SOME OF THE OXIDATION-REDUCTION PROPERTIES OF THE CHORIONIC GONADOTROPIC HORMONE

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Gurin, Bachman, and Wilson (1–3) have recently made important contributions to our knowledge of the chemical nature of the gonadotropic hormone of pregnancy urine. They have given a method for the preparation of the hormone in a highly purified form and have described many of its outstanding chemical and physical properties. We have been especially interested in the reference of these authors to the unexplained, continuous, and apparently spontaneous inactivation of their highly purified material which takes place, particularly in aqueous solution, without detectable loss or rupture of certain portions of the molecule (3).

Earlier communications (4–6) have called attention to our observations relative to the oxidation and inactivation of a reducing factor of pregnancy urine which appears to be the chorionic gonadotropic hormone. Some of our more recent findings appear to shed light upon the observations of the above authors. The spontaneous oxidation of this reducing factor proceeds at a very slow rate but is more rapid in aqueous solution than in urine, where other stronger reducing agents tend to protect it. If activating agents are added and moderate heat is applied, the activity becomes of sufficient magnitude to bring about the reduction of dilute solutions of iodine or other oxidants. The object of this paper is to describe some of the rather unusual characteristics of this oxidation-reduction system.

EXPERIMENTAL

Preparation of Reducing Material—The experiments described in this report were undertaken some time ago and consequently

the procedure employed to separate the gonadotropic factor from pregnancy urine differs somewhat from that presented by Gurin, Bachman, and Wilson (2, 3); that is, Katzman and Doisy's method (7) was used with subsequent reprecipitation with acetone. Chilled protein-free urine of pregnant women was adjusted to pH 7.4 and the resulting precipitate was centrifuged and discarded. In this manner an appreciable amount of inert material was avoided at the outset. The urine was then acidified to pH 5 with glacial acetic acid and with vigorous stirring 75 cc. of acetone saturated with benzoic acid were added for each liter of urine. After the precipitate was centrifuged, it was washed three times with cold water saturated with benzoic acid and then with cold acetone to remove the benzoic acid. The acetone-insoluble fraction of the precipitate was repeatedly extracted with water at pH 7.4 and an equal volume of acetone was then added to the combined aqueous extract. The precipitate which formed at this point was centrifuged and discarded. After the pH of the acetone residue was adjusted to 5.0, the acetone concentration was increased to 70 per cent. The resulting insoluble fraction was centrifuged and again repeatedly extracted with water at pH 7.4. The fractional precipitation with 50 and 70 per cent acetone, detailed above, was carried out three times and the final precipitate was dried in a desiccator at 4°. The material so prepared assayed between 600 and 800 rat units per mg.

Potentiometric Studies—In addition to a marked gonadotropic effect, the material separated from pregnancy urine in the above manner invariably possessed characteristic reducing properties. In order to aid in identifying the reducing factor and to study some of its fundamental oxidation-reduction properties, it was titrated potentiometrically with iodine and potassium ferricyanide as oxidants.

In our earlier work potentiometric measurements were employed as a means of evaluating the efficiency of various methods of separating the pregnancy reducing factor from other urinary reducing substances (5). While these earlier studies aided in developing methods of separation, it became evident that the first titration curves were somewhat displaced from their correct position on the oxidation-reduction scale. This was apparently caused by a reaction at the platinum electrode in the presence of hy-

drogen. When the platinum electrodes were plated with gold, more correct potentials were observed.

The electrometric titrations were carried out in the usual manner. The solution to be titrated was placed in a conventional type of titration chamber to which the oxygen-free oxidant was introduced from a small sealed-in burette. In the work reported

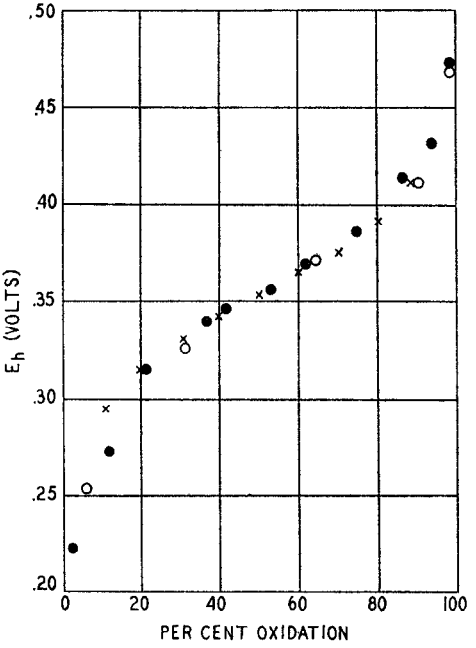

FIG. 1. Electrode potentials. ● represents the pregnancy reducing factor; ○ international standard gonadotropin; × the calculated values for an oxidation-reduction system which depends upon a single electron reaction.

here duplicate gold-plated platinum electrodes were employed and purified nitrogen was used to replace air in the apparatus. Before a titration was started, the entire sealed apparatus was repeatedly evacuated and flushed with nitrogen, with the solutions in place. The titration vessel was immersed in a water bath which was maintained at 38°. It was customary to titrate 5 mg. of the gonadotropic preparation dissolved in 10 cc. of M/15 Sørensen's phosphate buffer having a pH of 5.9.

The relation of electrode potential, E_h, to per cent oxidation is given in Fig. 1, which shows the potentials observed in the titration of the pregnancy reducing factor prepared as described above, the values observed in the titration of gonadotropin (international standard), and the calculated values for an oxidation-reduction system which depends upon a single electron reaction and which has an E_0 value equivalent to that represented by the mid-point in the titration curves. It appears that the same reducing agent is present in the two preparations and that the E_0 is +0.354 volt at 38°. In view of these findings and the constant association of this potential reducing property with preparations of the chorionic

TABLE I

Relation between Reaction Rate and Buffer Salt Concentration

Buffer salt* added to 5 mg. of preparation	Time required to decolorize 5 cc. 0.0005 N iodine at 38°
mg.	
0	18 hrs.
4.6	340 min.
9.3	120 "
18.6	8.5 "
27.9	5.0 "
37.3	4.25 "
46.4	4.0 "
92.9	4.0 "
139.3	4.0 "

* Prepared in a ratio of 9 moles of KH_2PO_4 to 1 mole of Na_2HPO_4 (pH 5.9).

gonadotropic hormone, and also since the urinary concentration of this factor has an unvarying relationship to pregnancy, it seems safe to conclude that the hormone itself or some substance always associated with it possesses reducing power. This is in agreement with Gurin, Bachman, and Wilson's indication (2) that their highly purified material slowly reduces ammoniacal silver nitrate in the cold.

Oxidation of Reducing Factor and Its Restoration—While many of the characteristics of this oxidation-reduction system are quite unusual, this fact in itself aids in differentiating the system from other reducing materials of biological origin. In aqueous solution it undergoes slow spontaneous oxidation. This oxidation can be

prevented by the addition of certain reductants. On the other hand, the oxidation is not greatly accelerated by the addition of strong oxidants unless the temperature is increased and phosphate buffer is present. The striking influence of the latter two factors will become apparent from an inspection of Tables I and II in which illustrative data are presented. The iodine titrations were made in the presence of 0.2 cc. of 2 per cent soluble starch (Merck) prepared according to Lintner. The reducing factor, buffer, and starch were adjusted to uniform volumes and heated to the designated temperature before the iodine, which was warmed to the same temperature, was added. From Table I it will be seen that with 5 mg. of the material in the absence of Sørensen's phosphate buffer 18 hours were required to reduce 5 cc. of 0.0005 N iodine

TABLE II

Relation of Temperature to Rate of Reaction

Temperature	Time required for 5 mg. of preparation to reduce 5 cc. 0.0005 N iodine*
°C.	*min.*
20	225
30	30
40	4
50	0.5

* In the presence of 50 mg. of Sørensen's phosphate buffer salt (pH 5.9).

at 38°. On the other hand, in the presence of phosphate buffer (pH 5.9) the reduction time was markedly reduced. It will be noted further that there is a limit above which further increase in phosphate has no added accelerating effect. The influence is presumably due to the phosphate ion, since with the substitution of sodium chloride or potassium chloride for the buffer (at the same pH, 5.9) no change in the reduction time was evident. From the data presented in Table II, it will be observed that in the presence of an optimum concentration of phosphate buffer the oxidation-reduction reaction is markedly accelerated by moderate increases in temperature.

Since a time factor is involved in the reaction and because the presence or absence of phosphate has such a marked effect upon the oxidation-reduction reaction, consideration was given to the

possible influence of other chemical agents employed in the titration. Potassium iodide is used to keep the iodine in solution. It was somewhat surprising to find that this salt retards the reaction rate. That this is true will become evident from an inspection of illustrative data given in Table III. Another surprising finding has been the effect of the soluble starch employed as an indicator. In the presence of starch the oxidation-reduction reaction is altered; and starch from several sources was found to behave differently. Thus, at equal concentrations, Kahlbaum soluble starch increases the rate of reduction about 3-fold over that observed with starch prepared according to Lintner, although neither

TABLE III

Retarding Action of Potassium Iodide

KI added to 5 mg. of preparation*	Time required to reduce 5 cc. 0.0005 N iodine at 38°
mg.	*min.*
0	4
0.14	5
0.28	8
0.56	12
1.12	24
1.68	24
2.0	24

* In the presence of 50 mg. of Sørensen's phosphate buffer salt (pH 5.9).

starch has any significant direct action upon the iodine. Just why such a difference exists is not entirely clear.

Although a more desirable preparation of soluble starch may be forthcoming, starch prepared according to Lintner is recommended, because it appears to be uniform and is readily accessible. In order to maintain a suitable and a uniform iodine solution which contains very little excess iodide the 0.0005 N iodine can be conveniently prepared by combining the following in order: 1 cc. of a stock potassium iodate solution (3.567 gm. of KIO_3 per liter of stock solution), 1 cc. of a stock potassium iodide solution (13.835 gm. of KI per liter of stock solution), 15 cc. of water, 0.5 cc. of 1 N sulfuric acid, and, after thorough mixing, sufficient water is added to provide a total volume of 200 cc.

The hormone tends to become biologically inactive when allowed to undergo spontaneous oxidation or following oxidation by means of strong oxidants; and the decline in biological activity parallels the decreased ability to reduce iodine under standardized conditions. A finding which appears to be of considerable interest and of practical importance is that the biological activity can be restored by treatment with certain strong reductants. That this is the case becomes evident from the results of the following illustrative experiments. The reducing factor was separated as described above. An aliquot of an aqueous solution assaying 12 rat units per cc. was heated, in the presence of phosphate buffer salt (pH 5.9), in a boiling water bath for 30 minutes. A relatively large surface was exposed to the air. After the solution was restored to its original volume, one-half was retained as a control and sufficient quinol was added to the other half to bring the concentration to 1 per cent.

10 per cent sodium hydroxide was added to the fraction containing quinol to bring the pH to approximately 8.5. After the material had stood for 30 minutes, the hormone was precipitated with 10 volumes of acetone at pH 5 and the precipitate was repeatedly washed with acetone. The dried material was dissolved in water and diluted to its original volume.

The heated control assayed more than 4 and less than 6 rat units per cc., while the "restored" fraction gave a slightly stronger reaction than the original unheated control when injected in equal amounts.

The reducing activity of the heated material as determined by iodine titration was a little less than one-half that of the original preparation, while the "restored" fraction was about 20 per cent stronger than the original preparation.

In another characteristic experiment the biological activity of the gonadotropic substance obtained from boiled pregnancy urine was increased more than 4-fold by reduction with quinol. Thus, 500 cc. of pregnancy urine were boiled for $1\frac{1}{2}$ hours in an open beaker. The original volume was restored and the gonadotropic factor obtained as described above. Part of the material so obtained was reduced with quinol and reprecipitated as described before. The material present in the non-reduced fraction was found to assay more than 125 and less than 250 rat units per liter

of the original urine, while the reduced fraction contained more than 1000 and less than 2000 rat units per liter of urine. The increase in reducing power as determined by iodine titration was similar in magnitude to the increase in biological activity.

That non-pregnancy specimens are relatively free of the reducing factor which is characteristic of pregnancy urine has been observed repeatedly. The protein-free specimens from a large group of normal adult males and young non-pregnant female subjects were compared with similar specimens from a comparable group of women in early pregnancy. The gonadotropic substance was extracted from the urine and then brought to its maximum reducing

TABLE IV

Relative Strength of Pregnancy and Non-Pregnancy Extracts

Specimen No.	Sp. gr.	Time required to reduce 5 cc. 0.0005 N iodine at 38° *
113-A. Non-pregnancy	1.029	More than 4 hrs.
113-B. "	1.028	" " 4 "
113-C. "	1.030	" " 4 "
113-D. "	1.025	" " 4 "
114-A. Pregnancy	1.028	8 min.
114-B. "	1.025	8.5 "
114-C. "	1.019	10 "
114-D. "	1.030	12 "

* In the presence of 1 cc. of 0.2 per cent soluble starch prepared according to Lintner.

power by treatment with quinol according to the procedures described above. The relative reducing capacity of each preparation was estimated by determining the time required for 5 cc. of 0.0005 N iodine to be reduced by the amount of material obtained from 50 cc. of urine. Typical results are given in Table IV.

DISCUSSION

The results of the potentiometric measurements and the constant association of a reducing property with chorionic gonadotropic preparations obtained from pregnancy urine or placental extracts strongly suggest that the reducing power can be attributed to the hormone itself.[1] The E_0 of this oxidation-reduction system

[1] Since this paper was prepared for publication, an opportunity has been afforded to study one of Gurin, Bachman, and Wilson's preparations

as well as the indication that the reaction depends upon a single electron exchange leads to the belief that the oxidation-reduction activity may be due to a bound metal ion or a derivative of tyrosine. Work now in progress points quite definitely to the hydroxyl group of a moderately labile derivative of tyrosine as the group which undergoes oxidation. If this is the case, it would appear to be in accord with the findings of Li, Simpson, and Evans (8) who have pointed out that the potency of human chorionic gonadotropin decreases as the phenolic hydroxyls of the tyrosine of the protein molecule are acetylated. It does not appear that the reducing action referred to here can be attributed to a carbohydrate group.

An account of further work in which the reducing capacity of the hormone was used as the basis of the chemical diagnosis of pregnancy will appear in a later paper.

SUMMARY

Studies of the oxidation-reduction characteristics of the chorionic gonadotropic hormone prepared by the benzoic acid method of Katzman and Doisy with subsequent reprecipitation with acetone have been made and may be summarized as follows:

1. Potentiometric studies upon the material so obtained and upon gonadotropin (international standard) lead to the conclusion that the oxidation-reduction properties can be attributed to the hormone itself. The E_0 of the system (at pH 5.9 and 38°) is +0.354 volt and the potential curve indicates 1 electron exchange.

2. The preparation undergoes slow spontaneous oxidation which can be inhibited by the addition of reductants and which is not accelerated appreciably by the addition of strong oxidants unless phosphate ion is present and moderate heat is applied.

3. The biological activity which has been decreased by oxidative changes can be restored by treatment with strong reductants.

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through the kindness of Dr. D. Wright Wilson. This preliminary study indicates that the reducing property attributed to the preparation by these authors (2) is the same as that referred to in the present paper. The reversibility of the oxidation and the conditions under which it is accelerated appear to bear this out.

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