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THE ACTION OF INHIBITORS ON ENZYMES FROM HUMAN PLACENTAE

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It has been known for some considerable time that oxytocin and vasopressin are destroyed by homogenate of various organs and by body fluids (Heller, 1957). However, the effect on rates of hormone destruction by homogenate treated with enzyme inhibitors has not been studied to any appreciable extent. Werle & Semm (1956) found that the inhibition of serum oxytocinase by disodium ethylenediaminetetraacetate and 8-oxyquinoline could be reversed by Mn^{2+}, Co^{2+} and Zn^{2+}. Page (1946) stated that 0·1 M iodoacetate did not inhibit plasma 'Pitocinase', and Birnie (1953) found that liver homogenate was unable to inactivate vasopressin when treated with 5×10^{-4} M Cu^{2+} or Zn^{2+}.

Homogenate of human placenta contains separate enzyme systems destroying oxytocin and vasopressin (Hooper & Jessup, 1959). The work presented here describes the action of various inhibitors on these enzyme systems, and some conclusions concerning the nature of oxytocinase and vasopressinase are discussed.

METHODS

Preparation of enzymes

Hooper & Jessup (1959) found vasopressinase activity mainly in the mitochondrial and microsome fractions. The mitochondrial preparations used in the present work were made by suspending particles in a volume of NaCl solution 0·9% (w/v) equal to $\frac{1}{3}$ vol. of the homogenate from which they were sedimented.

The particle-free supernatant containing oxytocinase activity (Hooper & Jessup, 1959) obtained by centrifuging homogenate at 28,000 *g* for 2 hr was cooled to 0° C. 0·8 vol. of saturated $(NH_4)_2SO_4$ solution was added slowly, and the mixture stirred for a further 30 min in order to reach equilibrium. The inactive precipitate was removed and oxytocinase precipitated from the supernatant by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The active precipitate was sedimented and dissolved in pH 8 buffer.

Incubations. Oxytocin and vasopressin (Pitocin, Pitressin; Parke, Davis & Co.) were incubated with enzymes at 37° C for 30 min in pH 7·5 buffer at an initial substrate concentration of 400 m-u./ml. Reactions were terminated by boiling after the addition of 1/10 vol. N-HCl. The boiled

mixtures were neutralized with $NaHCO_3$ and NaCl concentration made up to 0·9% (w/v) by adding water.

Most inhibitors were used at a concentration of 10^{-3}M and were allowed a contact time of 15 min with enzymes before addition of substrate. Concomitant incubations in which boiled enzyme preparations were used were made, in order to show whether or not inhibitors affected either substrates or bio-assays.

Biological assays. Oxytocin was measured either by the avian depressor method or by the isolated rat uterus method. Pressor activity of vasopressin was measured according to the procedure described by Dekanski (1952). Assays were of the bracketing type; two dose levels of 'standard' being given before and after two dose levels of 'unknown', the unknown being diluted until responses were approximately equal to those of the standard. Residual substrate concentrations were converted to enzyme concentrations by means of reference curves (Hooper & Jessup, 1959). The fiducial limits at a probability of 0·95 were calculated according to the method of Burn (1952).

RESULTS

Oxytocinase and vasopressinase were treated with compounds representing the more important classes of enzyme inhibitor.

TABLE 1. Effect of metals on oxytocinase and vasopressinase

	Oxytocinase			Vasopressinase		
Inhibitor (10^{-3}M)	Residual substrate concn. (%)	Enzyme (u./ml.)	Inhibition (%)	Residual substrate concn. (%)	Enzyme (u./ml.)	Inhibition (%)
Enzyme	1·0	9·0	*	30	1·3	—
Enzyme + Ag^+	52	1·3	86	33 (24–43)	1·2	0
Enzyme + Ba^{2+}	0·9	9·0	0	34 (27–41)	1·2	0
Enzyme + Ca^{2+}	2·0 (1·2–3·0)	8·0	11	24 (19–29)	1·5	11
Enzyme + Cu^{2+}	65	0·9	90	90 (78–100)	0·08	94
Enzyme + Fe^{3+}	1·0	9·0	0	16 (11–20)	2·0	0
Enzyme + Mg^{2+}	1·5 (1·0–2·0)	8·7	0	34 (22–46)	1·2	0
Enzyme + Mn^{2+}	0·5	>9·0	0	—	—	—
Enzyme + Pb^{2+}	0·8	9·0	0	29 (21–38)	1·3	0
Enzyme + Zn^{2+}	53	1·3	86	47 (33–61)	0·9	31
Enzyme	—	—	—	5	1·9	—
Enzyme + Mn^{2+}	—	—	—	4 (3·1–5·1)	2·0	0

Oxytocinase and vasopressinase prepared as described in Methods. Enzymes suspended in pH 7·5 buffer and treated with 10^{-3}M inhibitor for 15 min. Substrate then added to give concn. 400 mu./ml., mixture incubated at 37° C for 30 min, boiled and assayed as described in text. Fiducial limits shown in parentheses.

Effect of metals. Metals were used at a concentration of 10^{-3}M. Excepting Hg^{2+}, metals had little effect on substrates or bio-assays and values obtained for control experiments by using boiled enzyme preparations are not recorded. Neither oxytocin nor vasopressin activity was found in control experiments with Hg^{2+} as inhibitor; the inhibitory effect of this ion could not therefore be determined. Table 1 shows that both enzymes were inhibited by Cu^{2+}, and the effect of different concentrations of Cu^{2+} on oxytocinase is shown in Fig. 1. In addition to Cu^{2+}, oxytocinase was inhibited by Ag^+ and Zn^{2+}, the latter ion also having a small but significant inhibitory effect on vasopressinase. Fiducial limits are given in instances where inhibitors caused small differences in substrate concentrations,

Effect of respiratory poisons. Azide, carbon monoxide (coal gas) and cyanide were chosen as inhibitors representative of this class. Control samples of azide produced a depressor effect on intravenous injection in test animals and it was impossible to assay hormones reliably under such conditions. Control samples of cyanide at 10^{-2}M did not interfere with bio-assays. A possible

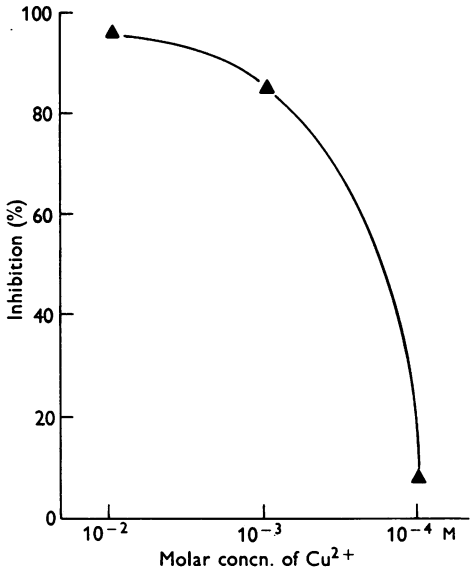

Fig. 1. Inhibition of oxytocinase by Cu^{+2}. Oxytocinase treated with Cu^{2+} pH 7·5 for 15 min at the concentrations indicated. Oxytocin added, incubated at 37° C for 30 min and residual substrate measured. Semilog. scale.

TABLE 2. Effect of respiratory inhibitors on oxytocinase and vasopressinase

	Oxytocinase			Vasopressinase		
Inhibitor	Residual substrate concn. (%)	Enzyme (u./ml.)	Inhibition (%)	Residual substrate concn. (%)	Enzyme (u./ml.)	Inhibition (%)
Enzyme	10	2·9	—	12	3·4	—
Enzyme + Coal gas (CO)	10	2·9	0	14 (10–18)	3·1	0
Enzyme + N_2	5·5 (4·4–6·6)	3·6	0	15 (8–21)	3·0	0
Enzyme	1·0	9·0	—	30	1·3	—
Enzyme + CN^- (10^{-3}M)	1·4 (0·9–1·9)	8·7	0	31	1·3	0

For explanation, see Table 1.

explanation of this is that HCN was expelled by boiling with HCl, as described under Methods. The effect of incubation under anaerobic conditions is also included in Table 2. Clearly, inhibitors of this class have no effect on oxytocinase or vasopressinase.

Effect of reagents for thiol groups. Iodoacetic acid, iodoacetamide and *p*-chloromercuribenzoate were selected from the large number of inhibitors of

this group. Results obtained with *p*-chloromercuribenzoate are not given, owing to the unfavourable reactions of test animals to this compound. It is apparent from Table 3 that thiol reagents are ineffective on the enzymes.

Effect of ethylenediaminetetraacetic acid and fluoride. Ethylenediaminetetraacetic acid is an effective chelating agent while fluoride is specific for Mg^{2+}. These reagents did not affect enzyme activity (Table 4).

TABLE 3. Effect of thiolalkylating reagents on oxytocinase and vasopressinase

Inhibitor (10^{-3} M)	Oxytocinase: Residual substrate concn. (%)	Oxytocinase: Enzyme (u./ml.)	Oxytocinase: Inhibition (%)	Vasopressinase: Residual substrate concn. (%)	Vasopressinase: Enzyme (u./ml.)	Vasopressinase: Inhibition (%)
Enzyme	1·0	9·0	—	30	1·3	—
Enzyme + iodoacetic acid	1·0	9·0	0	27 (20–35)	1·3	0
Enzyme + iodoacetamide	< 1	>9·0	0	—	—	—
Enzyme	—	—	—	6	1·9	—
Enzyme + iodoacetamide	—	—	—	7 (3·5–9·2)	1·7	0

For explanation see Table 1.

TABLE 4. Effect of ethylenediaminetetraacetic acid and fluoride on oxytocinase and vasopressinase.

Inhibitor (10^{-3} M)	Oxytocinase: Residual substrate concn. (%)	Oxytocinase: Enzyme (u./ml.)	Oxytocinase: Inhibition (%)	Vasopressinase: Residual substrate concn. (%)	Vasopressinase: Enzyme (u./ml.)	Vasopressinase: Inhibition (%)
Enyzme	1·0	9·0	—	30	1·3	—
Enzyme + ethylenediaminetetraacetic acid	< 1	>9	0	24 (16–33)	1·5	0
Enzyme + F^-	< 1	>9	0	27 (21–33)	1·3	0

For explanation see Table 1.

Effect of organophosphorus inhibitors. Some of these compounds appear to be specific inhibitors of enzymes having esterase activity. Di-*iso*propylphosphofluoridate (DFP) and tetraethylpyrophosphate (TEPP) are two of the more active and commonly used members of this group. In order to remove as much inhibitor as possible from test solutions, they were evaporated to dryness *in vacuo* and diluted to the original volume before bio-assay. DFP was removed completely by this procedure. TEPP, however, was not completely removed, and it was necessary to use atropinized animals for assay. Table 5 shows the striking difference in behaviour of oxytocinase and vasopressinase with these phosphorylating reagents.

The significance of the effect of DFP and TEPP on the two enzymes is shown by the *t* test. As can be seen from Table 5, there is no significant difference between the amounts of residual oxytocin obtained after incubating the hormone with boiled enzyme or enzyme treated with DFP or TEPP

(t values of 1·4 and 0·6). It may be inferred therefore that DFP, TEPP and boiling have the same effect on oxytocinase; that is, complete inhibition. Similarly, there is no significant difference between untreated vasopressinase and enzyme treated with DFP or TEPP (t values 0·9 and 0·3). The loss of vasopressin after incubation with enzyme plus DFP or TEPP is due to enzyme action; the fiducial limits of control experiments with boiled enzyme include 100%.

TABLE 5. Effect of DFP and TEPP on oxytocinase and vasopressinase.

	Oxytocinase			Vasopressinase		
Inhibitor (10^{-3} M)	Residual substrate concn. (%)	Group comparison (t test)	Inhibition (%)	Residual substrate concn. (%)	Group comparison (t test)	Inhibition (%)
Enzyme	8 (5·9–10·1)	5·9	100	9 (6–12)	0·9	0
Enzyme + DFP	58 (30–85)			17 (11–22)		
Boiled enzyme + DFP	71 (58–84)	1·4		92 (59–124)	5·3	
Enzyme	8 (5·9–10·1)	21·1	100	18 (12–23)	0·3	0
Enzyme + TEPP	70 (60–80)			17 (15–20)		
Boiled enzyme + TEPP	75 (60–89)	0·6		69 (32–105)	4·3	

Oxytocinase and vasopressinase treated with TEPP for 15 min and with DFP for 2 min before incubation with hormones. Solutions boiled, neutralized and evaporated *in vacuo* for 20 hr to remove inhibitors. Control experiments with boiled enzyme preparations show loss of substrate by the procedure; t calculated according to Snedecor (1950).

TABLE 6. Regeneration of oxytocinase activity by cyanide and ethylenediaminetetraacetic acid

Inhibitor	Residual substrate concn. (%)	Enzyme (u./ml.)	Inhibition (%)
Enzyme	1·0	9·0	—
Enzyme + Cu^{2+}	65	0·9	90
Enzyme + Cu^{2+} + CN^-	23 (20–26)	3·0	66
Enzyme + Cu^{2+} + ethylenediaminetetraacetic acid	43 (23–63)	1·7	80
Enzyme + Zn^{2+}	53	1·3	86
Enzyme + Zn^{2+} + ethylenediaminetetraacetic acid	61 (49–74)	1·0	89
Enzyme + Ag^+	52	1·3	86
Enzyme + Ag^+ + CN^-	0·3	> 9	0
Enzyme + Ag^+ + ethylenediaminetetraacetic acid	70 (56–85)	0·5	95

Oxytocinase treated with inhibitor (10^{-3} M) for 5 min; cyanide (10^{-2} M) and ethylenediaminetetraacetic acid (4×10^{-3} M) then added; after 15 min substrate added and solution incubated. Fiducial limits in parentheses.

Attempted reversal of inhibition by cyanide and ethylenediaminetetraacetic acid. The activity of heavy metal inhibited enzymes is frequently restored by chelating agents. Oxytocinase was inhibited with 10^{-3} M Cu^{2+}, Ag^+ and Zn^{2+}. After a contact time of 5 min, 10^{-2} M cyanide and 4×10^{-3} M ethylenediaminetetraacetic acid were added, the solutions kept for 15 min and then incubated with substrate. Table 6 shows that cyanide is more effective than ethylenediaminetetraacetic acid in restoring enzyme activity. The inhibitory action of

Ag^+ is completely reversed but only some 30% reversal occurred with the Cu^{2+} inactivated system.

The reactivation of Cu^{2+} inhibited oxytocinase by CN^- is shown as a function of time in Fig. 2. Control experiments showed that loss of substrate due to factors other than enzyme action was equivalent to 2% regeneration of enzyme activity.

The biological activity of oxytocin is destroyed by reduction of the disulphide group. Air oxidation of the reduced hormone restores a considerable proportion of the original activity (Audrain & Clauser, 1958). It seemed pertinent therefore to see if air oxidation of enzyme-inactivated oxytocin and vasopressin regenerated their biological activities. Solutions of enzyme-inactivated oxytocin and vasopressin were treated with a trace of ferric chloride and aerated for 20 hr. On assay it was found that no regeneration had in fact occurred.

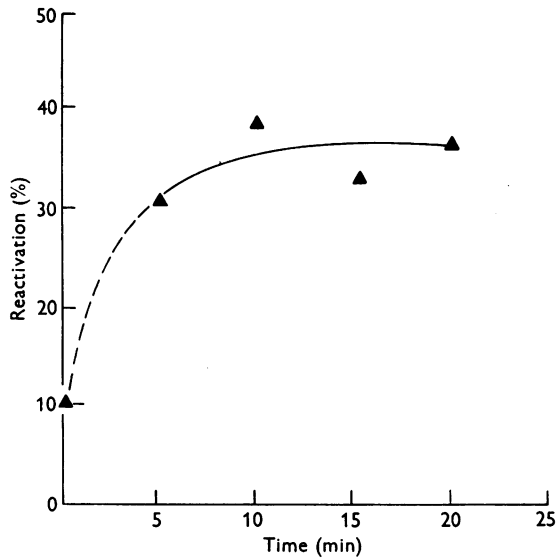

Fig. 2. Reactivation by CN^- of Cu^{2+}-inhibited oxytocinase. Oxytocinase treated with 10^{-3}M Cu^{2+} at pH 7·5 for 5 min, 10^{-2}M CN^- then added. Equal portions withdrawn after intervals of time, incubated with oxytocin at 37° C for 30 min, and residual substrate assayed.

DISCUSSION

Inhibitors were mixed with enzymes in the absence of substrate in order to permit reaction between inhibitor and free active centres of enzymes. It is known that some inhibitors which are ineffective at a certain hydrogen ion concentration become effective at a different concentration, owing to ionization of reactive groups in the enzyme. 7·5 is approximately the optimum pH for both oxytocinase and vasopressinase (Hooper & Jessup, 1959). Contact

between enzymes and inhibitors was therefore allowed at this hydrogen ion concentration. It is possible that inhibitors ineffective at pH 7·5 for 15 min would be effective at different hydrogen ion concentrations, or with a contact time greater than 15 min.

Oxytocinase and vasopressinase are non-dialysable (Hooper & Jessup, 1959). It may be assumed therefore that co-factors and easily removable metals are not necessary for activity. The absence of co-factors is a characteristic of proteolytic enzymes. The inability of ethylenediaminetetraacetic acid or fluoride to cause inactivation is further evidence that metals generally, and Mg^{2+} in particular, do not play important parts.

The absence of inhibition by anaerobic incubation and also by respiratory poisons indicates probably that oxytocinase and vasopressinase do not possess oxidase or dehydrogenase activity; in addition, it seems unlikely that they are metallo-enzymes. The inability of the —SH alkylating reagents iodoacetic acid and iodoacetamide to cause inhibition shows that —SH groups are not essential for activity. This result agrees with that obtained by use of respiratory poisons, since active—SH groups are present in many dehydrogenase systems. There are however several enzymes with dehydrogenase activity which are not inactivated by thiol reagents.

Inhibition of both enzymes by Cu^{2+}, and of oxytocinase by Ag^{+} and Zn^{2+} also, does not reveal any fundamental property. Cu^{2+}, Ag^{+} and Zn^{2+} belong to the class of mercaptide-forming metals; however, since Pb^{2+} and thiolalkylating reagents do not inactivate the enzymes, the mode of inhibition by metals is unknown. Substantially complete reversal of Ag^{+} inhibition by cyanide suggests that Ag^{+} reacts differently from Cu^{2+} and Zn^{2+}.

The action of organophosphorus inhibitors is informative and reveals a difference between oxytocinase and vasopressinase. DFP inhibits esterases. It also inhibits the proteolytic activity of chymotrypsin and trypsin, which possess both esterase and proteolytic actions. TEPP has a similar action to DFP. It does not however invariably inhibit esterases, since trypsin is unaffected at pH 7·5 (Jansen, Fellows Nutting, Jang & Balls, 1949). Inhibition of oxytocinase by both compounds suggests that the enzyme is a peptidase with esterase activity. Absence of inhibition of vasopressinase probably indicates that the enzyme is a peptidase devoid of esterase activity.

SUMMARY

1. Crude preparations of oxytocinase and vasopressinase were treated with enzyme inhibitors at pH 7·5 for 15 min before incubation with substrates.

2. Respiratory poisons, chelating agents, thiol alkylating reagents and many metals were without effect on enzyme activity.

3. Cu^{2+} inhibited both enzymes.

4. DFP and TEPP inhibited oxytocinase but vasopressinase remained unaffected.

5. It is suggested that oxytocinase and vasopressinase are both peptidases, but the former in addition shows esterase activity.

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