INACTIVATION OF GONADOTROPHINS

III. INACTIVATION AND MODIFICATION OF SERUM GONADOTROPHIN BY PERIODATE IONS

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Summary

Serum gonadotrophin was rapidly but not completely inactivated by dilute solutions of potassium periodate. The effect of time and of concentration of periodate on the reaction were studied. No evidence was obtained of an increase in activity after treatment with still greater dilutions of periodate.

The residual activity, after treatment with periodate, showed evidence of modification as it produced a greater degree of luteinization than comparable doses of normal hormone in both normal and hypophysectomized animals. In addition, it was no longer inactivated by influenza virus. No departure from parallelism was observed between the dose response curves of treated hormone and those produced by untreated gonadotrophin.

The activity of the oxidized hormone was not altered by subdivision of the dose, which indicated that the inactivation was not due to change which permitted excretion.

Unlike luteinizing hormone and chorionic gonadotrophin, the treated serum gonadotrophin produced significant growth of the ovaries of hypophysectomized rats.

I. INTRODUCTION

In the first paper of this series (Whitten 1948) it was shown that serum gonadotrophin was inactivated by preparations of influenza virus and receptor-destroying enzyme from *Vibrio cholerae* and it was concluded that the inactivation was enzymic. Other substrates of these enzymes have been shown to be highly susceptible to the action of dilute aqueous periodate. Hirst (1949) showed that this reagent inactivated the "receptor-substance" of red cells and McCrea (1948) found that "Francis inhibitor" was similarly affected. Comparable results were obtained by Burnet (1948) with cyst mucoid except that after treatment with low concentrations of periodate an increase in inhibitory titre, both with regard to infection and haemagglutination was observed when tested against one strain of virus.

In view of these findings and those of Pearse (1948), who demonstrated gonadotrophins histologically after periodic oxidation, it was decided to investigate the action of periodate on the biological properties of serum gonadotrophin.

The response elicited by serum gonadotrophin in the ovaries of normal immature female rats is mainly one of follicle stimulation but various degrees

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of luteinization of the *membrana granulosa* occur, particularly at high dose levels. The response in hypophysectomized animals is similar but luteinization is less in evidence (Noble *et al.* 1939; Rowlands and Williams 1940). The last two authors postulated the presence in serum gonadotrophins of two active components, follicle-stimulating which predominates, and luteinizing.

Evans *et al.* (1936) and Hellbaum (1937) claimed to have separated serum gonadotrophin into two fractions. Cole, Pencharz, and Goss (1940) were unable to confirm these findings nor could they detect any qualitative difference between crude and highly purified preparations. Li, Evans, and Wonder (1940) prepared serum gonadotrophin in an electrophoretically homogeneous form, the action of which was indistinguishable from that of the original serum. From these findings it was concluded that the activity of serum gonadotrophin was dependent on a single hormone. Nevertheless, Rimington (1946) was unable to arrive at the same conclusion since he separated from an electrophoretically homogeneous preparation a fraction which was about four times as active as the original material.

One of the preparations used in this study produced an unusually high degree of luteinization. Rowlands and Williams (loc. cit.) describe one, and probably two, similar preparations. It is perhaps unfortunate that one of these was used by Noble *et al.* (loc. cit.) in their description of the action of the hormone. These preparations indicate that the follicle-stimulating and luteinizing activities of serum gonadotrophin may be, to some extent, independent, and the evidence produced by Kupperman, Meyer, and McShan (1941) by the use of suitable antisera supports this view. However, Cartland and Nelson (1937) were unable to detect any alteration in the nature of the response to serum gonadotrophin after partial inactivation with formaldehyde.

As the action of periodate is relatively mild and selective, it was considered that a study of its reaction with serum gonadotrophin might yield some useful information regarding the hormone substance.

II. Materials and Methods

Serum gonadotrophin.—Two preparations of serum gonadotrophin, Nos. 1 and 2, were supplied by Organon and assayed about 37 I.U. per mg. A third preparation, No. 3, was kindly supplied by Dr. C. W. Emmens of the Physiology Department, School of Veterinary Science, University of Sydney. This had been prepared by Professor C. Rimington several years previously and then assayed 2,000 I.U. per mg. It was assayed prior to use against the International Standard, at two dose levels and with separation of litter mates, when it contained 611 I.U. per mg. with 1 per cent. fiducial limits of 506 and 746 I.U. per mg. Histological examination of the ovaries of these groups showed that it produced an unusually high degree of luteinization. There was, however, no significant departure from parallelism in the dose response curves. All solutions of these hormones were prepared in distilled water before use.

Potassium periodate.—Potassium periodate of A.R. purity was prepared fresh and at the required concentration in M/15 phosphate buffer at pH 6.0.

Influenza virus.—Allantoic fluid of high titre from chick embryos which had been infected with LEE. B. influenza virus was kindly supplied by Mr. J. H. Whittem, of the Pathology Department, School of Veterinary Science, University of Sydney.

Hypophysectomized animals.—Female rats weighing 60-70 g. were hypophysectomized by the retropharyngeal approach under ether anaesthesia. Body weight records and examination of the *sella turcica* at death served as checks of the completeness of the operation. Injections were commenced 6-8 days after the operation.

Biological assay.—The gonadotrophin was assayed according to the method of the British Pharmacopoeia (1948) and the animals used were 23-25 day old female Wistar rats bred in this laboratory. The numbers of animals used, together with information regarding litter distribution and dose levels, are given in the tables. Preliminary observations were carried out and ovarian weight *per se* was used as the criterion of hormonal activity.

Experimental procedure.—Equal volumes of hormone and periodate solutions were mixed at room temperature and the reaction allowed to proceed for the specified time, when any excess periodate was reduced by the addition of glucose. The concentration of periodate is expressed as the molarity after mixing.

III. Observations

Preliminary tests showed that serum gonadotrophin was rapidly inactivated by M/1000 potassium periodate in M/15 phosphate buffer at pH 6.0, at room temperature. Previous reduction of the periodate by glucose prevented the inactivation as shown in Table 1.

Table 1

MEAN OVARIAN WEIGHTS OF GROUPS OF TEN RATS INJECTED WITH 25 I.U. OF SERUM GONADOTROPHIN (PREPARATION No. 1), UNTREATED, TREATED WITH PERIODATE, AND WITH REDUCED PERIODATE

Treatment	Mean Ovarian Wt. (mg. ± S.E.)
Untreated	68 ± 5
M/500 periodate	19 ± 1
M/500 periodate (reduced)	65 ± 4

(*a*) *Effect of Concentration of Periodate*

The concentration of periodate necessary to give maximum inactivation in 30 minutes of a solution of preparation No. 1 containing 25 I.U. per ml. was determined. The treated hormone was injected into groups of seven animals at a dose equivalent to 25 I.U. of the original hormone. The mean ovarian weights are given in Table 2, from which it is evident that no further significant inactivation occurs after the concentration of M/10,000 is reached. Similar results were obtained with preparation No. 2. There is no evidence from these figures of any increase in gonadotrophic activity corresponding to the increase of inhibitory titre observed by Burnet (loc. cit.) with cyst mucoid.

(*b*) *Effect of Time on the Reaction*

Serum gonadotrophin was permitted to react with periodate for 5, 10, 30, and 60 minutes, when the reaction was stopped by the addition of excess glucose. The reaction mixture contained 25 I.U. of serum gonadotrophin (No. 1) per ml. of M/1000 KIO_4. One ml. of these solutions was injected into each of the groups of six rats. The mean ovarian weights of these groups are

TABLE 2

MEAN OVARIAN WEIGHT OF GROUPS OF SEVEN RATS INJECTED WITH 25 I.U. OF SERUM GONADOTROPHIN (PREPARATION No. 1) AFTER TREATMENT WITH INCREASING CONCENTRATIONS OF PERIODATE

Concentration of Periodate	Mean Ovarian Wt. (mg. ± S.E.)
0	73 ± 6
M/40,000	60 ± 3
M/30,000	65 ± 8
M/20,000	46 ± 2
M/15,000	32 ± 3
M/10,000	21 ± 2
M/5,000	22 ± 2
M/1,000	18 ± 1
M/500	19 ± 1
Untreated animals	15 ± 1

given in Table 3. Similar results were obtained with the other hormone preparations. From these results it is evident that no significant reduction of hormone activity occurred after an interval of five minutes. Additional observations indicated that subsequent loss did occur but at a much slower rate.

TABLE 3

MEAN OVARIAN WEIGHTS OF GROUPS OF SIX RATS INJECTED WITH 25 I.U. OF SERUM GONADOTROPHIN AFTER EXPOSURE TO M/1000 PERIODATE FOR VARIOUS TIMES

Time (min.)	Mean Ovarian Wt. (mg. ± S.E.)
0	148 ± 7
5	34 ± 3
10	31 ± 2
30	25 ± 1
60	25 ± 2

The initial rapid inactivation resembles that observed by McCrea (loc. cit.) with "Francis inhibitor" and it is considered that this may be due to oxidation of the carbohydrate portion of the hormone, whereas the subsequent loss may correspond to the slow inactivation of the biological properties of proteins reported by Goebel, Olitsky, and Saenz (1948). However, no attempt was

made to determine the nature of the reaction or reactions involved. In all but one of the subsequent experiments the reaction was stopped by the addition of glucose after an arbitrary period of 30 minutes.

(*c*) *Residual Activity of Periodate-treated Serum Gonadotrophin*

By the use of the information described earlier, the amount of hormonal activity remaining after treatment with excess periodate for 30 minutes was determined for the three samples of gonadotrophin. The treated material was assayed against untreated hormone from the same sample. Unfortunately, the high doses required proved toxic with one preparation and no accurate estimate

TABLE 4

PERCENTAGE OF ACTIVITY OF SERUM GONADOTROPHIN LOST AFTER TREATMENT WITH EXCESS PERIODATE FOR 30 MIN.

Preparation No.	No. of Rats per Dose	Loss (%)	Fiducial Limits of Error $P = 0.99$
1	10	95[1] approx.	
1	8[2]	96.4	94.9–97.4
2	9	93.1	91.6–94.8
2	9	93.0	85.6–99.2
3	8[3]	83.4	77.4–87.2

[1] Preparation toxic at high doses.
[2] Litter mates segregated.
[3] Four dose levels of treated and untreated hormone used.

of potency could be made. The results are given in Table 4 and a typical analysis of variance is given in Table 5. It is evident that preparations No. 1 and No. 2 retained 4-7 per cent. of their original activity whereas 17 per cent. of preparation No. 3 was recovered. This apparent difference may be due to the fact that considerable loss of activity had occurred before use in these experiments. However, if the residual activity is calculated on the potency of No. 3 when originally prepared (2000 I.U./mg.), the result, viz. 6.0 per cent., is in closer agreement with those obtained for Nos. 1 and 2. This will be further considered when the qualitative nature of the residual hormone is discussed.

An analogous small amount of residual activity was observed by Burnet (1949) when mucin was treated with periodate and tested against Newcastle Disease virus.

(*d*) *Nature of Residual Gonadotrophin*

(i) *Dose Response Curves.*—A complete examination of the dose response curve for periodate-treated gonadotrophin has not been made. However, in the assay of residual activity of preparation No. 3, four dose levels of treated and control hormone were administered to groups of eight rats. The results obtained are shown in Table 6. The group injected with untreated hormone at

the lowest dose and that receiving the highest dose of treated hormone were responsible for departure from linearity and were omitted from analysis. This analysis confirmed the absence of departure from parallelism observed in the previous assays.

TABLE 5

DATA AND ANALYSIS OF VARIANCE OF ASSAY OF TREATED SERUM GONADOTROPHIN (PREPARATION No. 2) AGAINST UNTREATED HORMONE

Untreated		Treated	
Dose 16 I.U. Ovarian Wt. (mg.)	Dose 24 I.U. Ovarian Wt. (mg.)	Dose 267 I.U. Ovarian Wt. (mg.)	Dose 400 I.U. Ovarian Wt. (mg.)
67	79	57	93
52	59	74	128
56	109	47	116
43	122	77	128
52	110	72	91
41	76	50	149
45	114	79	100
71	78	75	118
38	80	38	94
Mean 51.7	91.9	63.2	113.0

ANALYSIS OF VARIANCE

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Ratio	P
Between samples	1	2401.0	2401.0	7.7	< 0.05
Slope	1	18225.0	18225.0	58.4	< 0.001
Departure from parallelism	1	205.4	205.4	–	–
Error	32	9976.4	311.8	–	–
Total	35	308.08			

(ii) *Histological Response in Normal Animals.*—Ovaries from the groups of animals used in the assays were examined after sectioning and staining with haematoxylin and eosin. An estimate of the degree of luteinization ranging from one to five was allotted. The results obtained for preparation No. 2 are given in Table 7. Analysis revealed that a significantly greater degree of luteinization ($P < 0.001$) occurred in the ovaries of rats injected with oxidized hormone. In addition, no significant difference was detected between the nature of the response of the two doses of the treated hormone, whereas, with the untreated gonadotrophin the higher dose produced a significantly greater degree of luteinization ($P < 0.001$). This latter finding confirms the accepted increase in luteinization which occurs with increased doses of normal serum gonadotrophin. Plate 1, Figures 1 and 2, shows typical ovaries from animals injected with treated and untreated hormone. Similar results were obtained with preparation No. 1. With preparation No. 3, as indicated earlier, a high

degree of luteinization was observed with the untreated hormone—however, all follicles of animals injected with this oxidized hormone were completely luteinized.

TABLE 6

DATA OF ASSAY OF TREATED AGAINST UNTREATED SERUM GONADOTROPHIN (PREPARATION No. 3) AT FOUR DOSE LEVELS

	Untreated				Treated			
Dose (I.U.)	5	10	20	40	70	140	280	560
	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)	Ovarian Wt. (mg.)
	27	44	108	150	56	102	237	204
	30	54	110	174	45	100	182	253
	30	52	92	141	71	56	143	219
	25	34	80	104	63	93	157	251
	29	62	65	215	56	90	140	228
	30	30	58	160	57	118	192	226
	25	26	99	124	65	76	154	218
	33	44	90	140	59	80	148	178
Mean	28.6	43.3	87.8	151.0	51.8	90.5	168.0	222.1

The high degree of luteinization prior to treatment suggests that changes similar to those produced by periodate have already occurred in preparation No. 3. This evidence supports the calculation made in Section III (*c*) of the residual activity using as a basis the original activity before storage.

TABLE 7

DATA OF GRADING OF HISTOLOGICAL NATURE OF RESPONSE TO TREATED AND UNTREATED SERUM GONADOTROPHIN (PREPARATION No. 2)

	Treated		Untreated	
Dose (I.U.)	267	400	16	24
Mean ovarian wt. (mg.)*	63.2	113.0	51.7	91.9
Histological Grading				
Mean	4.8	4.8	1.5	2.9
Range	4-5	4-5	1-3	2-3

* See Table 5.

(iii) *Response in Hypophysectomized Animals.*—The response produced by two comparable doses of both treated and untreated hormone (preparation No. 1) was examined. The doses and results are given in Table 8. Analysis revealed no significant difference between the response to the two samples, at the selected dose rates, a significant slope ($P < 0.01$) for the combined response curve, and no departure from parallelism of the response curves of the samples.

On section it was seen that the untreated hormone produced almost pure follicle stimulation. The ovaries of the animals injected with the treated hormone showed similar evidence of follicular growth but the *membrana granulosa* of almost all follicles with atria was partly or completely luteinized. Plate 1, Figures 3 and 4, shows typical responses to these substances in litter mates, the ovaries of which weighed 50 and 52 mg. respectively. These findings confirm those observed in intact animals and show that the altered response is not mediated through the anterior pituitary.

TABLE 8

RESPONSE IN HYPOPHYSECTOMIZED RATS TREATED WITH NORMAL AND OXIDIZED SERUM GONADOTROPHIN (PREPARATION No. 1) AT TWO COMPARABLE DOSES

Dose (mg.)	Normal Gonadotrophin 0.5 Ovarian Wt. (mg.)	Normal Gonadotrophin 1.0 Ovarian Wt. (mg.)	Oxidized Gonadotrophin 10.0 Ovarian Wt. (mg.)	Oxidized Gonadotrophin 20.0 Ovarian Wt. (mg.)
	56	51	33	43
	48	42	37	42
	55	61	16	60
	43	66	28	57
	45	40	44	87
	33	46	42	50
	35	83	39	41
	36	52	44	102
	29	57	28	42
	20	28	50	48
	48	23	26	28
	33	46		23
				46
Mean	40.1	49.6	35.2	51.5

A similar modification of the response to serum gonadotrophin in hypophysectomized animals treated with oestrogens post-operatively has been reported by Williams (1945). In a limited number of animals the author has not observed as great a degree of luteinization by this procedure as with the oxidized hormone. Nevertheless, it is possible that endogenous oestrogen may be responsible for the increased luteinization.

(iv) *Effect of Subdivision of the Dose.*—Since serum gonadotrophin is not excreted and thus produces a maximum response following a single injection, it was considered that the reduction in activity produced by periodate may have resulted from an alteration which permitted excretion. In order to examine this possibility, two groups of ten animals were injected with equal quantities of treated hormone. One group received the total dose on the first day, whereas the other was injected with one-fifth the dose for five successive days. Both groups were killed on the sixth day, and the ovaries removed, fixed, and weighed. The mean ovarian weight of the group which received the single

injection was 91.0 mg. whereas that of the other group was 61.8 mg., the difference being significant ($P < 0.01$). Thus subdivision of the dose did not increase the response.

(v) *Effect of Influenza Virus.*—Anderson (1947) has shown that cells treated with periodate are altered with respect to their reaction with influenza virus, so it was decided to examine the effect of the virus on treated gonadotrophin. Two samples of periodate-treated gonadotrophin (preparation No. 1) were incubated with active influenza virus and assayed against duplicate aliquots which had been incubated with inactivated virus. The results in Table 9 show that no loss occurred. A qualitative test carried out concurrently showed that the action of influenza virus on intact gonadotrophin was not inhibited by the presence of an equivalent concentration of glucose-reduced periodate.

TABLE 9

PERCENTAGE OF ACTIVITY OF OXIDIZED GONADOTROPHIN RECOVERED AFTER TREATMENT WITH INFLUENZA VIRUS

Preparation No.	No. of Rats per Dose	Per cent. Recovered	Fiducial Limits of Error $P = 0.99$
1	10	101.7	70.4-148.9
1	7*	107.8	66.5-159.7

* Litter mates segregated.

IV. DISCUSSION

As there has been no substantial evidence of the separation of serum gonadotrophin into follicle-stimulating and luteinizing components it has been accepted generally that this hormone is a single mucoprotein or a mucoprotein complex, which exhibits a combination of both actions. This dual nature of the hormone has been used as an argument against the concept of two anterior pituitary hormones. In this regard, it is of interest that treatment of serum gonadotrophin with periodate ions modifies it so that luteinization becomes more evident or even predominates.

The most obvious explanation of the inactivation and modification of serum gonadotrophin by periodate is that the follicle-stimulating component is reduced whereas that responsible for luteinization is relatively unaltered. The present assay methods do not permit determination of the absolute amounts of those components so it is impossible to verify this assumption. If, however, it is correct, it indicates that follicle stimulation depends on groupings susceptible to periodic oxidation. The conditions under which the modification occurred and the mucoid nature of the hormone suggest that these are the carbohydrate groups of the hormone.

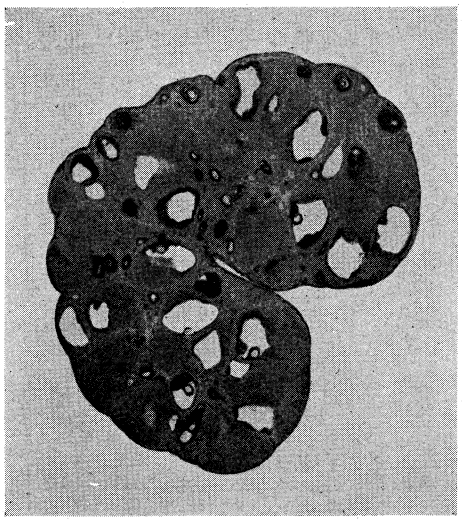

Fig. 1

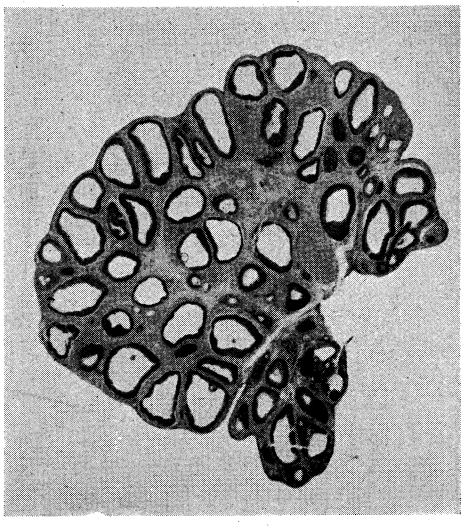

Fig. 2

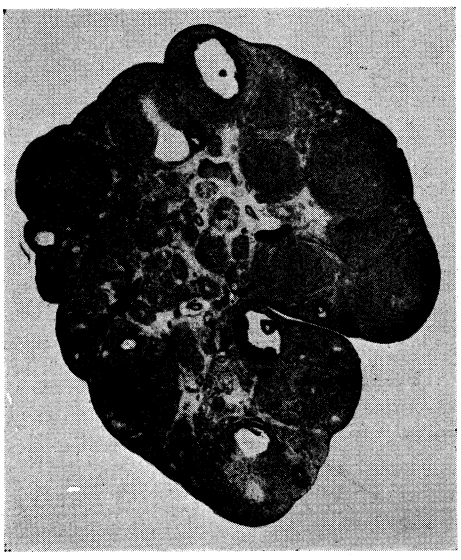

Fig. 3

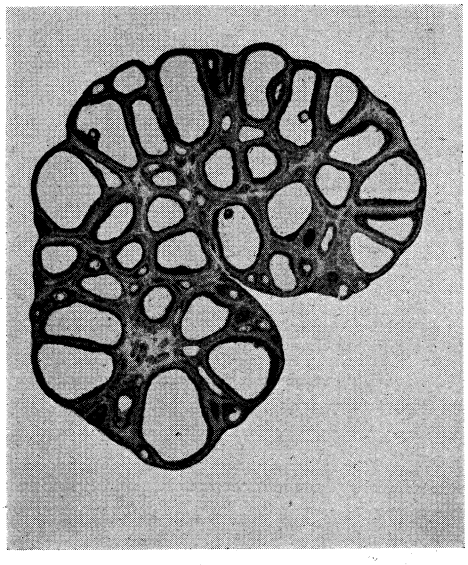

Fig. 4

Fig. 1.—Photomicrograph of section of ovary of immature rat injected with 16 I.U. of serum gonadotrophin (preparation No. 2). x 16. Ovarian wt. 52 mg.

Fig. 2.—Photomicrograph of section of ovary of immature rat injected with 267 I.U. of serum gonadotrophin (preparation No. 2) treated with periodate ions. x 16. Ovarian wt. 57 mg.

Fig. 3.—Photomicrograph of section of ovary of hypophysectomized rat injected with 1 mg. of serum gonadotrophin (preparation No. 1). x 16. Ovarian wt. 52 mg.

Fig. 4.—Photomicrograph of section of ovary of hypophysectomized rat injected with 20 mg. of serum gonadotrophin (preparation No. 1) treated with periodate ions. x 16. Ovarian wt. 50 mg. The animals used to obtain Figures 3 and 4 were litter mates.

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