Reports

Presence, Formation, and Metabolism of Normetanephrine in the Brain

The neurohumoral agent norepinephrine has been shown to be highly localized in the central nervous system (*1*), yet little is known about its metabolism in the brain. Although the role that this catechol amine plays in the action of the central nervous system is still conjectural, knowledge concerning its metabolism may shed some light on its mode of action. In the past, norepinephrine was thought to be transformed by deamination, yet the possibility of there being other pathways has been recognized (*2*). Recent work in this laboratory has shown that the principal pathway for the metabolism of norepinephrine in the rat involves O-methylation to yield normetanephrine (*m*-O-methylnorepinephrine) (*3*) and that this reaction requires S-adenosylmethionine as the methyl donor (*4*). This report describes the presence of normetanephrine in the rat brain as well as the enzymes involved in its formation and metabolism.

Four adult male rats, Osborne-Mendel stock, were given 50 mg of iproniazid phosphate (a monoamine oxidase inhibitor) per kilogram twice daily, intraperitoneally, to block the further metabolism of normetanephrine. After 3 days of treatment with iproniazid, the rats were decapitated, and the brains were removed, chilled, pooled, and immediately homogenized with 1 volume of 0.1*N* HCl. The homogenate was adjusted to *p*H 10.0 with borate buffer and extracted three times with 5 volumes of isoamyl alcohol. After clarification of the isoamyl extract by centrifugation, it was reextracted twice with 0.05 volume of 0.05*N* HCl. The acid extract was evaporated to dryness in vacuum, taken up in methanol, and evaporated to a small volume under nitrogen. The methanol extract was subjected to two-dimensional chromatography (ascending technique) with Whatman No. 1 filter paper; isopropanol:ammonia (5 percent), 8:2, was used as the first solvent system and *n*-butanol : acetic acid : water, 8 : 2 : 2, as the second. After spraying of the chromatogram with 0.1 percent 2,6-dichloroquinone chlorimide in alcohol followed by 0.1*M* *p*H 10.0 borate buffer, a faint but distinct blue spot (R_f's 0.50 and 0.45) appeared. An authentic sample of normetanephrine treated in the same manner had the same R_f's and color reaction (*5*). From the intensity of the blue spot, it was estimated that 0.1 to 0.2 μg of normetanephrine per gram of brain tissue was present. No normetanephrine could be detected in brains of untreated rats (*6*). These observations suggest that rat brain transforms endogenous norepinephrine to normetanephrine and that the latter compound is metabolized further by deamination.

Enzymes in the brain concerned with the above metabolic processes were then studied. Incubation of *l*-norepinephrine *d*-bitartrate with the soluble fraction of rat brain, magnesium chloride, and S-adenosylmethionine resulted in the formation of normetanephrine (Table 1). In the absence of S-adenosylmethionine, or when mitochondria were substituted for the soluble fraction, no O-methylation occurred. Enzymatically formed normetanephrine, isolated and chromatographed as described above, had the same R_f's as a synthetic sample. The rate of O-methylation of norepinephrine in the brain was the same in normal and iproniazid-treated rats.

When normetanephrine was incubated with brain mitochondria, considerable amounts of the amine were metabolized (Table 1). No disappearance of normetanephrine occurred when it was incubated with the soluble fraction. The ability of brain mitochondria, prepared from rats pretreated with the monoamine oxidase inhibitor iproniazid, to metabolize normetanephrine was markedly reduced (Table 1). Further evidence that normetanephrine was deaminated by brain mitochondria was obtained by oxidizing the deaminated product enzymatically to 3-methoxy-4-hydroxymandelic acid. After incubation of normetanephrine, brain mitochondria, aldehyde dehydrogenase (*7*), and diphosphopyridine nucleotide for 2 hours, the reaction mixture was acidified with HCl and extracted with 10 volumes of *n*-butanol. The butanol layer was reextracted with 5 percent sodium bicarbonate solution, and the aqueous layer was reextracted into ethyl acetate at *p*H 1. The ethyl acetate extract was reduced to a small volume under nitrogen and subjected to two-dimensional chromatography as described above. When the chromatogram was sprayed with dichloroquinone chlorimide reagent, the resulting blue spot had the same color and R_f's (0.30 and 0.60) as an authentic sample of 3-methoxy-4-hydroxymandelic acid. Incubating normetanephrine with brain mitochondria and soluble fraction also resulted in the formation of a compound having the same R_f's as 3-methoxy-4-hydroxymandelic acid.

The observations described above indicated the following metabolic pathway for norepinephrine in the brain:

$$\text{Norepinephrine} \xrightarrow[\text{S-adenosylmethionine}]{\text{O-methylation}} \text{normetanephrine} \xrightarrow[\text{oxidation}]{\text{deamination}} \text{3-methoxy-4-hydroxymandelic acid}$$

At present, conclusive evidence concerning the principal pathway for the

Table 1. Formation and metabolism of normetanephrine in the brain. Intracellular fractions obtained from 100 mg of rat brain were incubated with 0.2 μmole of substrate and 50 μmole of *p*H 7.8 phosphate buffer at 37°C*. After 1 hour of incubation, the reaction mixture was assayed for normetanephrine (*3*).

Cell fraction	Substrate	Additions	Normetanephrine	
			Formed (μmole)	Disappeared (μmole)
Soluble	*l*-Norepinephrine	None	0.00	
Soluble	*l*-Norepinephrine	S-adenosylmethionine	0.02	
Mitochondria	*dl*-Normetanephrine	None		0.10
Mitochondria	*dl*-Normetanephrine	Iproniazid†		0.02

* Magnesium chloride (10 μmole) was added to the incubation mixtures for O-methylation of norepinephrine.
† Iproniazid (200 mg/kgm) was given to rats 1 hour before sacrifice and removal of the brain.

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metabolism of norepinephrine in the central nervous system is lacking. However, from experiments described here and elsewhere (*3*), it appears likely that O-methylation constitutes an important route for the metabolism of the norepinephrine in the brain.

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References and Notes

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5. Under the conditions described above, metanephrine (*m*-O-methyl epinephrine) has markedly different R_f's from normetanephrine. No metanephrine could be detected in brain extracts of iproniazid-treated animals.
6. Normetanephrine and metanephrine were found to be present in the adrenal glands of iproniazid-treated rats.
7. Purified aldehyde dehydrogenase was prepared by a procedure described by H. Weissbach, B. C. Redfield, and S. Udenfriend (*J. Biol. Chem.*, **229**, 953 (1957) and kindly supplied by H. Weissbach.

3 December 1957

Extraction of an Osteogenic Inductor Factor from Bone

The hypothesis that osseous tissue contains an extractable substance which is capable of inducing the formation of new bone has been presented in detail by Bertlesen (*1*), Levander *et al.* (*2*), Willestaedt *et al.* (*3*), and La Croix (*4*) and has been denied by Heinen *et al.* (*5*) and Danis (*6*).

The percentage of all positive experimental results obtained to date, in rabbits and dogs, chiefly by alcoholic or acidic bone extracts (30.8 percent; that is, in 294 of 955 animals reported in the literature), when compared with the percentage of positive results in control animals (22.6 percent; that is, in 74 of 328 animals reported), while statistically significant, leaves room for refinement of techniques (*6*).

Intracerebral implantation (*7*) of a paste of bovine bone (*8*) in the young rat produced endocranial fusion of the normally patent coronal suture after 15 days. This paste had been stored for several months at 4°C in bovine plasma or physiological saline. Histologic examination of all paste samples showed that no living osteocytes or osteoblasts were present. In order to isolate the factors responsible for such osteogenic activity in a relatively resistant host, the following extractive procedures were undertaken.

Either 9 or 18 g of bone paste was incubated for 24 hours at 37°C in a solution consisting of 50 ml of Ringer–Tyrode and 20 ml of distilled water. The mixture was subsequently centrifuged and filtered. The resulting solution was metachromatic to toluidine blue. This reaction was lost after the solution was concentrated on a steam bath to one-fourth of its original volume. The clear brown solution was then stored at 4°C. Storage up to 30 days did not diminish its osteogenic activity.

Pieces of Gelfoam sponge were impregnated with the solution, either before or after concentration, and placed intracerebrally, under parietal bone flaps, in 22 seven-day-old Long-Evans rats. There were ten controls. Fifteen days after intracerebral implantation the animals were sacrificed, and the implantation sites were examined grossly and fixed with 10 percent Formalin. Decalcification with formic acid-sodium citrate solution was followed by paraffin embedding and hematoxylin and eosin staining of 10-μ serial sections.

The results were uniformly positive (Fig. 1). All cases of intracerebral implantation showed extensive osteogenic activity at the site of impregnated Gelfoam exclusively. Osteogenesis was present within the tissues which had invaded the sponge. This inductor activity was shown by all solutions tested, both concentrated and unconcentrated, of either strength, derived from plasma- or saline-stored paste. Grossly, the implants were firm and fused to the host calvaria. They gave a distinctly calcareous impression to a fine steel probe. Histologically, immature trabeculated bone was observed throughout the implant. Some of this new bone was fused to the host endocranial plate. Most of the bone was observed on serial section to have no such continuity. It consisted of isolated islands of osseous tissue in all stages of development. A very strong impression was obtained that these areas of bone had arisen *in situ*. At implantation sites the effective thickness of the calvaria was often four times that of adjacent areas. Active osteogenesis was underway on all surfaces of newly formed bone at sacrifice. The formation of new marrow spaces was frequent. The area of induced osteogenesis never extended beyond the area of implantation. Connective tissue and vascular infiltration and proliferation in the implant area was marked. The sponge had virtually disappeared at this state.

Control animals never showed the slightest osteogenic stimulation or induction following operative procedures alone, or following implantation of either plain, Ringer–Tyrode- or plasma-impregnated Gelfoam.

It seems probable that part of the observed osteogenic response was due to stimulation of preexistent osteoblasts in calvarial implantation sites by some factor extracted from the bone paste. An additional inductive capacity of the extracted solution was also clearly indicated in the numerous sites of new bone formation, demonstrably unconnected with host bone. A spectrum of modulating cell types was noted, ranging from undifferentiated connective tissue cells to mature osteocytes. Intermediate cell types showed increasing basophilia with accompanying cytoplasmic and nuclear enlargement. The intermediate cell type associated with the onset of bone matrix formation possessed an eccentric nucleus, intense basophilia, and a somewhat vacuolated cytoplasm.

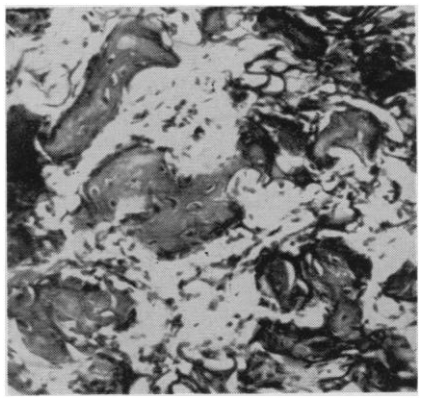

Fig. 1. Island of induced bone, 15 days after intracerebral implantation of Gelfoam impregnated with the extracted solution. Note the typical osteocytes and the sites of continued osteogenesis. This island is unconnected with and well removed from the host bone. In the immediate area several stages of connective tissue metaplasia were observed (× 750).

Biochemical analysis (*9*) of an osteogenically active solution derived from saline-stored bone paste demonstrated a concentration of 0.29 mg/ml of Chondroitin sulfate A or C, or both. This recovered material was redissolved in Ringer–Tyrode in a concentration of 0.1 mg percent. Gelfoam sponges impregnated with this solution were implanted as above and uniformly produced osteogenesis in eight rats. Additional fractions continue to be tested in a variety of heterotopic sites.

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