INFLUENCE OF LOW LEVELS OF METAL ION CONTAMINANTS ON THE MITOCHONDRIAL-SWELLING ACTIVITY OF 8-LYSINE-VASOPRESSIN PREPARATIONS

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Lehninger and Neubert (1961) reported that oxytocin and 8-arginine-vasopressin, at concentrations of 2 x 10^{-5} M and higher, stimulated the water uptake of isolated rat liver mitochondria in a manner that resembled the swelling action of simple disulfides such as oxidized glutathione. They suggested that this effect on mitochondrial membranes might be a useful experimental model for studying the action of the posterior pituitary hormones on their target tissues, presumably by reactions involving the disulfide group of the hormones. This work was repeated and extended by Greenbaum and Dicker (1963a, b) in whose hands oxytocin, 8-arginine-vasopressin, 8-lysine-vasopressin, and 8-lysine-vasotocin, at much lower concentrations, produced a more rapid increase in water uptake. Greenbaum and Dicker concluded that definite target organ sensitivity and specificity existed and that the relatively small differences in amino acid composition of the four polypeptides were associated with striking differences in potency.

In the present study we attempted to repeat the work of Greenbaum and Dicker on 8-lysine-vasopressin, which they found to be effective at concentrations below 10^{-6} M. After exhaustive efforts with rat liver mitochondria that swelled as expected under the influence of oxidized and reduced glutathione (Neubert and Lehninger, 1962), L-thyroxine (Lehninger, 1959), Fe^{++} (Hunter *et al.*, 1963), Ca^{++} (Tapley, 1956), and inorganic phosphate (Recknagel and Malamed, 1958) we could not demonstrate a swelling effect of 8-lysine-vasopressin at

concentrations as low as 10^{-6} M. However, we did obtain swelling with some preparations of the hormone at concentrations of 2 x 10^{-5} M and higher.

In these experiments we noted considerable variation in the swelling activity of different lots of 8-lysine-vasopressin. Reassay of the samples indicated that the variations in swelling activity were not associated with differences in pressor activity. These observations and the knowledge that the 8-lysine-vasopressin samples contained small and variable amounts of non-combustible substances suggested to us that metal ion contaminants might be responsible for part or all of the swelling action of the hormone preparations.

To test this suggestion, we subjected samples of 8-lysine-vasopressin to combustion and determined the swelling activities of the non-combustible residues. In several experiments with different samples of 8-lysine-vasopressin, the swelling curves for the hormone at 2 x 10^{-5} M and for the ash derived from an equal amount of the same hormone preparation were essentially identical. In contrast, the swelling activity of thyroxine was destroyed almost completely when it was subjected to the same procedure. Results of a typical experiment are presented in Fig. 1.

Data from a calcium determination on the ash derived from sample No. 1 in Fig. 1 indicated that a 2 x 10^{-5} M solution of this 8-lysine-vasopressin preparation contained Ca^{++} at a concentration of 4 x 10^{-6} M. Since Ca^{++} accounted for only 22% of the total ash, other inorganic substances were also present. In an attempt to reproduce the swelling effect of this hormone sample and of its ash, we studied the swelling activity of 4 x 10^{-6} M Ca^{++} in the presence of 1 x 10^{-6} M concentrations of either Fe^{+++}, Cu^{++}, or Zn^{++}. As shown in Fig. 2, the effect of the metal ion pairs was considerably greater than that of Ca^{++} alone. Although the ash was not analyzed for metals other than Ca^{++}, it is likely that one or more of the metals studied were present in quantities

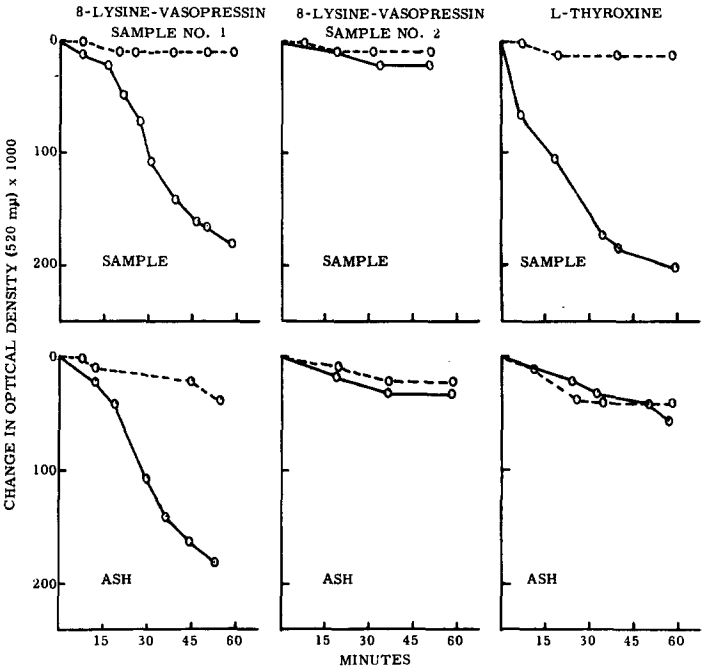

Fig. 1 — Water uptake by isolated rat liver mitochondria in the presence of 8-lysine-vasopressin preparations, L-thyroxine, and their respective non-combustible residues. Medium, 0.125 M KCl — 0.02 M tris, pH 7.4. Temperature, 20-21°. Hormone concentrations, 2 X 10^{-5} M. Ash concentrations, equivalent to 2 X 10^{-5} M hormone concentrations. The broken curves represent the water uptake in the control experiments. All experiments were performed in duplicate on the same mitochondria preparation. The curves shown above were in very close agreement with their duplicates.

such that their combination with 4 x 10^{-6} M Ca^{++} could account for the swelling activity of sample No. 1.

Some 8-lysine-vasopressin preparations, such as sample No. 2 in Fig. 1, did not cause appreciable mitochondrial swelling at hormone concentrations of 2 x 10^{-5} M. The swelling effect of sample No. 2 was detectable at 1 x 10^{-4} M, but was still much lower than the effect of sample No. 1 at 2 x 10^{-5} M (Fig. 3). Because of a limited supply of sample No. 2, we were not able to compare its swelling activity with that of its ash at the higher concentration. Non-combustible substances were present in sample No. 2 in an amount equivalent to about 1/25 the amount in sample No. 1. Since a 1 x 10^{-4} M solution of sample No. 2

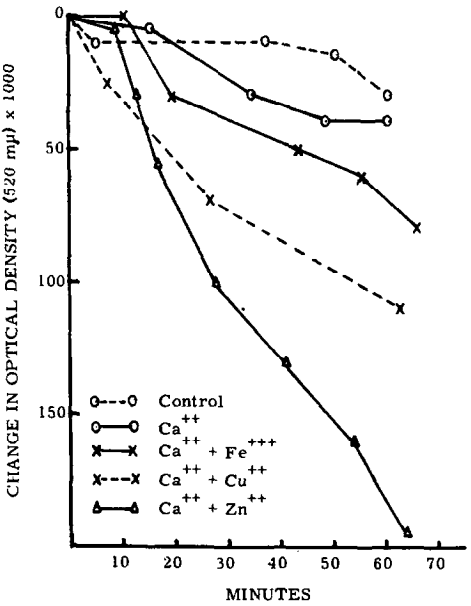

Fig. 2. — Water uptake by isolated rat liver mitochondria in the presence of Ca^{++} and of combinations of Ca^{++} with Fe^{+++}, Cu^{++}, and Zn^{++}. Experimental conditions, the same as in Fig. 1. Ca^{++} concentration, 4×10^{-6} M. Fe^{+++}, Cu^{++}, and Zn^{++} concentrations, 1×10^{-6} M. All experiments were performed in duplicate on the same mitochondria preparation used for the experiments in Fig. 1. The curves shown above were in good agreement with their duplicates. Zn^{++} alone and Fe^{+++} alone at 1×10^{-6} M caused no swelling; Cu^{++} alone at this concentration produced about the same degree of swelling as did the combination of Ca^{++} and Fe^{+++}.

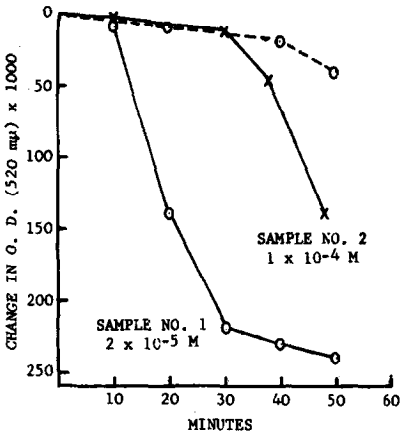

Fig. 3. — Water uptake by isolated rat liver mitochondria in the presence of two samples of 8-lysine-vasopressin with the same pressor activity but with different contents of non-combustible substances. Experimental conditions, the same as in Fig. 1. The residue after combustion was equivalent to 3.0% of the original sample weight for sample No. 1 and 0.13% for sample No. 2.

would contain 1/5 the amount of inorganic substances as a 2×10^{-5} M solution of sample No. 1, it is likely that a part or all of the low swelling activity observed at the higher concentration was the result of metal ions and not of reactions involving the disulfide group of the hormone. However, before the involvement of the disulfide group can be

eliminated completely, it will be necessary to study the swelling activity of an 8-lysine-vasopressin sample that is as free as possible of metal ions.

The results of the present study suggest that reports on the mitochondrial-swelling activity of other polypeptides and proteins should be interpreted with caution until the role of low levels of metal contaminants can be evaluated.

EXPERIMENTAL PROCEDURE

Male Sherman rats (200-300 g) that had been allowed free access to food and water were sacrificed by decapitation. The liver was removed immediately, washed briefly with cold 0.25 M sucrose—10^{-4} M EDTA, and cut into small pieces with stainless steel scissors. The chopped liver was suspended in 30 ml of cold 0.25 M sucrose—10^{-4} M EDTA and homogenized in a cooled Teflon-pestle homogenizer (clearance, 0.006-0.009 inch). The homogenate was made up to a volume of 10 ml per g of liver with cold 0.25 M sucrose—10^{-4} M EDTA and centrifuged at 600 x g for 15 min at 0°. The supernatant was removed and centrifuged at 10,000 x g for 10 min at 0°. The mitochondrial pellet was washed twice by resuspension in cold 0.25 M sucrose without added EDTA (2.5 ml per g of liver). The supernatant and lightly-packed surface layer were allowed to drain off completely each time. Removal of all supernatants and resuspension of the pellet were carried out in a cold room at 4°. The mitochondria were suspended finally in cold 0.125 M KCl—0.02 M tris, pH 7.4 (2.0 ml per g of liver). This stock suspension was placed in an ice bath and used within 1 hr.

Mitochondrial swelling was measured by following changes in apparent optical density at 520 mμ (Tedeschi and Harris, 1958) with a Bausch and Lomb Spectronic 20 spectrophotometer. The experiments were performed at 20-21° in 0.125 M KCl—0.02 M tris, pH 7.4. Matched Pyrex test tubes (15 x 125 mm) that had been washed with nitric acid were used as

cuvettes. Sufficient mitochondria stock suspension (approximately 0.10 ml) was added to 6 ml of the buffer to provide an initial optical density of 0.50-0.60. This amount of stock suspension was equivalent to 0.15-0.20 mg of total mitochondrial nitrogen. Solutions of the substances to be tested for swelling activity were added in volumes of 0.10 ml or less.

The combustions were carried out by heating the samples in air in a micro platinum boat for 5-10 min at 600-650°. The non-combustible residue was dissolved in conc. HCl (0.03 ml) and the resulting solution was transferred with rinsing to a small beaker. Water and HCl were removed in an evacuated desiccator that contained KOH pellets. The residue was dissolved in water and the resulting solution was used in the swelling experiments. Before combustion of each sample, a control solution was prepared by heating the empty boat, adding 0.03 ml of conc. HCl, and proceeding as just described. The ash derived from sample No. 1 in Fig. 1 was devoid of pressor activity.

The 8-lysine-vasopressin samples were synthesized and purified in this laboratory by Dr. Johannes Meienhofer. When freshly prepared, their pressor activities were in the range of 250 units per mg. However, during storage prior to use in the present experiments, the pressor activities declined to about 75% of their original values.

Water from an all-glass still was used for the preparation of all solutions and for the final rinsing of glassware. Tris (reagent grade) and L-thyroxine were obtained from the Sigma Chemical Co. All other chemicals, including the salts used as sources of the metal ions (calcium chloride, ferric ammonium sulfate, cupric chloride, and zinc chloride) were reagent grade commercial preparations.

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REFERENCES

Greenbaum, A. L. and Dicker, S. E., Biochim. Biophys. Acta 74: 519, 1963a.

Greenbaum, A. L. and Dicker, S. E., Biochem. Biophys. Res. Commun. 12: 402, 1963b.

Hunter, F. E., Jr., Gebicki, J. M., Hoffsten, P. E., Weinstein, J. and Scott, A., J. Biol. Chem. 238: 828, 1963.

Lehninger, A. L., J. Biol. Chem. 234: 2187, 1959.

Lehninger, A. L. and Neubert, D., Proc. Natl. Acad. Sci. U. S. 47: 1929, 1961.

Neubert, D. and Lehninger, A. L., J. Biol. Chem. 237: 952, 1962.

Recknagel, R. O. and Malamed, S., J. Biol. Chem. 232: 705, 1958.

Tapley, D. F., J. Biol. Chem. 222: 325, 1956.

Tedeschi, H. and Harris, D. L., Biochim. Biophys. Acta 28: 392, 1958.