rates of formation of dopamine and norepinephrine and to show that the rates differ in different parts of the brain.

By use of both techniques, these researchers also showed that rates of catecholamine synthesis are controlled by a feedback inhibition. Catecholamine levels play a role in this control, Costa said.

Environmental Response Shown

"We are discouraged by the failure of our attempts to show the role of neurohormones by direct drug experiments," Bernard Brodie told the final panel. "We are now trying a game of poker with nature, trying to force her to show her closed cards. Which neuronal system is used under a particular environmental stimulant? What neuronal circuits become active when, for example, you put a rat in the cold? We have been able to measure norepinephrine turnover and to find a 70-percent increase after 1 hour in the cold. There is no increase in serotonin. This suggests that the adrenergic system is involved in the animal's defensive action. After 1 hour in a hot room, the rat's rate of serotonin synthesis is up 70 percent and the animals look as if they have received reserpine. This suggests that other circuits involving serotonin are needed to get rid of the heat."

Holger Hydén, University of Göteborg neurobiologist, reported that, in RNA from glial cells removed from the globus pallidus of patients with Parkinson's disease, the ratio of adenine to uracil was much higher than the ratio in RNA samples from patients who did not have this disease. Working with G. Gomirato of the University of Pisa, Hydén was able to make this comparative RNA analysis by using 3- to 8-millimeter specimens of tissue removed in brain surgery.

As discussant for Hydén's paper, Columbia's Erwin Chargaff said that "RNA is a generic name for a very large number of different compounds of very diverse sorts. Transport or messenger RNA is a particularly shady group. Messenger RNA can have any composition according to the protein being carried."

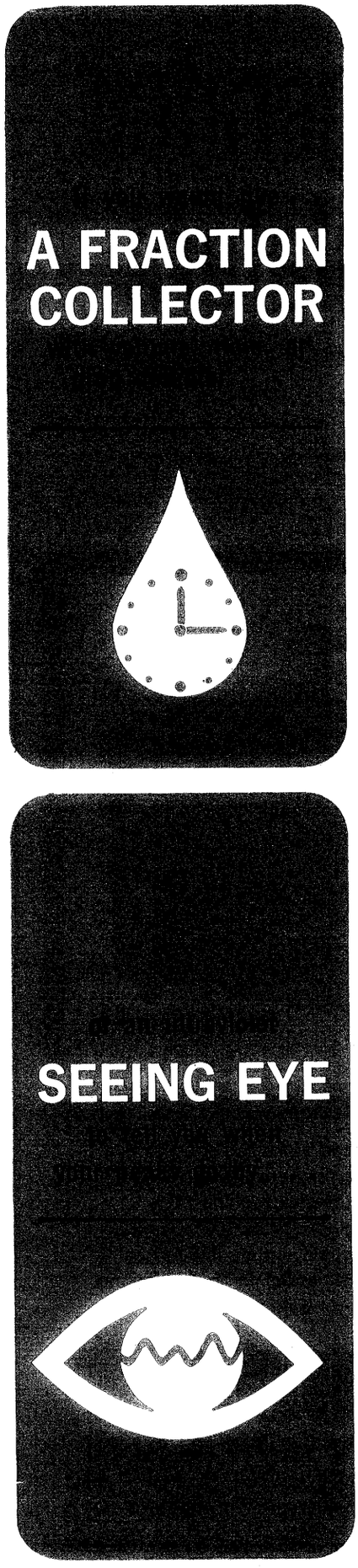
In response, Hydén said he would soon publish results of current work in which he is using tritium labeling to trace messenger RNA and is varying *p*H values in order to identify locations in the cell body where messenger RNA and ribosomal RNA are concentrated.

Hydén Reports S100 Higher in Glia

Hydén's widely known studies of normal brain cells have suggested that the glial cells, which account for half of total brain volume and which were thought, before his work, to function only as supporting structure, may in fact be the control or programming units of nerve action.

At the final panel session Hydén described recent work indicating that the *S*100 protein, recently reported for the first time by B. W. Moore and thought to be specific to the brain, is contained in the cell bodies of the glial cells but only in the nuclei of the neurons. Many workers are now studying *S*100 and other recently found brain proteins whose turnover is rapid and whose function is unknown. Since cell growth is very limited in adult brain neurons, nerve cell renewal cannot account for the high rate of turnover.

Hydén's technique, as he described it, gives some impression of the delicacy of the microanalyses for which he is noted. Using the large vestibular nerve from rabbit brain stem, he separated glia from neuron by free-hand dissection. Some 20 samples of each cell type were homogenized and centrifuged. Then agar was introduced by capillary force in the middle third of a capillary 40 millimeters long. The appropriate fraction of the neuron sample was introduced in the top part of the capillary. Antiserum to *S*100 was placed in the bottom third. Tubes with glial centrifugate



were similarly prepared. After 4 days, precipitates were found in capillaries containing the fraction from glial cells but not in tubes containing the neuron fraction.

Using fluorescent-antibody technique with frozen cell samples, Hydén found *S*100 localized in the nuclei of glial cells and in scattered spots of the membrane system. By this means he also found *S*100 in the nuclei of nerve cells. Because the nucleus amounts to only about 3.5 percent of the mass of this nerve cell, nuclear concentration of *S*100 did not show up in precipitation analysis.

Does *S*100 move from glial nuclei to the nuclei of nerve cells? If so, this unidentified protein might be an inducer substance (*1*).

Acetylcholine Esterase Studied

Some workers have reported that norepinephrine is a transmitter in the reticular formation of the brain stem. Others have reported that acetylcholine is the transmitter in reticular pathways. Most of these reports have come from electrophysiologists; few biochemical studies have been made.

Rudi Pavlin, Ljubljana University, Yugoslavia, told the symposium that he used a new magnetic microdiver technique (*2*) to study choline esterase activity in single nerve cells and in clumps of glial cells dissected from reticular nuclei of rat. The dissection was made according to the method of Hydén, with whom Pavlin had worked earlier. In most experiments the giant cells from the nucleus reticularis pontis caudalis were used. Certain nerve fibers descending from the cortex and the corpus striatum terminate in this nucleus. The microdiver technique was described at the symposium by one of its inventors, Miro Brzin, also of Ljubljana University (*2*).

Pavlin said he found evidence of active choline esterase in all cells examined. Enzyme activity of single cells from the same nucleus varied widely, but he found no correlation of activity with either cell volume or cell surface.

Acetylcholine and three higher choline esters are known to occur in rat brain. Selective esterase inhibitors were used to measure, differentially, enzyme activity specific for each choline ester.

In young rats, acetylcholine esterase activity is higher in glial cells than in neurons, Pavlin said. After 80 days of life, acetylcholine esterase activity had increased by 300 percent in neurons and by only 10 percent in glial cells.

In other experiments Pavlin studied LSD as a possible inhibitor of acetylcholine esterase. While LSD in low concentration inhibited hydrolysis of acetylcholine in homogenates of brain stem tissue, inhibition in single nerve cells was found only at concentrations higher than 20 micromoles per liter. Since concentrations used to produce the LSD effect in humans are much lower, inhibition of acetylcholine esterase in the brain stem can probably be ruled out as the mechanism of LSD effect.

Pavlin said he will develop the microdiver technique to study the action of several enzymes operating simultaneously in a single nerve cell. Further work by this and other techniques, he said, might lead to the "recognition that both cholinergic and adrenergic fibers terminate in the same neuron."

The above is only a sampling of a rich program. The full report of the symposium will be published by the Foundation.

T. L. CAMPBELL

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References and Notes

1. For a full report of this work, see H. Hydén and B. McEwen, *Proc. Nat. Acad. Sci. U.S.* **55**, 354 (1966).
2. The magnetic microdiver is described in Brzin, Dettbarn, Rosenberg and Nachmansorn, *J. Cell Biology* **26**, 353 (1965).

Forthcoming Events

April

15–16. Iowa **Acad. of Science**, Pella. (G. W. Peglar, Dept. of Mathematics, Iowa State Univ., Ames)

15–16. Minnesota **Acad. of Science**, annual mtg., Macalester College, St. Paul. (W. Larson, The Academy, 3100 38th Ave. S., Minneapolis 55406)

15–16. Montana **Acad. of Sciences**, Missoula. (L. H. Harvey, Univ. of Montana, Missoula 59801)

15–17. American Soc. of **Internal Medicine**, New York, N.Y. (A. O. Whitehall, 3410 Geary Blvd., San Francisco, Calif.)

16–18. **Lateral Line Detectors**, intern. conf., New York, N.Y. (P. H. Cahn, Stern College, Yeshiva Univ., 253 Lexington Ave., New York 10016)

17–20. **Electron and Ion Beam Science and Technology**, 2nd intern. conf., American Inst. of Mining, Metallurgical, and Petroleum Engineers, New York, N.Y. (H. N. Appleton, 345 E. 47 St., New York 10017)

18–19. American **Otological** Soc., San Juan, P.R. (W. H. Bradley, 1100 E. Genessee St., Syracuse, N.Y.)

18–20. Thermodynamics of **Ceramic**