*EFFECT OF OXYTOCIN, VASOPRESSIN, AND OTHER DISULFIDE HORMONES ON UPTAKE AND EXTRUSION OF WATER BY MITOCHONDRIA**

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In earlier reports from this laboratory it was shown that reduced glutathione (GSH), cysteine, and certain other thiols stimulate water uptake by isolated rat liver mitochondria.[1-3] More recently it has been found that certain disulfides such as oxidized glutathione (GSSG) also cause mitochondrial swelling, and are sometimes more active than the corresponding thiols.[3] Moreover, a combination of a thiol and a disulfide may be far more active in stimulating water uptake than either compound alone; in fact, low concentrations of GSH and of GSSG may be chosen which have no significant swelling action tested singly, but when combined produce rapid uptake of water.[3] Both the water uptake by mitochondria and the action of C-factor, a mitochondrial protein involved in the ATP-dependent extrusion of water from mitochondria[2, 4-6] are dependent on the ratio of —SH to —S—S— in the medium. Water uptake stimulated by thiols and disulfides depends on the action of the respiratory chains in the mitochondria, since it is blocked under anaerobic conditions or by respiratory inhibitors.[1, 3] Other aspects of water uptake and extrusion by mitochondria have been reviewed.[7, 8]

The pronounced swelling action of simple disulfides prompted us to investigate the action of some peptide hormones containing disulfide linkages on water uptake and extrusion by isolated rat liver mitochondria. We have found that highly purified specimens of vasopressin and oxytocin, as well as insulin, greatly accelerate water uptake by mitochondria; on a molar basis these hormones are much more active than simple disulfides such as GSSG. Their action, like that of simple disulfides, is greatly potentiated by reduced glutathione. Preliminary accounts of these experiments have been presented.[9, 10]

Experimental Details.—The uptake and ATP-linked extrusion of water by freshly isolated rat liver mitochondria (well-fed Wistar rats) suspended in media of 0.125 *M* KCl-0.02 *M* tris(hydroxymethyl)aminomethane at pH 7.3 were measured by following changes in light absorption at 520 mμ as described before.[3, 11] The synthetic oxytocin was a gift of Dr. A. Z. Lane of Parke, Davis Co., Detroit, and assayed 480 International units per mg, or approximately the activity given by the best preparations described. The vasopressin employed was purified by chromatography on carboxymethylcellulose according to Ward and Guillemin,[12] using as starting material a specimen of beef vasopressin, also obtained through the courtesy of Dr. A. Z. Lane, which assayed 80 pressor units per mg. The fractions collected from the column were assayed for pressor activity in the pithed cat as well as for activity in promoting mitochondrial swelling. The bioassay of pressor activity was kindly carried out by Dr. Morris Rosenfeld, Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins School of Medicine. The vasopressin isolated from the peak tubes (Fig. 1) assayed over 400 pressor units per mg and can thus be considered to be essentially pure. This peak also possessed the greatest activity in causing mitochondrial swelling.

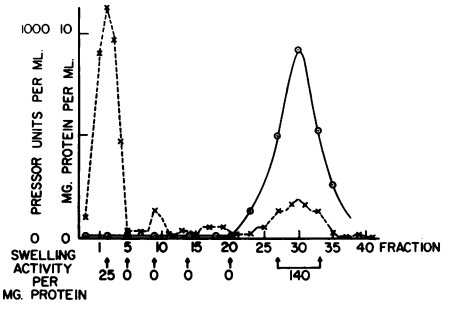

FIG. 1.—Chromatographic purification of vasopressin and mitochondrial swelling activity. Five hundred mg of crude vasopressin were chromatographed on carboxymethylcellulose with an elution gradient of 0.02 *M* to 0.2 *M* ammonium acetate, pH 6.0–7.0. The dotted line gives protein concentration and the solid line pressor activity.

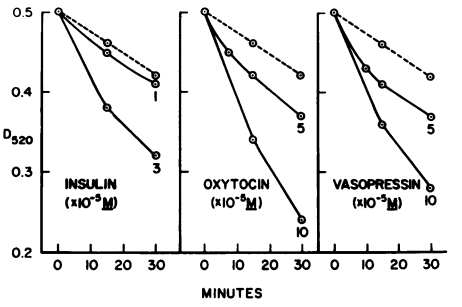

FIG. 2.—Water uptake by rat liver mitochondria in the presence of disulfide hormones. The medium was 0.125 *M* KCl-0.02 *M* tris-HCl buffer, pH 7.3; temperature was 21°. The 5.0 ml system contained 0.5 mg mitochondrial protein. The dotted line gives the rate of swelling in absence of hormone.

The insulin was an amorphous low-zinc preparation (Lot W1282) kindly provided by Dr. Otto K. Behrens of the Lilly Research Laboratories, Indianapolis.

Stimulation of Mitochondrial Water Uptake by Disulfide Hormones.—The data from typical experiments in Figure 2 show the effect of oxytocin, vasopressin, and insulin on the rate of swelling of rat liver mitochondria in a KCl-tris medium. From these and many similar experiments it was found that both oxytocin and vasopressin are potent mitochondrial swelling agents, both having the same order of activity. Easily detectable swelling was given by a concentration of 2×10^{-5} *M* and a level of 6×10^{-5} *M* hormone produced about the same swelling effect as 5–10 mM GSSG.[3] The neurohypophyseal hormones are thus 50–100 times more active than the simple disulfide GSSG.

Also shown in Figure 2 is the swelling effect of a low-zinc insulin preparation. It is seen that 1×10^{-5} *M* insulin gives a detectable effect, and 3×10^{-5} gives a very high rate of swelling. Insulin at this pH, concentration, and ionic strength occurs largely as the hexamer (Mol. wt. = 36,000)[13]; the monomer contains 3 disulfide bridges. Since it is not known which molecular species is active, and whether all the disulfide bridges participate, the molar activity of insulin is difficult to assess. However, it is clear that it has an activity of the same order of magnitude as that of the neurohypophyseal hormones, if not greater. Actually, independent experiments of Melhuish and Greenbaum[14] had shown that zinc-insulin causes mitochondrial swelling. However, since Zn^{++} itself is a very potent swelling agent,[15] the significance of their observations was not entirely clear. The present experiments with an insulin preparation containing less than 0.04 per cent zinc provide more satisfactory and clear-cut evidence for the inherent activity of insulin itself in stimulating water uptake by mitochondria.

Similar experiments with the three hormones on mitochondria suspended in a medium of 0.3 *M* sucrose-0.02 *M* tris rather than KCl-tris gave about the same results. In addition it was observed that all three hormones stimulated the swelling of rat kidney mitochondria, with approximately the same potency.

The swelling induced by the hormones is maximal at pH 7.0–7.3, as is that induced by GSSG.[3] The swelling induced by thiols, on the other hand, is maximal

between pH 7.5 and pH 8.0. Serum albumin and ATP inhibit the swelling induced by the hormones as well as by simple disulfides such as GSSG. On the other hand, swelling induced by thiols is not inhibited by serum albumin or ATP.[1, 3, 16]

Potentiation of Action of Disulfide Hormones by Reduced Glutathione.—Data in Table 1 demonstrate that reduced glutathione, in low concentrations which give no significant swelling action alone, may greatly potentiate the swelling action of oxytocin, vasopressin, and insulin. Tested in the most sensitive and critical range, GSH brings about enhancement of the action of these hormones of tenfold or more. For example, 1×10^{-5} *M* insulin gave no significant increase in swelling rate. However, addition of 1×10^{-3} *M* GSH, which has just barely measurable activity itself (this is somewhat less than the normal concentration in whole liver), greatly potentiates the action of this concentration of insulin.

Such potentiation of the swelling action of the disulfide hormones by thiols is in agreement with earlier experiments showing potentiation by combinations of simple thiols and disulfides, such as the homologous mixture GSSG + GSH or mixed combinations such as cysteine + GSSG.[3] The normal tissue concentrations of GSH and other thiols are thus sufficient to potentiate the swelling action of the disulfide hormones by an order of magnitude or more. This potentiation effect is critically dependent on the concentration and the ratio of the thiol and disulfide; at certain ratios it may be absent or even shown an inhibition, in the case of either simple compounds[3] or the hormone systems studied here (see Exp. 13 and 14, Table 1).

TABLE 1

POTENTIATION OF ACTION OF DISULFIDE HORMONES BY GSH ON WATER UPTAKE BY MITOCHONDRIA

				Water uptake $-\Delta O.D._{520} \times 10^3$
1.	Oxytocin	1.6×10^{-5} *M*		10
2.	"	5×10^{-5} *M*		50
3.			GSH 3×10^{-6} *M*	10
4.			" 3×10^{-4} *M*	5
5.	Oxytocin	1.6×10^{-5} *M* +	GSH 3×10^{-4} *M*	115
6.	"	5×10^{-5} *M* +	" 3×10^{-6} *M*	160
7.	Vasopressin	2.4×10^{-5} *M*		10
8.	"	7.2×10^{-5} *M*		195
9.			GSH 2×10^{-5} *M*	0
10.			" 2×10^{-6} *M*	0
11.	Vasopressin	2.4×10^{-5} *M* +	GSH 2×10^{-5} *M*	80
12.	"	2.4×10^{-5} *M* +	" 2×10^{-6} *M*	120
13.	"	7.2×10^{-5} *M* +	" 2×10^{-5} *M*	10
14.	"	7.2×10^{-5} *M* +	" 2×10^{-6} *M*	155
15.	Insulin	1×10^{-5} *M*		0
16.	"	3×10^{-5} *M*		140
17.			GSH 1×10^{-3} *M*	15
18.	Insulin	1×10^{-5} *M* +	" 1×10^{-3} *M*	130
19.	"	3×10^{-5} *M* +	" 1×10^{-3} *M*	220

Data are given in terms of the decrease in optical density ($\times 10^3$) at 520 mμ which occurs after 60-min exposure of mitochondria to hormones and GSH as shown, corresponding to water uptake.

Reversal of Hormone-Induced Swelling by ATP.—Figure 3 shows that addition of ATP causes reversal of oxytocin-induced swelling of rat liver mitochondria, as well as swelling induced by the "mixed" combination of oxytocin and GSH. This finding agrees with earlier experiments showing that water uptake induced by

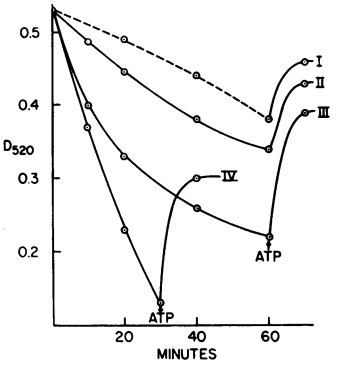

FIG. 3.—ATP-dependent water extrusion from oxytocin-swollen mitochondria. Conditions as in Figure 2. At the arrows ATP, $MgCl_2$, and bovine serum albumin added to give concentrations of 5 mM, 5 mM, and 0.2 mg per ml, respectively. Curve I, control (no hormone); II, 5×10^{-5} *M* oxytocin added as swelling agent; III, 5×10^{-5} *M* oxytocin + 3×10^{-6} *M* GSH; and IV, 1.5×10^{-4} *M* oxytocin. GSH at 3×10^{-6} *M* gives no stimulation of swelling tested alone.

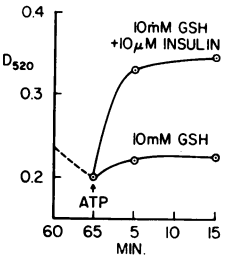

FIG. 4.—Stimulating effect of insulin on the ATP-dependent water extrusion in the presence of GSH. After a swelling period of 60 minutes, ATP, Mg^{++} and serum albumin were added to the swollen mitochondria. The swelling induced by 10 mM GSH is not reversed by ATP, but addition of 10 μM insulin to the medium restores the contraction effect.

GSSG or GSSG + GSH is reversed by ATP.[3] On the other hand, swelling induced by high concentrations of GSH is not reversed by ATP, but can be reversed if a specific mitochondrial protein, namely C-factor, is added to the test medium.[2, 4–6] This protein is detached from mitochondria by GSH, but the detachment is largely prevented if GSSG is also present in the medium; detachment of the C-factor is a function of the ratio of —SH to —S—S— in the medium.[3]

The experiment in Figure 4 shows that mitochondria extrude water in the presence of ATP when swelling is stimulated by insulin + GSSH, but not when it is stimulated by GSH alone. Similar effects were given by oxytocin and vasopressin, and it may be concluded that the hormones act exactly like GSSG in preventing C-factor detachment by GSH.

Discussion.—The data presented in this paper demonstrate that the peptide hormones vasopressin, oxytocin, and insulin, all of which contain disulfide bridges, are potent swelling agents showing striking similarity in their mode of action to simple disulfides such as oxidized glutathione and cystamine. It appears very significant that these hormones are some 100 times more active in stimulating water uptake than the simple disulfide GSSG. With the additional tenfold enhancement of potency given by the presence of very small concentrations of GSH, these hormones may be as much as 1,000 times more potent than pure GSSG. It is not proven by these experiments that it is the disulfide groups of the hormones which are specifically and primarily responsible for the stimulation of water uptake; however the hormones behave in all respects like the simple disulfides, which in turn differ strikingly in their action from other types of swelling agents such as phosphate, thyroxine, etc.[7, 8] In particular, potentiation by thiols is a characteristic of disulfide-induced swelling and this effect is as pronounced with the hormones as in the case of simple disulfides.

It may be suggested therefore that the active group of these hormones is the disulfide group, and that the activity of this group in stimulating water uptake is greatly enhanced above that of simple disulfides by the specific amino acid sequence and peptide chain conformation on either or both sides of the disulfide bridge(s) of the hormones. This suggestion is in full agreement with our earlier finding that even simple disulfides show large differences in swelling activity depending on their structure,[3] indicating that modifications in structure in the neighborhood of the disulfide group can produce large changes in swelling activity. Furthermore, it is also known that hormonal activity depends on the presence of certain amino acids in an appropriate sequence; only limited amino acid replacements are possible for retention of activity.[17]

It is very significant that beef anterior pituitary growth hormone, which contains four disulfide linkages, has also been found by Melhuish and Greenbaum[14] to cause mitochondrial swelling at very low concentrations, i.e., 2×10^{-6} *M*. These authors did not comment on a possible role of disulfide groups in the action of growth hormone or insulin, but in view of the data reported in this and other papers[1, 3] the relationships developed here may very likely include the case of growth hormone.

We have also examined a large number of other proteins and peptides for their ability to promote mitochondrial swelling, but in general these have little or no activity, and often inhibit swelling. It therefore appears that the swelling reaction is not given by all peptides or proteins containing disulfide groups. Melhuish and Greenbaum, however, reported that adrenocorticotrophic hormone (which contains no sulfhydryl or disulfide groups) also causes mitochondrial swelling at low concentrations.[14] This appears to be an important exception, and requires further examination. It may be noted, however, that this hormone shows an anomalously high sensitivity to oxidizing agents.[18]

It appears probable that the concentrations of the disulfide hormones required *in vitro* to produce significant stimulation of water uptake in liver mitochondria over the short reaction periods studied here are considerably higher than the intracellular concentration of these hormones in their target tissues in the intact normal animal. For this and other reasons it would be wholly premature to conclude that the effects described here represent the mode of action of these hormones. Furthermore, even though swelling of mitochondria is stimulated by only a relatively few physiologically occurring compounds (inorganic phosphate, Ca^{++}, GSH, GSSG, higher fatty acids, ascorbic acid),[7, 8] it would seem highly unlikely that the six different hormones now known to stimulate swelling of mitochondria *in vitro* (thyroxine,[7] growth hormone, oxytocin, vasopressin, insulin, and adrenocorticotrophic hormone) all act physiologically in their endocrine function on mitochondria, even if different specific receptor sites on the mitochondria were postulated. However, it is a purpose of this paper to propose the concept that the swelling action of the disulfide hormones on the membranes of mitochondria, if not actually a normal physiological event, may at least be a very useful experimental model of the action of these hormones on those target structures, presumably membranes, on which they normally act in their endocrine function. These findings also have a second point of significance: they suggest that all the disulfide hormones may have basically the same mode of attack via the disulfide group, but through differences

in peptide structure each is presumably specific for a given site in one or another specific membrane.

There is increasing experimental support, which cannot be quoted in full, for the now widely held view that many hormones, in particular insulin, vasopressin, and oxytocin, act primarily by modifying the properties of certain membranes. In the case of insulin, many investigations,[19] beginning with those of Levine and his colleagues, have established that this hormone increases the penetration of certain substances into cells. Furthermore, Barrnett and Ball have shown that insulin increases the rate of pinocytosis in adipose tissue.[20] The neurohypophyseal hormones also alter membrane functions. For example, they promote passage of water and other small molecules such as acetamide through isolated toad skin[21] or bladder.[22] Their antidiuretic effect on mammalian kidney has been attributed to increased permeability of the distal tubule to water, especially on the basis of micropuncture experiments.[23] There are therefore strong points of evidence which support the membrane hypothesis for the action of these hormones.

The membrane hypothesis has recently been given much more direct chemical support. Fong *et al.*[24] have demonstrated that tritium-labeled vasopressin when administered to rats can be recovered again, attached by a covalent linkage to a kidney protein, which presumably originated from a membrane. The properties of the linkage indicated it to be a disulfide and it was suggested that the vasopressin had formed a mixed disulfide by reaction with a sulfhydryl group of a specific kidney receptor protein, by means of a disulfide-sulfhydryl interchange reaction. It was also postulated that formation of such a mixed disulfide between hormone and membrane initiates a "chain" of disulfide-sulfhydryl interchange reactions in the membrane, such as first described by Huggins, Tapley, and Jensen,[25] which could lead to alteration of permeability by changes in the conformation or arrangement of protein molecules in the membrane. Cadenas *et al.*[26] have presented similar evidence that insulin is bound by tissues via a mixed disulfide linkage.

The suggestion made in this paper that all the disulfide hormones may have their basis of action primarily through their disulfide groups, under modification by a specific amino acid sequence and peptide chain conformation, has been given striking support by an independent experimental finding. Mirsky and Perisutti have recently reported that oxytocin has an insulin-like action on the rate of lipogenesis from carbohydrate and on the rate of carbohydrate oxidation in adipose tissue *in vitro*.[27]

It is also becoming increasingly evident that many different biological membranes, such as the plasma membrane, endoplasmic reticulum, perinuclear envelope, the mitochondrial membranes, myelin sheath, erythrocyte membrane, etc., possess the same basic stratified lipid-protein arrangement, with approximately the same behavior toward osmic acid and other stains and with comparable dimensions. The disulfide hormones may therefore attack all types of membranes under suitable conditions (i.e., when present in high concentrations) by fundamentally the same mechanism, because of the chemical and structural similarity of the membranes. However, each of the hormones may show its true biological specificity by being able to attack under conditions of low concentration only one specific type of membrane of perhaps a very specific cell type. Actually, the action of these hormones should not be thought of as limited to the cell or plasma membrane but

may occur with the membranes of internal structures, as pointed out by Peters[28] and also by Hechter *et al.*[29]

Finally, it should be pointed out that the potentiation of the swelling action of the disulfide hormones by the simple sulfhydryl compound GSH described here provides an additional element through which biological control can be exerted. Obviously the intracellular concentration of GSH can become an important determinant of the activity of a given concentration of the disulfide hormones. It is therefore of some interest that the concentration of both GSH[30] and glutathione reductase[31] in some tissues is a function of endocrine state. Furthermore, glutathione reductase is very strongly inhibited by the free B chain of insulin in the reduced state, as shown by Mize and Langdon.[31, 32] It appears possible then that GSH performs an important "buffering" function in regulating the attack of disulfides on membranes, which may itself be under feed-back control through glutathione reductase.

The characteristic action of the disulfide hormones on isolated liver mitochondria as described, considered as a model system for studying the interaction of these hormones with their specific target membranes, may provide new experimental approaches to locating their specific cellular and intracellular sites of action. We are continuing this approach to include chemical and kinetic studies of the reaction of labeled disulfides including hormones with mitochondrial membrane components and identification of the number and types of covalent linkages.

Summary.—The three disulfide hormones oxytocin, vasopressin, and insulin greatly stimulate respiration-dependent uptake of water by isolated rat liver and kidney mitochondria, and their action closely resembles the swelling action of simple disulfides such as oxidized glutathione. The disulfide hormones are at least 50–100 times more active, mole for mole, than the simple disulfide GSSG, suggesting the possibility that amino acid sequence and peptide chain conformation specifically enhance the swelling action of the disulfide group. A number of other proteins containing disulfide groups do not stimulate water uptake. The swelling action of the hormones is further potentiated about tenfold by low concentrations of reduced glutathione which have no effect tested alone. The swelling produced by the hormones is reversed again by ATP.

It is proposed on the grounds of chemical and ultrastructural similarities of biological membranes, as well as other recent findings, that the swelling action of the disulfide hormones on isolated rat liver mitochondria may be a useful experimental model for the action of these hormones on their normal target sites, which much recent evidence indicates to be membranes of one type or another.

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*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE**

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The problem of coding in protein biosynthesis, i.e., of how a certain sequence of four different nucleotides in an RNA[1] chain can specify a given sequence of 20 different amino acids in a polypeptide chain, has been considered by several investigators in the last decade. Until now these studies have been either theoretical[2, 3] or statistical[4–6] in nature.