XLVI. THE OXYTOCIC HORMONE OF THE POSTERIOR LOBE OF THE PITUITARY GLAND.

VI. FURTHER STUDIES OF THE ACTION OF OXIDISING AND REDUCING AGENTS.

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IN Part V [Gulland and Randall, 1935] it was shown that the molecule of the oxytocic hormone certainly contains one, and may possibly contain two, oxidation-reduction systems, although the existence of the second is not definitely established.

The present communication describes the actions on the hormone of some oxidising and reducing agents, which were studied in the hope that they might throw light on the nature of the groups forming the oxidation-reduction system, or systems.

EXPERIMENTAL.

Sulphur dioxide and sodium sulphite (Fig. 1). Sodium sulphite at p_H 8.4 caused irreversible and complete inactivation of the hormone in 5 minutes. Sulphur dioxide at p_H 3.5, on the other hand, caused a slower inactivation to about 60 %, which was followed by reactivation and finally by inactivation to 3.6 % after 24 hours.

Fig. 1. Action of sulphur dioxide and sodium sulphite.

SO₂ at p_H 3.5

Na₂SO₃ at p_H 8.4

Fig. 2. Action of sodium cyanide and hydrogen cyanide.

HCN at p_H 3.5
(2 expts.)

NaCN at p_H 7.8
(2 expts.)

At p_H 3.5. The following solutions were placed in this order in a 2.5 ml. graduated flask: *M*/10 sodium acetate buffer at p_H 3.5 (0.5 ml.), *N*/10 hydrochloric acid (0.25 ml.), water (0.9 ml.), *N*/10 sodium sulphite (0.5 ml.), and a

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Kamm¹ solution of the hormone (0.1 ml.), containing 300 oxytocic units associated with 5.65 mg. of solid per ml. (called Kamm solution 1 throughout this paper). The mixture was diluted to 2.5 ml. with water, the final p_H being 3.5, and the stoppered flask was placed in a thermostat at 20°. Samples were diluted to a convenient volume for assay².

At p_H 8.4. Palitzsch's sodium borate buffer [Clark, 1928] at p_H 8.4 (0.5 ml.), *N*/10 sodium sulphite (0.5 ml.), water (1.0 ml.) and Kamm solution 1 (0.1 ml.) were mixed, diluted to 2.5 ml. (p_H 8.4) and placed in a thermostat at 21°.

Sodium cyanide and hydrogen cyanide (Fig. 2). The hormone underwent a slow, irreversible and probably complete inactivation when treated with sodium cyanide at p_H 7.8. Hydrogen cyanide at p_H 3.5 slowly caused a temporary decrease in activity; this was followed by a slight reactivation and then by a fall to an oxytocic value of about 82 %, at which level the activity seemed to remain constant. In both series of experiments the samples were freed from hydrogen cyanide by aeration at p_H 3.5, a procedure adopted before the susceptibility of the reduced form of the hormone had been recognised. It is possible that some alteration of activity may have occurred during this aeration in the case of the experiments at p_H 3.5, though probably not in the case of those at p_H 7.8. Even if this were so the essential nature of the results at p_H 3.5 remains clear.

At p_H 3.5. *M*/10 sodium acetate buffer at p_H 3.5 (0.5 ml.), *N*/10 hydrochloric acid (0.45 ml.), *N*/10 sodium cyanide (0.5 ml.) and Kamm solution 1 (0.1 ml.) were mixed, diluted to 2.5 ml. and placed in a thermostat at 20°. The p_H was 3.5. Samples were aerated with a current of moist air for 30–45 minutes until free from cyanide (Prussian blue test; sensitivity, 1 in 5×10^4) and were suitably diluted and assayed.

At p_H 7.8. Palitzsch's sodium borate buffer at p_H 8.2 (0.5 ml.), *N*/10 hydrochloric acid (0.37 ml.), *N*/10 sodium cyanide (0.5 ml.) and Kamm solution 1 (0.1 ml.) were mixed, diluted to 2.5 ml. and kept in a thermostat at 20°. Samples were adjusted to p_H 3 by means of *N*/10 hydrochloric acid and were then aerated as before, diluted and assayed.

Hydrogen peroxide (Fig. 3). The general character of the interaction of the hormone with hydrogen peroxide was the same at p_H 3.5 and at p_H 8.2. A period of inactivation was followed by one of reactivation, after which the activity again declined. The only essential difference between the courses of these reactions was the increased rate at which the changes occurred in the alkaline medium. It should be noted that estimations of the concentrations of hydrogen peroxide made by the permanganate method [Treadwell and Hall, 1928] throughout the experiments showed that the concentrations remained unchanged within the limits of the experimental error of the method.

At p_H 3.5. *M*/10 sodium acetate buffer at p_H 3.5 (0.5 ml.), *M*/10 hydrogen peroxide (0.2 ml.) and a Kamm hormone solution (0.1 ml.) containing 90 units associated with 0.37 mg. of solid were mixed and diluted to 2.5 ml. The mixture was kept in a thermostat at 21°, and samples were diluted for assay.

At p_H 8.2. The procedure was similar to that used above, except that Palitzsch's borate buffer at p_H 8.2 (0.5 ml.) replaced the acetate buffer.

¹ For definition of this term, see Gulland and Newton [1932].

² Assays were carried out by the guinea-pig uterus method, following the "bracketing" technique described by Gulland and Newton [1932]. It should be mentioned that throughout the work described in this paper the response of the isolated uterus was not influenced by the introduction into the testing bath of the reagents or their decomposition products in amounts much greater than those actually used in the experiments. The sole exception to this statement is discussed in the description of the experiments with hydroxylamine.

Iodine (Fig. 4). An aqueous iodine solution at p_H 3.5 caused the oxytocic activity to fall to 7 % of its initial value, at which it remained constant.

Fig. 3. Action of hydrogen peroxide.

Fig. 4. Action of iodine at p_H 3.5.

A mixture of $M/10$ sodium acetate buffer at p_H 3.5 (0.5 ml.), aqueous iodine solution saturated at 20° (1.0 ml., 0.28 mg. of iodine) and Kamm solution 1 (0.1 ml.) was diluted to 2.5 ml. and kept in a thermostat at 20° . Samples were suitably diluted for assay. An excess of iodine, as shown by the starch-iodide test, was present throughout the experiments. The temperature coefficient of this reaction appeared to be considerably higher than those of the other reactions which have been studied.

Chlorine. An aqueous chlorine solution completely inactivated the hormone in acid solution in the course of 15 hours at 0° .

A mixture of $M/10$ sodium acetate buffer at p_H 3.5 (0.9 ml.), Kamm solution 1 (0.1 ml.) and freshly prepared chlorine water (1.0 ml., 5.6 mg. chlorine) was kept at 0° overnight. A sample was aerated with moist air till free from chlorine (starch-iodide test) and was then adjusted to p_H 7 with sodium hydroxide and suitably diluted for assay. The activity of the experimental solution was less than 0.3 % of the initial value.

Semicarbazide had no action on the hormone.

A mixture of a Kamm solution (5.0 ml., containing 10 units associated with 0.1 mg. solid), $M/3$ sodium phosphate buffer at p_H 7 (1.0 ml.) and semicarbazide hydrochloride (22.3 mg.) was diluted to 10 ml. at p_H 7. The concentration of semicarbazide was thus $M/50$. After the mixture had remained at 0° for 48 hours, the assay of a sample showed that no inactivation had occurred.

Hydroxylamine. The analysis of the effect of hydroxylamine at $M/50$ dilution at p_H 7 was not so straightforward as in the case of semicarbazide [cf. Gulland, 1933]. In some experiments an apparent inactivation of about 20 % or even 40 % was observed, whereas in others no inactivation could be detected. These discrepancies were finally explained by demonstrating that the responses of some, but not all, uterine horns to a series of standard doses of hormone solution or of histamine were depressed by the simultaneous introduction of hydroxylamine into the testing bath at a dilution of $M/50,000$, or sometimes even less. The conclusion was therefore drawn that hydroxylamine has no chemical action on the hormone.

DISCUSSION.

Several of the reagents of which the effects are described in this communication have already been employed by Guha and Chakravorty [1933] and by Das and Guha [1934, 1, 2]. Their results, however, can only be of qualitative value for the following reasons.

In the first place, these authors use the "equality" method of assay; thus Guha and Chakravorty state that "the comparison was made by finding the suitable dose which could produce a contraction in a virgin guinea-pig's uterus equal to that produced by the standard dose", and adherence to this technique is confirmed in the later papers. In our opinion, and we believe that here we share the view of others who have long experience of oxytocic assays, the "equality" method is unreliable and may lead to false conclusions. Its sole recommendation is the fact that the performance of an assay occupies relatively little time and trouble. The only satisfactory method of assay is the "bracketing" method; in this, the least dose of standard clearly greater than the unknown and the greatest dose of standard clearly less than the unknown are ascertained, and the activity of the sample is taken as the mean determined from these values [Burn and Dale, 1922; Burn, 1928; Gulland and Newton, 1932].

Secondly, although Guha and his collaborators recognise the obvious desirability of using hormone solutions which are as free as possible from material which has no oxytocic activity, such, for example, as the preparation of Kamm *et al.* [1928], they do in fact use what must be far less pure solutions of the hormone. There are two objections to the use of hormone solutions which contain much inactive material. In certain reactions the course of the changes undergone by the hormone varies according to the ratio of activity/solid, and in general, the risk is run that the concentration of a reagent may decrease markedly during a reaction owing to preferential interaction with concomitant substances. The consequences are, of course, that the true effect of the reagent on the hormone may not be discerned. Unfortunately, the Indian authors state neither the solid content nor the number of oxytocic units in their solutions, so that exact comparisons are impossible. Nevertheless, to illustrate the point at issue it may be mentioned that in our hands a Kamm solution prepared according to Kamm *et al.* [1928] contained 3.67 mg. of solid (dried at 105° to constant weight) per ml. with which were associated 900 oxytocic units. On the other hand, a solution prepared as described by Guha and Chakravorty [1933] contained 450 units per ml. associated with 28.3 mg. of solid, of which 3.9 mg. were ammonium sulphate.

Thirdly, the practice adopted by Guha and his collaborators of exposing the hormone to the chemical reagent for an arbitrary period of time can only show what the activity is at the end of that period. The observations recorded in this communication alone, to make no mention of the results quoted in previous members of the series, provide ample evidence that experiments made in this way cannot record correctly the progress of a reaction and may give a false impression of its final result. The oxytocic activity must be assayed at suitable intervals of time throughout the reactions.

However desirable it may be to develop at as early a date as possible a distinctive name for the oxytocic hormone, it is unfortunate that Das and Guha [1934, 1, 2] should attempt to designate it as "oxytocin", since this name was given by Messrs Parke, Davis and Co., to one of their commercial preparations of the hormone. It would be regrettable from various standpoints if a chemical entity were to share its name with a commercial preparation, and we prefer to continue to refer to the "oxytocic hormone" until sufficient is known of its properties to permit a rational name to be proposed.

The observations described in this communication cannot be correlated to any degree of probability with a definite structure for the oxidation-reduction systems of the hormone molecule, but it is noteworthy that none is actually opposed to the presence of a disulphide linkage; in fact, several of the results could have been predicted on such an assumption, by analogy with the effects

of the same reagents on cystine. Although we do not feel justified in making a definite suggestion that a disulphide linkage is present, it is of interest to compare the action of the reagents on the hormone and on cystine, all the more because Sullivan and Smith [1928] observed that high oxytomic activity of posterior pituitary preparations could be correlated with high content of labile sulphur, and Du Vigneaud *et al.* [1933] found that increasing activity in a series of pitocin (oxytomic hormone) preparations was accompanied by an increase in the sulphur content. The results of these authors may indicate that the sulphur-containing substances from which the hormone is formed are available in larger amounts in active posterior lobes and that the more fully developed forerunners of the hormone molecule resemble it in such a way that their elimination is not readily achieved by processes of purification.

Sodium sulphite and sodium cyanide both inactivate the hormone completely and irreversibly. Their action on cystine is to rupture the disulphide linkage, producing one molecule of cysteine and one molecule of sodium cysteine-*S*-sulphonate [Clarke, 1932; Lugg, 1932] or sodium α -amino- β -sulphocyanopropionate respectively [Pulewka and Winzer, 1928; Lugg, 1933; Bodansky, 1929]. Moreover, Clarke [1932] found that a solution of ammonium sulphite and cystine which was exposed to air ultimately contained cysteine-*S*-sulphonic acid but no cysteine, and he concluded that the cysteine initially formed underwent oxidation to cystine, which in turn reacted with more ammonium sulphite, and so on until the conversion was complete. If the hormone contained a disulphide linkage, analogous changes might lead to disruption of the molecule and consequent total loss of activity with both reagents.

Iodine effected marked inactivation of the hormone, chlorine complete loss of activity. The halogens oxidise cystine to cysteic acid by way of the corresponding sulphinic acid, thus again destroying the disulphide linkage [Shinohara, 1932, 1, 2; Lucas and King, 1932; Simonsen, 1933].

The correlation of the effects of the other reagents with the presence of a specific group is less simple. The successive changes in the activity of the hormone when treated with sulphur dioxide or hydrogen cyanide—decrease, increase and final decrease of activity—might be regarded as evidence for the presence of two oxidation-reduction systems; the reagents could then be regarded as reducing these systems and ultimately as destroying a disulphide-sulphydryl system. Since these reagents have little or no action on cystine, it would be necessary to invoke the well-known lability of sulphur linkages when combined in peptides as compared with the stability in cystine. On the other hand, such arguments lose their value when it is realised that hydrogen peroxide, an oxidising agent, effects the same series of changes—decrease, increase and finally decrease of activity. Hydrogen peroxide oxidises cystine to cysteic acid [Schöberl, 1933].

Finally, it is evident that the oxytomic hormone and insulin exhibit similarities in some of their reactions [see for the insulin reactions: Blatherwick *et al.*, 1927; Freudenberg *et al.*, 1931; 1932; Kuhn *et al.*, 1931; Du Vigneaud *et al.*, 1931–32]. There is, however, one marked difference between these hormones: so far, all attempts to reactivate reduced insulin have proved unsuccessful.

SUMMARY.

1. The action of the following reagents on the hormone has been studied: sodium sulphite, sulphur dioxide, sodium cyanide, hydrogen cyanide, chlorine, iodine, hydrogen peroxide, semicarbazide and hydroxylamine.

2. The results are discussed from the standpoint of the presence of a disulphide linkage in the hormone molecule.

REFERENCES.

- Blatherwick, Bischoff, Maxwell, Berger and Sahyun (1927). *J. Biol. Chem.* **72**, 57.
Bodansky (1929). *J. Pharm. Exp. Ther.* **37**, 463.
Burn (1928). *Methods of biological assay* (Oxford Medical Publications).
— and Dale (1922). *Med. Res. Coun. Lond. Sp. Rep. Ser.* No. 69.
Clark (1928). *Determination of hydrogen ions*, 3rd ed. (Baillière, Tindall and Cox, London.)
Clarke (1932). *J. Biol. Chem.* **97**, 235.
Das and Guha (1934, 1). *Indian J. Med. Res.* **21**, 765.
— — (1934, 2). *Indian J. Med. Res.* **22**, 157.
Du Vigneaud, Fitch, Pekarek and Lockwood (1931–32). *J. Biol. Chem.* **94**, 233.
— Sealock, Sifferd, Kamm and Grote (1933). *J. Biol. Chem.* **100**, xciv.
Freudenberg and Discherl (1931). *Z. physiol. Chem.* **202**, 193.
— — and Eyer (1931). *Z. physiol. Chem.* **202**, 128.
— and Eyer (1932). *Z. physiol. Chem.* **213**, 226.
Guha and Chakravorty (1933). *Indian J. Med. Res.* **21**, 429.
Gulland (1933). *Chem. and Ind.* **52**, 160.
— and Newton (1932). *Biochem. J.* **26**, 337.
— and Randall (1935). *Biochem. J.* **29**, 378.
Kamm, Aldrich, Grote, Rowe and Bugbee (1928). *J. Amer. Chem. Soc.* **50**, 573.
Kuhn, Eyer and Freudenberg (1931). *Z. physiol. Chem.* **202**, 97.
Lucas and King (1932). *Biochem. J.* **26**, 2076.
Lugg (1932). *Biochem. J.* **26**, 2144.
— (1933). *Biochem. J.* **27**, 668.
Pulewka and Winzer (1928). *Arch. Exp. Path. Pharm.* **138**, 154.
Schöberl (1933). *Z. physiol. Chem.* **216**, 193.
Shinohara (1932, 1). *J. Biol. Chem.* **96**, 285.
— (1932, 2). *J. Biol. Chem.* **97**, xxii.
Simonsen (1933). *J. Biol. Chem.* **101**, 35.
Sullivan and Smith (1928). *U.S. Pub. Health Rep.* **43**, 1334.
Treadwell and Hall (1928). *Analytical chemistry*, **2**, 533. (John Wiley and Sons, New York.)