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The Distribution of Thiamine Diphosphate-Phosphohydrolase in the Neurosecretory Nuclei of the Rat Following Osmotic Stress

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Summary. 1. The distribution of thiamine diphosphate-phosphohydrolase (TPPase) was studied by a random hit method in glyoxal fixed supraoptic (SON) and paraventricular (PVN) nuclei of the rat.

2. After 2 days of water deprivation the distribution of TPPase in SON had increased at least 40% while that of PVN was still at the control level. Both nuclei reacted with a progressive increase in TPPase distribution following longer dehydration periods.

3. The Golgi associated TPPase distribution in the neurosecretory cell is likely to be a reliable parameter for secretory activity.

Introduction

Electron microscopical studies indicate that in protein synthesizing gland cells secretory granules are formed by vesiculation of the cysternae of the Golgi apparatus (PALAY, 1958; KUROSUMI, 1961). This role of the Golgi apparatus in packaging the secretory substances into vesicles seems to apply equally to invertebrate neurosecretory cells (SCHARRER and BROWN, 1962; BERN, 1963). Ultrastructural investigation of the supraoptic nucleus of the rat during hypo- and hyperactivity of this mammalian neurosecretory cell complex has also given experimental support for the dynamic role of the Golgi apparatus in the formation of neurosecretory granules (NEMETSCHEK-GANSLER, 1965; ZAMBRANO and DE ROBERTIS, 1966).

The present investigation was undertaken in order to study at a light microscopical level the *changes in morphology of the Golgi apparatus in the process of neurosecretory hyperfunction*. For this purpose we studied histochemically the spatial distribution of thiamine diphosphate-phosphohydrolase (TPPase) in the supraoptic (SON) and paraventricular nuclei (PVN) of the rat following an osmotic stress induced by thirsting periods of different lengths. This enzyme that is considered to be specific to the Golgi apparatus (NOVIKOFF and GOLDFISCHER, 1961; NOVIKOFF, ESSNER, GOLDFISCHER and HEUS, 1962; GOLDFISCHER, 1964) is reported to be highly active in the neurosecretory hypothalamic nuclei (NAIDOO, 1962). Recent histochemical studies on the ontogenetic development of the enzyme pattern in rat neurosecretory hypothalamic centers indicate that TPPase is already present in these nuclei at the early stages of formation of neurosecretory material (PILGRIM, 1967).

Materials and Methods

Preparation of Animals and Fixation of Tissues

Male Wistar rats of 1.5 months (ca. 210 g) were used in these experiments and were kept in individual cages at 25° C. Control rats received both chow and tap water *ad libitum* while the experimental animals, randomly chosen, had free access to food but received no water. The rats were killed by decapitation at 11.00 a.m. and their brains were excised within 4 minutes. The hypothalamic area of each animal was fixed in a cold solution (4° C) of 4% glyoxal buffered with 0.2 M sodium cacodylate (pH 6.5) containing 0.22 M sucrose (SABATINI, BENSCH and BARRNETT, 1963).

After a fixing period of 24 hours the tissues were washed at 4° C for 24 hours in a solution of 0.1 M sodium cacodylate containing 0.2 M sucrose. The hypothalami were stored at 4° C in fresh washing solution for not more than 6 days.

Cutting and Staining Procedure

The chiasmatic area of the fixed hypothalami was cut serially on a Pearse cryostate kept at $-15°$ C. The sections were then put on warm albuminized slides and incubated with constant agitation in a TPPase incubation medium, a slight modification of the one used by NOVIKOFF (NOVIKOFF and GOLDFISCHER, 1961). It consists of: 0.1 M tris-maleate buffer pH 7.2; 4 mM Thiamine pyrophosphate tetrahydrate (Koch-Light), 5 mM $MnCl_2$, and 3.6 mM $Pb(NO_3)_2$.

The lead phosphate deposits in the sections were transformed into black lead sulphide by incubating the slides in 10% ammonium sulphide for 2 minutes. The slides were then passed through graded alcohols and the sections mounted in DEPEX Mounting medium (GURR).

Controls without TPP in the incubation medium, or in which the enzyme activity had been destroyed by pre-heating the sections for 10 minutes at 100° C in a waterbath, did not show any activity.

Quantitative Distribution of TPPase

The distribution of TPPase in SON and PVN was measured quantitatively by the random hit method using an integration ocular (HENNIG, 1957). In each 16 μ section the lead sulphide positive hits were counted three times under oil immersion by turning the integration ocular. The results were expressed as the percentage of hits in the integration ocular per magnocellular nucleus per animal.

Results

The activity of TPPase in the hypothalamus of control-animals was striking only in the supraoptic nucleus and the paraventricular nucleus. Within these nuclei the cells showed differences in staining capacity, some cells not showing any activity while in others a lamellar structure was visible in the cytoplasm adjacent to the nucleus (Fig. 1a, 2a). Some activity was also present in the parvicellular nuclei of the hypothalamus such as the arcuate nucleus, and the ventromedial nucleus. The enzyme activity of the blood vessels was rather low and could easily be distinguished from the neuronal TPP-ase staining.

After a six day's thirsting period there were striking changes in the amount and distribution of the lead sulphide deposits in the magnocellular nuclei. All cells were now TPP-ase positive and, as far as we could judge from these qualitative findings, there was a large increase in enzymatic activity both in the SON and PVN (Fig. 1 and 2). In contrast with the controls, the cytoplasmic lamellar structure around the nucleus had disappeared and was replaced in all cells by a thick fairly massive black band which filled nearly the whole perikaryon. Quantitative determination of the distribution of the lead deposit within each magnocellular nucleus by the "random hit" method revealed that after a thirsting period

of 6 days the deposit covered an area which was nearly twice that of the normal control (Fig. 3). The staining was strictly perikaryonal, in other words the axons and neuropile failed to show any activity.

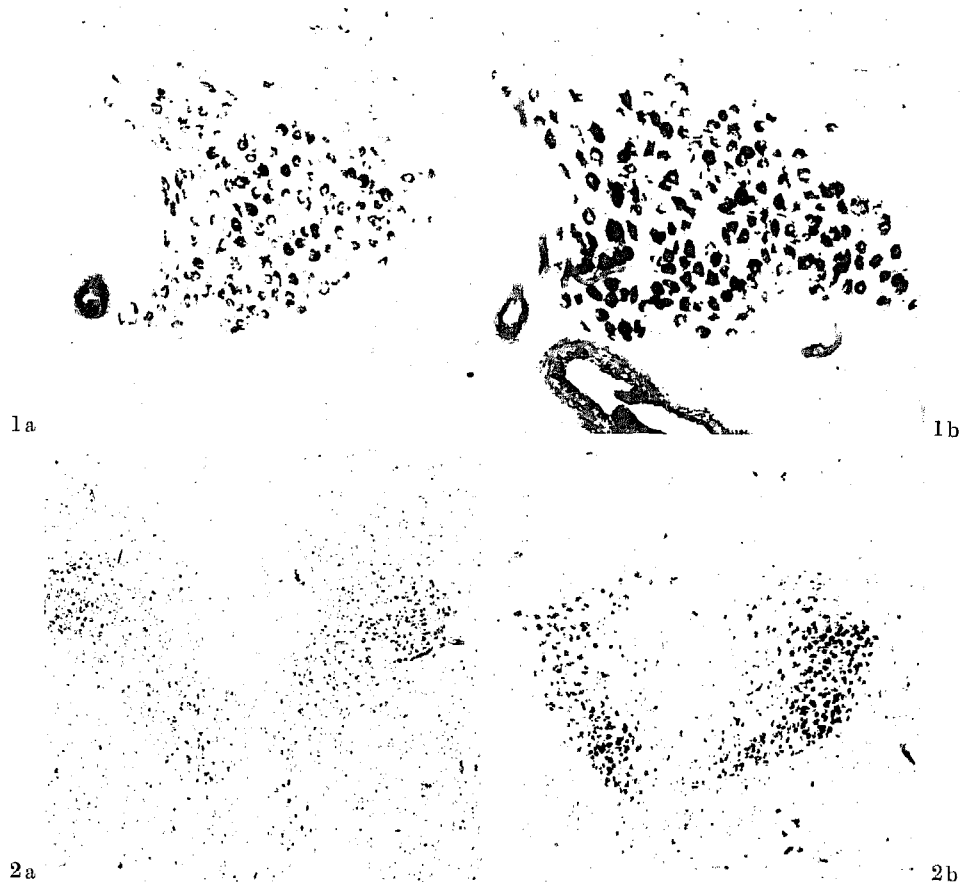

Fig. 1. TPPase activity in the supraoptic nucleus of a control rat (a) and of one which was subjected to a thirsting period of 6 days (b). Glyoxal fixation; ×115. Incubation time in TPPase medium: 10 min at room temperature

Fig. 2. TPPase activity in the paraventricular nuclei of a control rat (a) and of one which was subjected to a thirsting period of 6 days (b). Glyoxal fixation; ×50. Incubation time: 10 min at room temperature

At the end of the six day's thirsting period, the animals were still in a healthy condition.

The unexpected identical reaction of SON and PVN to this 6 day's thirsting period prompted us to study the TPPase-activity of these neurosecretory nuclei following less severe osmotic stress conditions. In this serie of experiments, in which the incubation time and temperature were changed to get better standard conditions, the distribution of TPP-ase was measured after thirsting periods of

1, 2, 3, 4, 5, and 6 days (Fig. 4a, b). In the SON a significant increase in TPP-ase distribution is already present after a 2 day's thirsting period, a distribution that is reached in the PVN only after 4 days of water deprivation.

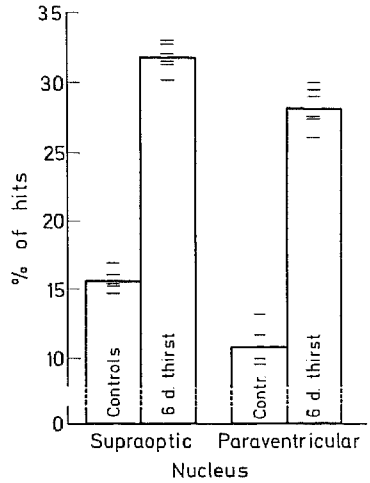

Fig. 3. The percentage of TPPase-produced lead sulphide-positive hits in the magnocellular nuclei of control rats and of rats which were deprived of water for 6 days. Each thin horizontal line represents the average hit percentage in the indicated nucleus of one single rat ($n > 30$). Incubation time: 10 min at room temperature

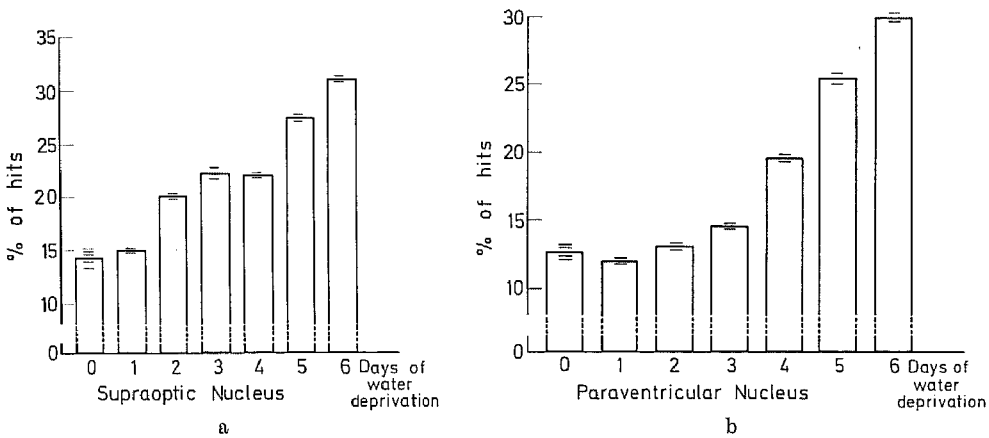

Fig. 4. The percentage of TPPase produced lead sulphide positive hits in the magnocellular nuclei of rats deprived of water for 0, 1, 2, 3, 4, 5 and 6 days. Each thin horizontal line represents the average hit percentage in the indicated nucleus (a: SON; b: PVN) of one single rat. Incubation time: 30 min at 4° C

Discussion

Although the specificity of TPPase for the Golgi apparatus has been in dispute (BARRON and TUNCBAY, 1962) ever since Novikoff and Goldfischer first introduced the staining method (NOVIKOFF and GOLDFISCHER, 1961; NOVIKOFF, ESSNER, GOLDFISCHER and HEUS, 1962), later observations support the original view

(GOLDFISCHER, 1964). In glutaraldehyde-fixed SON the sites of TPPase activity are not restricted to the smooth surfaced elements of the Golgi apparatus, but are also present in membranes of the small granules which are in close contact with the Golgi cysternae (OSINCHAK, 1964). Despite the fact that no definite role could be attributed to the TPPase, the presence of the enzyme in these vesicles together with the absence in mature neurosecretory vesicles indicate that TPPase can play a role in early vesicle formation. Another possibility is that TPPase only indicates the derivation of the early vesicle, a membrane property lost during the process of maturation of this neurosecretory vesicle.

Our study of the distribution of TPPase in the neurosecretory nuclei was in need of a reproducible staining method, which did not results in enzyme losses or diffusion of the enzyme. In our experience the use of glyoxal as a pre-fixative together with an incubation temperature of 4° C fulfilled these requirements[1]. The determination of the area occupied by the enzyme using the integration ocular of HENNIG (1957) does present serious difficulties due to the thickness of our sections. The transformation of "% of hits" into "relative area occupied" must then take into account the non-uniform shape of the TPPase distribution and the differences in staining intensity following different periods of dehydration, and would result in a very disputable parameter. The reproducibility of our "hit" countings together with the knowledge that the increase in this parameter underestimates the enlargement of the area occupied by the enzyme, prompted us to avoid such an unreliable transformation.

The increase of TPPase distribution in both SON and PVN after a period of prolonged osmotic stress — a condition which is known to be effective in producing signs of hyperactivity in these nuclei (HILLARP, 1949; WOLTER, 1956; EDSTRÖM, EICHNER and SCHOR, 1961) — indicates that this parameter can be used in the study of the neurosecretory cell complexes. Before 4 days of dehydration the PVN failed to show an increase in TPPase distribution, in contrast with the SON in which a significant increase is already present after 2 days of water deprivation. The magnitude of the reaction together with the role of SON and PVN in the production of respectively antidiuretic hormone and oxytocin (OLIVECRONA, 1957; NIBBELINK, 1961) make it probable that the PVN plays a yet unknown role in the maintenance of the water and electrolyte balance only after 4 days of thirsting.

Whether this TPPase parameter for hypothalamic magnocellular secretory activity is applicable to other neurosecretory and vesicle-forming systems as well remains to be proved, but it seems possible that elaborate nuclear and nucleolar measurements can be bypassed in the study of neurosecretory cell activity by applying this rather simple TPPase technique. Although no conclusions can be drawn from this study regarding differences in the SON and PVN enzyme levels of normal and thirsting animals, it seems apparent that such differences exist. This aspect is now under investigation by applying quantitative histochemical methods (LOWRY, 1964; SCHULZ, PASSONNEAU and LOWRY, 1967) on pure freeze-dried supraoptic material (JONGKIND, 1967).

[1] The influence of different fixation methods on TPPase recovery and staining properties in epididymus, cerebellum and hypothalamus will be published separately in a forthcoming paper.

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