CC. THE IDENTITY AND MECHANISM OF ACTION OF THE GLYCOTROPIC (ANTI-INSULIN) SUBSTANCE OF THE ANTERIOR PITUITARY GLAND

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THE experiments of Cope & Marks [1934] were the first to demonstrate that the injection of a crude anterior pituitary extract into a normal rabbit can induce complete insensitivity to the hypoglycaemic action of insulin administered many hours after the last injection of pituitary extract. In the experiments of these authors the blood sugar level of the treated rabbit was seldom significantly affected by the injections of the anterior pituitary preparation alone, and the "anti-insulin" activity of the extracts was therefore not due to a "diabetogenic" action. For the purpose of convenience the name "glycotropic factor" was suggested [Young, 1936, 1] for the anterior pituitary substance which induces insulin insensitivity without itself causing a rise of blood sugar level. Investigation showed [Young, 1936, 1] that the glycotropic factor was present in preparations of prolactin obtained by the method of Riddle *et al.* [1933], a fact which appeared to be of significance with respect to the action of anterior pituitary preparations on milk secretion [Young, 1936, 1, 2]. Subsequent observations [Young, 1937, 1; 1938] showed that prolactin and the glycotropic substance are not identical. The evidence presented in support of this differentiation, although conclusive, has previously been described only briefly, and it is one purpose of the present communication to supplement the description of the earlier work and to present further evidence that prolactin and the glycotropic factor are not identical. The results of experiments on the influence of the administration of anterior lobe extracts on the hyperglycaemic action of injected adrenaline and on sugar tolerance are also recorded in the present paper, and the significance of the results obtained is discussed with respect to the mechanism of action of the glycotropic substance.

EXPERIMENTAL

Animals

The animals used in the majority of the experiments in the present investigation have been unanaesthetized, healthy, young adult rabbits, male or female, weighing about 1.5 kg., from an inbred pure strain of Dutch rabbits bred at the farm laboratories of the National Institute for Medical Research. In our experience animals of this strain are particularly constant in their response to glycotropic pituitary extracts.

In the investigations on the influence of the subcutaneous injection or intravenous infusion of adrenaline it was convenient to use larger rabbits, so pure strain Chinchilla rabbits, and occasionally rabbits weighing about 3 kg. purchased from a dealer, were used for these experiments.

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All the rabbits received an ample diet of cabbage, bran, oats and hay. For experiments on the glycotropic factor it is essential to use rabbits which are in excellent condition and are eating well, in order to obtain consistent results.

Biological assays

Thyrotropic activity was assayed by the method of Rowlands & Parkes [1934]. The thyrotropic activity considered in the present investigations is therefore that causing an increase in size of the thyroid gland of the immature guinea-pig. It is not certain that the substance active in this respect is identical with that stimulating secretory activity of the thyroid gland.

Prolactin was assayed by the pigeon crop-gland method according to the technique described by Rowlands [1937]. The name prolactin is used to describe the substance active in causing increase in weight of the pigeon crop-gland, without reference to the question whether or not all preparations of this substance are truly lactogenic [cf. Folley & Young, 1938].

Method of expressing the results of biological assays

The question of establishing International standard preparations of prolactin and of the thyrotropic hormone, and of establishing International units of activity in terms of these preparations, is at present under discussion. Any method of expressing the physiological activity of pituitary substances in terms of units, in the present paper, must therefore be regarded as of temporary value only. In connexion with the question of establishing an International standard preparation of prolactin, Dr O. Riddle and Dr R. W. Bates have kindly made available a preparation of prolactin standardized by them. An assay curve for this prolactin, which contains 10 Riddle units/mg., has been constructed by the methods used in this laboratory. This curve has allowed us to express the results of our pigeon crop-gland assays in terms of Riddle units, and in the present paper this has been done.

Rowlands & Parkes [1934] defined a unit of thyrotropic activity as that amount of thyrotropic substance which, when injected daily for 5 days, would induce the thyroid glands of a 200 g. guinea-pig to double in weight. This unit has been adopted in the present paper, and will be described as the Rowlands-Parkes unit of thyrotropic activity.

Blood sugar estimations

Blood sugar was determined on 0.1 ml. of blood by the method of Hagedorn & Jensen. The figures in the present paper are therefore higher than those for "true" blood sugar.

In our experience the blood sugar level of a pituitary-treated rabbit is very easily raised on handling unless great care is exercised not to excite the animal. It is necessary to ensure that the rabbit is perfectly comfortable during the taking of blood samples. The ear vein from which samples are to be drawn is pierced some hours before blood is required for sugar estimations, so that frequent small samples can be obtained without the slightest reaction on the part of the rabbit.

In most instances the blood sugar curves given in the present paper represent the average response for 6 or more rabbits. Average, rather than individual, results are given because small individual variations are thereby minimized. The groups of animals for which average curves are presented sometimes constituted only a small proportion of the total number of rabbits used in a particular type

of experiment, and the individual experiments for which average results are given were chosen at random from the total number available. Where the difference between the average curves for two different groups might or might not be significant, standard errors of corresponding values in the average curves were calculated. In this way it was possible to determine whether or not corresponding points in the two average curves differed significantly.

Adrenaline infusion

Adrenaline was infused into the marginal ear vein of the unanaesthetized rabbit following the technique of Cori *et al.* [1930], and using a Burn-Dale burette pump. 1/1000 adrenaline (Parke Davis Ltd.) was diluted to a suitable volume with 0.9% NaCl solution containing 1 mg. of glutathione/5 ml., the latter being added to stabilize the dilute solution of adrenaline.

Anterior pituitary extracts

Extracts of ox, pig and horse pituitary glands were used in the present investigation. Most of the extracts were obtained from ox pituitary gland, however, and where the species-source is not stated it is to be assumed that the preparation was extracted from ox anterior lobe.

All the ox pituitary preparations were extracted from anterior lobe tissue which had been dissected as free as possible from posterior lobe tissue. In a number of instances, however, extracts of pig and horse pituitary tissues were made from the undissected glands.

(a) *Acetone desiccation of anterior lobe tissue.* Acetone desiccation of frozen ox anterior lobes was effected by mincing the frozen tissue into 20 vol. of ice-cold acetone. After remaining in acetone in the cold room overnight, the tissue was transferred at intervals of 24 hr. to 3 or 4 fresh portions of cold acetone. The dehydrated tissue was finally washed with ether and preserved in a vacuum desiccator at room temperature. Frozen pig or horse pituitary tissue was desiccated in acetone at room temperature. Commercial (B.D.H. Ltd.), acetone-desiccated ox anterior lobe tissue was used for a number of preparations.

(b) *Preparation of prolactin.* Six different types of prolactin were used. These differed in the source (ox, horse or sheep pituitaries; fresh gland or acetone-desiccated powder) and in the methods of extraction. The different types are distinguished by suffixes.

Prolactin (1) was obtained from fresh, frozen dissected glands by the method described by Young [1938]. The material designated Prolactin-C in the earlier paper is the Prolactin (1) of the present communication. In brief, the method of preparation consisted of alkaline extraction of the anterior lobe tissue at 0°, followed by precipitation and reprecipitation of the prolactin at pH 5.5. All these procedures were carried out in the cold room at 0° as far as possible, and the prolactin was stored in a frozen condition at -11°, in a solution of such concentration that 3 ml. were equivalent to 1 g. of fresh anterior lobe tissue.

Prolactin (2) was prepared from commercial acetone-desiccated ox anterior lobe by the method of Bates & Riddle [1935] using either 60% or 70% alcohol for the initial extraction, though a slight addition to the Bates-Riddle technique was made. This consisted in a number of reprecipitations from water at pH 5.5 of the material insoluble at pH 3.5 in the presence of Na_2SO_4 . In this way any Na_2SO_4 present in the original precipitate was removed. The product was then dried and passed through the final stage of the Bates-Riddle method of purification.

Prolactin (3) was prepared from acetone-desiccated fresh gland by a method essentially that of Riddle *et al.* [1933], i.e. aqueous extraction at pH 8 followed by precipitation (and reprecipitation a large number of times) of the prolactin fraction at pH 5.5. The prolactin was finally washed with alcohol and ether and dried.

Prolactin (4) was similar to Prolactin (3) except that it was extracted from commercial dried gland.

Prolactin (5) was prepared by the slight modification of the Bates-Riddle method described by Young [1938]. The modification consisted in the replacement of the final fractionation with 70 % alcohol in the presence of NaCl by 6–12 reprecipitations from a large volume of water at pH 5.5. The product was washed with alcohol and ether and dried.

Prolactin (6) was the product obtained by an application to Prolactin (5) of a method of purification based on that of Lyons [1937]. 1 g. Prolactin (5) was stirred for 1 hr. at room temperature with 40 ml. *N*/20 HCl contained in 80 % aqueous acetone; the residue was extracted twice in the same manner. The combined extracts were then brought to the point of maximum precipitation by the addition of strong ammonia solution (about 0.25 ml.) and two volumes of alcohol were added. After settling overnight the precipitate was filtered off and dissolved in water at pH 8, any material insoluble at this pH being spun off and discarded, and it was then reprecipitated at pH 5.5. This process of solution at pH 8 and precipitation at pH 5.5 was repeated, and the precipitated prolactin was washed with alcohol and ether and dried.

(c) *Preparation of thyrotropic extracts.* The material which remained in solution when the prolactin fraction from ox pituitary tissue had been precipitated "isoelectrically" during the preparation of Prolactins (1), (2), (3) and (4) constituted the thyrotropic fractions used in the present investigation. No attempt was made to separate the thyrotropic and gonadotropic hormones, and all the thyrotropic preparations used possessed gonadotropic activity.

In each instance a thyrotropic preparation is numbered in agreement with the corresponding prolactin preparation.

Thyrotropic (1), i.e. the pH 5.5-soluble material corresponding with Prolactin (1), is the fraction described by Young [1938] as "non-prolactin-C". This was not precipitated and dried, but stored in solution by freezing at -11° . The solution was of such strength that 3 ml. of it were equivalent to 1 g. of fresh gland tissue.

Thyrotropic (2), the thyrotropic fraction corresponding with Prolactin (2), was prepared and dried according to the method previously described [Young, 1938]. This fraction was designated "ordinary non-prolactin" in the previous paper.

Thyrotropic (3) and *Thyrotropic* (4) were precipitated by the addition of 6 vol. of cold alcohol, washed with alcohol and ether and dried *in vacuo*.

(d) *Average yields of the different fractions.* The yields of the different types of preparation used are given in Table I as percentages of acetone-desiccated anterior pituitary tissue.

(e) *Posterior lobe contamination of anterior pituitary preparations.* As the administration of posterior pituitary extracts is known to affect carbohydrate metabolism, it was necessary to be certain that the anterior pituitary preparations used were not grossly contaminated with posterior lobe principles. The results of assays for oxytocic activity (guinea-pig uterus method) on the different preparations used are given in Table II. In a few instances the pressor activity of the ox pituitary preparations was determined, and found to be close to the figure expected from the previously determined oxytocic activity.

Table I. *Yields of the different pituitary preparations used*

Source of gland	Method of preparation	Yield, expressed as % of desiccated anterior lobe	
		Prolactin fraction	Thyrotropic fraction
Ox	(1)	—*	—*
	(2)	1.0–1.6	0.8–1.3
	(3)	9.8–11.1	2.2–2.7
	(4)	5.3–9.3	2.2–3.0
	(5)	6.7–12.8	—
	(6)	About 2	—
Horse	(3)	8.0–12.0†	—
Pig	(3)	11.7–14.2†	—

* These fractions were not dried before use but stored frozen in solution.

† Yield expressed as % of whole pituitary gland.

Table II. *Oxytocic activity of pituitary preparations*

(The preparations were heated in a boiling water bath with 0.25 % acetic acid for 2 min. before assay.)

Source of gland	Method of preparation	Oxytocic activity (international units)	
		Prolactin fraction	Thyrotropic fraction
Ox	(1)	About 0.005 units/ml.	About 0.006 units/ml.
	(2)	<0.0001 units/mg.	0.25–0.75 units/mg.
	(3)	<0.0001 „	<0.0001 „
	(5)	<0.0001 „	—
	(6)	<0.0001 „	—
	(3)	About 0.0003 „	—
Horse	(3)	About 0.0003 „	—
Pig	(3)	About 0.0001 „	—

The results in Table II show that with the exception of Thyrotropic (2) the ox pituitary preparations used possessed negligible oxytocic activity.

RESULTS

1. *The influence of pituitary extracts on the hypoglycaemic action of insulin*

In many early experiments of the present investigation on the glycotropic action of anterior pituitary extracts, 2 injections of a suitable extract were given daily to a rabbit for 2–4 days, and the response to 2 units of crystalline insulin, injected intravenously after a fast of 21 hr., was determined on the day following the last injection of extract. In these experiments the blood sugar curve following the administration of insulin was usually of the form illustrated in Fig. 1 [cf. Young, 1936, 1]. Occasionally, however, the daily injection of the anterior pituitary extract itself raised the blood sugar level of the rabbit [cf. Young, 1938] so that on the 3rd or 4th day of injection the blood sugar was at a definitely diabetic level. In these instances a glycaemic response to the injection of insulin, somewhat resembling that illustrated in Fig. 1, was sometimes, but not always, obtained (Fig. 2). In later experiments only two injections of anterior pituitary extract were given, these being administered at the beginning and at the 16th hour of a short fast. Two units of crystalline insulin were injected intravenously at the beginning of the 21st hour of fasting, and the blood sugar level was determined at intervals. With suitable pituitary extracts the hypoglycaemic action of the insulin was completely abolished (Fig. 3), there being no sign of an

initial, transient hypoglycaemia. Indeed, in most instances the immediate effect of the insulin injection was a definite rise of blood sugar level to 3–5 mg./100 ml. above its original level. Such a rise was not present when a volume of saline equal to that of the insulin solution was injected intravenously into the pituitary-treated rabbits. It should be mentioned that in spite of the fact that crystalline insulin (Burroughs Wellcome) was used, a slight initial hyperglycaemic response to the intravenous injection of insulin was frequently observed in the control experiments on rabbits which had not been previously treated with pituitary extracts.

Fig. 1.

Fig. 1. The influence of treatment with the glycotropic substance on the hypoglycaemic action of insulin in the rabbit. *Curve A.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 7 normal rabbits which had fasted 18 hr. *Curve B.* Average blood sugar response to the same dose of insulin of the same rabbits after each had received 4 injections of glycotropic substance during 2 days.

Fig. 2.

Fig. 2. The response to the intravenous injection of 2 units of crystalline insulin of two rabbits which exhibited hyperglycaemia as the result of treatment with the glycotropic substance.

It should be pointed out that the experiments which yielded curves of the type shown in Fig. 1 were those in which the injections of the glycotropic substance were given over a period of some days, during which time the rabbits were feeding normally. Curves of the type shown in Fig. 3 were obtained in experiments with rabbits which received pituitary injections during a short period of fasting, but it is not clear how far the condition of the animal (fasting or fed) at the time the injections of pituitary extract were given, determined the type of response to insulin.

In all the above experiments insulin was administered intravenously, but it should be mentioned that similar results were obtained when insulin was injected subcutaneously [cf. Marks & Young, 1938].

Control experiments with extracts of muscle, liver, adrenal and pineal tissues, prepared in a manner similar to that for the extraction of pituitary gland, showed no detectable glycotropic activity in these tissues, under the conditions of our experiments (Fig. 4—Curve A).

Rabbits treated with glycotropic pituitary extracts have more liver glycogen at the end of a short fast than do control animals [Young, 1937, 2; Marks & Young, 1938]. Control experiments were therefore made on rabbits in which the fasting liver glycogen level was raised. It was found that if our rabbits were given

Fig. 3.

Fig. 3. The influence of treatment with the glycotropic substance on the hypoglycaemic action of insulin in the rabbit. *Curve A.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 6 normal rabbits which had fasted 21 hr. *Curve B.* A typical response to the intravenous injection of 2 units of crystalline insulin of a rabbit which had received 2 injections of glycotropic substance during the preliminary fast of 21 hr.

(In order to facilitate comparison, the blood sugar results are expressed as a percentage of the initial level. In the control group the initial level was 103 mg./100 ml. while in the treated animal it was 106 mg./100 ml.)

Fig. 4.

Fig. 4. The influence of treatment with non-specific tissue extracts and of feeding with glucose, on the hypoglycaemic action of insulin in the rabbit. *Curve A.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 6 rabbits which had received 2 injections of various tissue extracts during the preliminary fast of 21 hr. *Curve B.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin, of 18 rabbits which had received 24 g. of glucose by mouth at the beginning of the preliminary fast of 21 hr.

24 g. of glucose in 40 ml. of water by stomach tube at the beginning of a 21 hr. fast, the liver glycogen at the end of the fast averaged 3.2% as compared with 1.8% for untreated rabbits, and 5.1% for rabbits treated with glycotropic substance (Prolactin (1)) during the period of fasting. The blood sugar level of the glucose-fed rabbits was slightly, but significantly, raised, as the average initial blood sugar in 18 experiments was 118.0 ± 2.4 mg./100 ml.,¹ compared with a normal value (50 animals) of 103.6 ± 2.3 . However, a comparison of Curve A,

¹ 2.4 is the standard error of the mean value 118.0. The standard error of the mean is calculated

from the formula $\epsilon = \sqrt{\frac{\epsilon(\bar{x} - x)^2}{n(n-1)}}$ where $\bar{x}$ = the mean value of the series of observations, x = any individual observation, and n = the number of observations. The difference between two mean values is taken to be significant if the value of $\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$ is greater than 3.

Fig. 3, with Curve B, Fig. 4, shows that in these animals the hypoglycaemic action of insulin was but little altered.

When the glycotropic activity of pituitary extracts was tested according to the later technique described above, the blood sugar level of the rabbits was never more than very slightly raised by the injections of the pituitary extracts alone, provided that the animals had been handled very carefully, and the extracts were of a suitable type. In 50 such experiments with active preparations, the average blood sugar level immediately before the insulin injection was 107.4 ± 2.4 mg./100 ml. while in 50 untreated control animals, which had likewise fasted for 21 hr., the blood sugar level averaged 103.6 ± 2.3 . These results show that the administration of the anterior lobe extracts alone had no significant effect on the fasting blood sugar level of rabbits under the conditions of these experiments.

In order to facilitate the comparison of the glycotropic activities of different pituitary preparations, it was desirable to adopt a "unit" of glycotropic activity. As rabbits appear to be somewhat less sensitive to the anti-insulin action of glycotropic extracts during the middle of the summer, the results of assays carried out during the winter months only were used in this connexion. A unit of glycotropic activity was defined as that amount of extract which, on subcutaneous administration to a fasting rabbit in two equal doses according to the method described above, would just abolish, or almost abolish, the hypoglycaemic action of 2 units of crystalline insulin administered intravenously after a fast of 21 hr. The hypoglycaemic action of the insulin was considered to be almost abolished if the blood sugar level failed to fall more than 10 mg./100 ml. below its original value within 3 hr. of the insulin injection. An approximate value for the minimum amount of extract required could usually be determined in 3-5 experiments. Although no great quantitative merit can be claimed for the results of these assays, the differences observed between the activities of different types of extract were of such a magnitude as to be demonstrable by this method.

The evidence that prolactin and the glycotropic substance are different springs from two main sources: (a) a consideration of the widely differing glycotropic activities of different prolactin preparations obtained by different methods from pituitary glands of the same species, and by the same method from pituitary glands of different species; (b) the preparation of glycotropic extracts possessing no detectable crop gland-stimulating activity.

(a) *The relative glycotropic activities of different preparations of prolactin.* Table III gives the average prolactin and glycotropic activities of different prolactin preparations. It will be seen that Prolactin (6), although highly active as a pigeon crop gland stimulator, possessed no detectable glycotropic activity. On the other hand, Prolactin (1), extracted from pituitary glands of the same

Table III. *Average prolactin and glycotropic activities of prolactin preparations obtained by different methods*

Source of hypophysis	Method of extraction	Prolactin content Riddle units	Glycotropic activity units	Prolactin units Glycotropic units
Ox	(1)	15/ml.	8/100 ml.	190
	(2)	6/mg.	12/g.	500
	(3)	3.5/mg.	6/g.	580
	(4)	0.5/mg.	1/g.	500
	(5)	3.5/mg.	5/g.	700
	(6)	6/mg.	<1/g.	>6000
Horse	(3)	0.2/mg.	12/g.	17
Pig	(3)	0.3/mg.	6/g.	50

species, possessed substantial glycotropic activity. Horse and pig pituitary tissues are poor in prolactin activity, but possess glycotropic activity comparable with that of ox pituitary tissue. It will be seen from Table III that the maximum value of the ratio prolactin activity/glycotropic activity (6000) is over 350 times the minimum value (17), which shows that there can be no relationship between the pigeon crop gland activity and glycotropic activity, i.e. prolactin and the glycotropic factor cannot be identical.

From the data in Tables II and III one can calculate the total prolactin and glycotropic activities of 100 g. of acetone-desiccated ox pituitary gland, as indicated by the results for the different preparations. The results obtained, which are given in Table IV, enable a comparison to be made of the different proportions of the total activities present in the desiccated gland which are carried through into these preparations. Prolactin preparations (1), (3) and (5) contain similar proportions of the total prolactin activity present in the gland tissue, but Prolactin (1) contains at least twice the total glycotropic activity of the other preparations. Prolactin (1) is obtained by a method involving only one extraction of the anterior lobe tissue [Young, 1938], so that a loss of active material must occur in the primary extraction. This probably accounts for the lower proportion of the total crop gland-stimulating material found in Prolactin (1), but renders the greater proportion of glycotropic activity found in this fraction more striking.

Table IV. *Prolactin and glycotropic activities contained in 100 g. of acetone-desiccated ox anterior lobe, as calculated from the data for the different specimens of prolactin*

Method of extraction	100 g. of ox acetone-desiccated gland calculated to contain	
	Prolactin Riddle units	Glycotropic activity units
(1)	22,500*	120*
(2)	7,800	16
(3)	36,400	63
(4)	3,650	7
(5)	34,000	49
(6)	12,000	<2

* Calculated on the assumption that 5 g. of fresh tissue = 1 g. of acetone-desiccated gland.

(b) *The preparation of glycotropic extracts possessing no detectable crop gland-stimulating activity.* A number of preparations of the thyrotropic hormone from ox pituitary gland were found to possess substantial glycotropic activity. The relevant data are summarized in Table V.

Table V. *Glycotropic activity of preparations of the thyrotropic hormone*

(All preparations extracted from ox hypophysis.)

Method of preparation	Thyrotropic activity Rowlands-Parkes units	Glycotropic activity units	Prolactin activity Riddle units
(1)	2/ml.	5/100 ml.	<0.3/ml.
(2)	1.5/mg.	<1/g.*	<0.03/mg.
(3)	1.0/mg.	5/g.	—
(4)	0.3/mg.	<1/g.*	—

* These preparations were contaminated with posterior lobe hormones. The results of the glycotropic tests are therefore unreliable (see below).

As preparations of the thyrotropic hormone which contain no amount of prolactin detectable by our methods possess substantial glycotropic activity, it is

again emphasized that prolactin and the glycotropic substance cannot be identical.

(c) *Differentiation of the glycotropic substance from the thyrotropic and gonadotropic hormones.* A number of the prolactin preparations which possessed glycotropic activity contained only traces of the thyrotropic and gonadotropic hormones. In particular Prolactin (2) and Prolactin (5) possessed so little thyrotropic activity that the administration of 20 mg. daily for 5 days failed to induce any enlargement of the thyroid gland of the immature guinea-pig under the conditions of our test. Similarly, the intravenous administration of 100 mg. of preparations of these types to an oestrous rabbit failed to cause ovulation. The amounts of thyrotropic and gonadotropic hormones contaminating these prolactin preparations must therefore have been very small, although their glycotropic activities were substantial. It is clear that there is no relationship between the gonadotropic and thyrotropic activities of pituitary extracts and their glycotropic activity, so that the glycotropic substance cannot be identical with the gonadotropic and thyrotropic hormones.

(d) *Differentiation of the glycotropic substance from the oxytocic and pressor substances of the posterior pituitary gland.* As seen above, anterior lobe extracts which contain only a trace of the oxytocic substance possess substantial glycotropic activity. Fig. 5 gives the average blood sugar response to 2 units of

Fig. 5. The influence of treatment with pituitrin on the hypoglycaemic action of insulin in the rabbit. *Curve B.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin, of 6 rabbits each of which had received 2 injections of 30 units of pituitrin (Parke Davis) during the preliminary fast of 21 hr.

Curve A is the control curve from Fig. 4, which is inserted for the purpose of comparison.

crystalline insulin in a group of 6 rabbits, each of which received 2 injections of pituitrin (pressor + oxytocic substance—Parke Davis) containing together 60 units of oxytocic activity (Curve B). The average response to the same dose of insulin in control experiments (Curve A, Fig. 4) is included for comparison. The initial blood sugar level of the animals treated with posterior lobe extract was usually slightly below that of control animals (average value in 6 experiments = 84.0 ± 5.3), but the hypoglycaemic action of insulin was normal, the blood sugar level falling to about 60% of its initial value in both groups of rabbits.

Burn [1923] found that when a posterior lobe extract and insulin were simultaneously injected subcutaneously into a rabbit, the hypoglycaemic action

of the insulin was almost abolished. Many other investigators have also found that pituitrin administration inhibits the hypoglycaemic action of subcutaneously administered insulin and depresses the sugar tolerance [cf. Cohen & Libman, 1937]. As Lambie & Redhead [1929] point out, the circulatory changes produced by the injection of pituitrin are profound and these may greatly modify the rate of diffusion into the tissues of injected material. The depressed rate of absorption of subcutaneously administered insulin may possibly account partly for the inhibitory action of simultaneously administered pituitrin. In the present investigation the second dose of pituitrin was given some hours before the intravenously injected insulin, and it is clear that the results of these experiments are not directly comparable with those of Burn and others. Our results do show, however, that the anti-insulin activity of the anterior lobe extracts was not due to the presence of traces of the oxytocic and vasopressor substances of the posterior lobe.

Anterior lobe extracts heavily contaminated with the oxytocic and vasopressor substances have always failed to exhibit glycotropic activity, even when there was reason to suspect that glycotropic substance was present in the extract. In a number of instances it was also found that the glycotropic activity of anterior lobe preparations was diminished when posterior lobe extracts were added to them. All these results are explicable on the assumption that when the anterior lobe extracts are heavily contaminated with the oxytocic and vasopressor substances, the diminished rate of absorption of the anterior lobe extracts caused by the intense vasoconstriction decreases the effectiveness of the administered glycotropic substance.

2. *The influence of injections of the glycotropic factor on the sugar tolerance curve in the rabbit*

It was briefly reported last year [Young, 1937, 1] that in a rabbit which has received 2 injections of a suitable preparation of the glycotropic substance and which has thereby become insensitive to the hypoglycaemic action of 2 units of insulin, the sugar tolerance curve is not significantly affected. Fig. 6 gives the

Fig. 6. The influence of treatment with the glycotropic substance on the sugar tolerance curve in the rabbit. *Curve A.* The average blood sugar response to the intravenous injection of 2 g. of glucose in 10 ml. of water of 6 normal rabbits which had fasted 21 hr. *Curve B.* The average blood sugar response to the same intravenous dose of glucose of the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

average blood sugar response of a group of 6 rabbits which had fasted 21 hr. to the intravenous injection of 2 g. of glucose in 10 ml. of water, administered at 0 min. (Curve A). Curve B is the average response of the same group after each rabbit had received 2 injections of a glycotropic pituitary extract (in most instances Prolactin (1)) which contained in all 1 unit of glycotropic activity. The blood sugar response to the intravenous administration of 2 g. of glucose is remarkably little affected by previous treatment with an amount of glycotropic substance which induces complete insensitivity to the hypoglycaemic action of insulin. For the first hour after the injection of glucose there is practically no difference between the average fall of blood sugar level in the control group of animals and that in the group treated with the glycotropic extract. The blood sugar in the pituitary-treated rabbits does not reach quite such a high level as it does in the control animals, and appears to fall a little more quickly for the first 60 min., though no difference between the two curves is statistically demonstrable. After 60 min. the blood sugar level in the control group falls faster than that of the experimental group so that at 90 min. a difference exists between the two curves which is possibly statistically significant ($\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}} = 2.6$). The sub-

normal rate of fall of blood sugar in the experimental group, beginning about one hour after the glucose injection, could be explained in two ways. First, the glycogen stores of a rabbit which has received injections of a glycotropic extract during a 21 hr. fast are considerably higher than those of control animals [Young, 1937, 1]. It is therefore possible that one hour after the injection of glucose the glycogen stores of the pituitary-treated animals are so full that a diminution in the rate of glycogen storage occurs with a consequent decreased rate of removal of sugar from the blood. Second, as Fisher *et al.* [1936] have shown, pituitary extracts may exert a depressing action on carbohydrate oxidation during a fast. If, in the normal animal, the fall of blood sugar for about one hour after glucose is predominantly due to storage of glycogen, while the subsequent fall is primarily associated with carbohydrate oxidation, then the observed facts would find an explanation, though it must be confessed that at present there seems little evidence to support such a theory.

If a crude saline extract of ox anterior lobe is used as the source of glycotropic activity, the sugar tolerance of the injected rabbit may be slightly, though definitely, decreased. This is also true for a number of cases in which Thyrotropic (1) is used, but does not detract from the value of the observation that the administration of other preparations of glycotropic factor, in a dose which is sufficient to abolish the hypoglycaemic action of 2 units of insulin, has very little effect on the sugar tolerance curve. It is probably of significance that those extracts (crude saline extract and Thyrotropic (2)) which are sometimes found to diminish the sugar tolerance in the rabbit under the conditions of the above test, are those which were found to be diabetogenic in the dog [Young, 1938].

Himsworth & Scott [1938, 1] have recently investigated the influence of various conditions on the increase in sugar tolerance which occurs in the rabbit receiving a high carbohydrate diet as the result of consecutive intravenous injections of 1 g. of glucose at intervals of half-an-hour (Staub-Traugott effect). They found that the hypophysectomized rabbit receiving a high carbohydrate diet showed a Staub-Traugott effect, in that each successive injection of glucose resulted in a smaller rise of blood sugar level than the previous one. When the hypophysectomized rabbit received preliminary treatment with a preparation of the glycotropic substance, the sugar tolerance was diminished, as shown by the absence of Staub-Traugott effect when successive injections of glucose were

given. The impairment of sugar tolerance was similar to that which occurs when a normal rabbit receives a diet low in carbohydrate. In view of the results of Himsworth & Scott with hypophysectomized rabbits it was necessary to determine whether or not the Staub-Traugott effect was abolished in an intact rabbit treated with the glycotropic substance. Although extensive investigations have not been carried out on this point, the results show that, as with hypophysectomized rabbits, the Staub-Traugott effect is abolished in normal rabbits by treatment with the glycotropic substance (Fig. 7). This decrease of tolerance,

Fig. 7. The influence of treatment with the glycotropic substance on the sugar tolerance in the rabbit. *Curve A.* The blood sugar response to three intravenous injections of 1 g. of glucose, administered at half-hourly intervals to a normal rabbit. *Curve B.* The blood sugar response in the same rabbit after two injections of the glycotropic substance. 1 g. of glucose in 10 ml. of water was injected at the times indicated by the arrows.

which is explicable on the basis of either of the suggestions put forward above, is similar to that which occurs in rabbits fed on a low carbohydrate diet [Himsworth & Scott, 1938, 1]. The sugar tolerance of rabbits which have been rendered completely insensitive to the hypoglycaemic action of injection of insulin by treatment with the glycotropic substance, is therefore only very slightly decreased, and remains within the range of variation to be expected in normal rabbits receiving different diets.

3. *The influence of injections of the glycotropic factor on the hyperglycaemic response to adrenaline in the rabbit*

Corkill *et al.* [1933] and Cope & Marks [1934] found that in the rabbit hypophysectomy greatly decreased the hyperglycaemic action of subcutaneously administered adrenaline, and conversely, that the administration of a crude anterior lobe extract greatly increased the blood sugar response to the subcutaneous injection of adrenaline. Administration of a suitable preparation of prolactin to the normal rabbit also greatly increases the glycaemic response to the subcutaneous injection of adrenaline [Young, 1936, 1, 2].

Curve A (Fig. 8) gives the average hyperglycaemic effect of the subcutaneous injection of 20 μ g. of adrenaline in a group of 10 normal rabbits which had fasted for 21 hr. Curve B (Fig. 8) is the average response of the same group to the same

dose of subcutaneous adrenaline after each rabbit had received two injections of prolactin (totalling 1 unit of glycotropic activity/rabbit) during the preliminary period of fasting. The difference in average response in the two groups is statistically significant, e.g. at 120 min. the average blood sugar level of the control group was 121 ± 3.5 mg./100 ml. while that for the experimental group was 165 ± 14 ; similar results were obtained for other points in the middle of the curves. An increase in the hyperglycaemic response to the subcutaneous injection of adrenaline was also found in a rabbit which had received injections of the glycotropic substance twice daily for 2-4 days.

Fig. 8.

Fig. 8. The influence of treatment with the glycotropic substance on the hyperglycaemic action of subcutaneously administered adrenaline in the rabbit. Curve A. The average blood sugar response to the subcutaneous administration of $20 \mu\text{g.}$ of adrenaline in 10 normal rabbits which had fasted 21 hr. Curve B. The average blood sugar response to the same subcutaneous dose of adrenaline in the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

Fig. 9.

Fig. 9. The influence of treatment with the glycotropic substance on the hyperglycaemic response to the intravenous infusion of adrenaline in the rabbit. Curve A. The average blood sugar response to the intravenous infusion of adrenaline at the rate of $0.05 \mu\text{g./kg. body weight/min.}$, for 2 hr., in 7 normal rabbits which had fasted for 21 hr. Curve B. The average blood sugar response to the intravenous infusion of adrenaline at the same rate in the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

As stated above, Corkill *et al.* [1933] and Cope & Marks [1934] found that the hyperglycaemic effect of subcutaneously administered adrenaline was greatly diminished in hypophysectomized rabbits. Russell & Cori [1937] have recently shown that the effect of the administration of adrenaline on the blood sugar level of the anaesthetized hypophysectomized rat was somewhat diminished when the adrenaline was given subcutaneously, but was normal when the adrenaline was given by slow intravenous infusion. These authors suggest that hypophysectomy diminishes the rate of absorption of adrenaline from the subcutaneous tissues of the rat. A comparison of the hyperglycaemic action in the normal rabbit treated with glycotropic substance, of adrenaline administered subcutaneously and by slow intravenous infusion, was therefore of interest.

Fig. 9 gives the average result for seven experiments in which a normal rabbit which had fasted 21 hr. received a slow intravenous infusion of adrenaline for 120 min. at the rate of $0.05 \mu\text{g./min./kg. body weight}$ (curve A), and the average response to adrenaline infused for the same time and at the same rate in the same group, when each animal had received two injections of Prolactin (1), containing altogether 1 unit of glycotropic activity, during the preliminary fast (Curve B).

The slight differences between corresponding points on these two curves are not statistically significant. The results show, therefore, that although the hyperglycaemic response to subcutaneously administered adrenaline is enhanced in the rabbit treated with the glycotropic substance, the response to the slow intravenous infusion of adrenaline was, under the conditions of our experiments, unchanged within the limits of experimental error.

As hypophysectomy diminishes the rate of absorption from the subcutaneous tissues [Russell & Cori, 1937; Heinbecker & Weichselbaum, 1937], it is possible that the administration of pituitary extracts also influences the rate of subcutaneous absorption. According to Heinbecker [1937] removal of the pituitary gland increases the action of adrenaline on the smooth muscle of the cat, and Heinbecker & Weichselbaum [1937] suggest that in the hypophysectomized animal adrenaline would cause an accentuated contraction of the blood vessels at the site of the subcutaneously injected adrenaline, with a consequent diminution in the rate of absorption. These authors consider that this diminution may account for the decreased hyperglycaemic action of subcutaneously administered adrenaline. If the injection of our pituitary extracts has an action on the response of smooth muscle to adrenaline, which is the converse of that of hypophysectomy, then the administration of glycotropic pituitary preparations might be expected to increase the rate of absorption of adrenaline from the subcutaneous tissues. The curves in Fig. 8 show that the action of subcutaneous adrenaline on the blood sugar is more prolonged in the pituitary-injected animals, and it would seem impossible to explain this effect by a more rapid absorption of adrenaline from the subcutaneous tissues. At present there seems to be no obvious explanation as to why the hyperglycaemic effect of subcutaneously injected adrenaline is so greatly enhanced in the pituitary-treated animals. The slight increase in response to the intravenous infusion of adrenaline in the pituitary-treated animals, although not statistically significant on the basis of the number of experiments carried out, may nevertheless indicate a slightly greater sensitivity to the hyperglycaemic action of adrenaline which has reached the blood stream, as the result of treatment with the glycotropic substance. As the treated rabbits have more glycogen in their livers at the end of the preliminary fasting period than do control animals [Young, 1937, 2] a slight increase in sensitivity to the hyperglycaemic action of adrenaline would be explicable on this basis alone, without recourse to a hypothesis that the liver glycogen becomes more easily mobilized under the influence of the pituitary extract [cf. Cope & Marks, 1934].

It should be recorded in this connexion that in our experience rabbits treated with the glycotropic substance are very liable to exhibit hyperglycaemia as the result of a slight emotional stimulus. No special investigation has been made on this point, and it is impossible to say whether or not observations of this kind would be found consistently in all rabbits, but it has become increasingly clear during a long series of experiments with the glycotropic substance, that in general, careful handling of the pituitary-treated animals is required if the blood sugar is to remain at its normal level. It is conceivable that the hyperglycaemic action of nerve stimuli is enhanced in these animals. The action of sympathin on the blood sugar has recently been investigated by Bodo & Benaglia [1938], but the action of pituitary extracts on the degree of hyperglycaemia following nerve stimulation in animals with denervated adrenal glands, has apparently not yet been investigated.

Collip [1938] finds that the injection of his pituitary anti-insulin substance inhibits the hyperglycaemic response to injected adrenaline. In our experiments there has been no indication of an inhibition of adrenaline hyperglycaemia in the pituitary-treated rabbits.

DISCUSSION

The results of the present investigation confirm the assumption that the pituitary gland contains a substance, the injection of which does not significantly alter the blood sugar of the fasting rabbit under the conditions of our experiments, but induces a complete insensitivity to the hypoglycaemic action of insulin. It was previously found that the daily injection of large doses of this "anti-insulin" substance into normal dogs did not induce glycosuria or ketonuria and that the "diabetogenic" and "anti-insulin" principles were therefore not identical [Young, 1936, 1, 2; 1938].

The name "glycotropic factor" was suggested as a convenient one for the pituitary substance which antagonizes the hypoglycaemic action of insulin without itself inducing a hyperglycaemia [Young, 1936, 2]. The term "glycotropic" was not intended to imply any specific mechanism of action of the "anti-insulin" factor, although at the time when the name was suggested it was believed that one action of the glycotropic substance was a facilitation of hepatic glycogenolysis, particularly the glycogenolytic action of adrenaline. Even at that time it was realized that this view was not altogether satisfactory [Young, 1936, 1], and the results since obtained do not support it. At present the mechanism whereby the anti-insulin action is exerted is not clear (see below), but until more is known about the nature and mechanism of action of the principle concerned, it is proposed to retain the name "glycotropic" factor or substance to describe it.

The identity of the glycotropic substance

The results of the present investigation differentiate the glycotropic substance from prolactin (pigeon crop gland-stimulating substance), the thyrotropic and gonadotropic hormones, and the oxytocic and pressor substances of the pituitary gland. Tissues other than the pituitary gland have failed to yield active extracts. So far there is no evidence as to whether or not the glycotropic substance is identical with the adrenotropic, ketogenic or glycostatic [Russell & Bennett, 1936] pituitary substances, or with the pituitary substance which increases the fat content of the liver [Best & Campbell, 1936].

According to a recent preliminary communication by Collip [1938], the pituitary melanophore-expanding substance, the ketogenic and anti-insulin substances, and a pituitary substance which antagonizes the hyperglycaemic action of adrenaline, are all identical. The single principle responsible for all these effects is believed to be produced in the pars intermedia of the pituitary gland. The full evidence for the belief that only one principle is responsible for so many different effects is not yet available. The results of the present investigation throw no light on the question whether or not the glycotropic substance is identical with the substance or substances responsible for the effects considered by Collip, and a discussion of this question is therefore not profitable at present.

In our experience loss of activity always occurs when preparations of the glycotropic substance are dried, and, as shown in Tables III and IV, Prolactin (1), which is an undried extract, contains a much higher proportion of glycotropic activity than the other preparations used. Our extracts containing the glycotropic substance can be dialyzed in cellophane bags (Visking) without undergoing appreciable loss of activity, but Collip [1938] finds that a substance capable of neutralizing the hypoglycaemic action of insulin will pass through a cellophane or collodion membrane, if the solution has been previously adjusted to pH 10 and heated on a boiling water bath for one hour. Further investigations on the

stability of the glycotropic substance towards heating in alkaline solution are required before the relation between the results of the present investigation and Collip's interesting findings is clear.

The most active preparations of the glycotropic substance were obtained from anterior lobe which had been carefully dissected from posterior lobe tissue, so that these preparations were virtually free from oxytocic activity (Table II). It is therefore safe to assume that the glycotropic substance is present in either the pars anterior or the pars intermedia, both of which may be present in the anterior lobe as obtained by our method of dissection. Collip [1938] believes that his anti-insulin substance is formed in the pars intermedia, but at present no evidence is available to determine whether or not this is true for the glycotropic substance.

The mechanism of action of the glycotropic substance

Cope & Marks [1934] found that in hypophysectomized rabbits the blood sugar often fell to a very low level and the animals exhibited symptoms of hypoglycaemia in spite of the presence of ample reserves of liver glycogen. They also found that the hyperglycaemic action of subcutaneously injected adrenaline was decreased in hypophysectomized rabbits and increased in rabbits treated with suitable anterior lobe extracts. These authors therefore put forward the attractive hypothesis that the anterior lobe of the pituitary gland contains a principle which renders the glycogen stores of the liver more susceptible to the mobilizing action of adrenaline [cf. also Young, 1936, 1]. More recently Cope [1937] has found that in the fasting hypophysectomized young rabbit, the blood sugar level is maintained until the liver glycogen stores are depleted; in the absence of liver glycogen there is a rapid fall of the blood sugar to convulsive levels. As the hyperglycaemic action of intravenously infused adrenaline is probably unaffected both by hypophysectomy [in the rat—Russell & Cori, 1937] and by treatment with pituitary extracts [in the rabbit—present communication] the hypothesis of Cope & Marks is not supported. This hypothesis is also not in agreement with the observations of Himsworth & Scott [1938, 2] who found that the glycotropic substance was active in abolishing the hypoglycaemic action of insulin administered to adrenalectomized rabbits.

The injection of insulin lowers the blood sugar level of the intact animal because the production of sugar by the liver is inhibited [Cori *et al.* 1923; Cori, 1931], and because the processes of glycogen formation in the muscles and sugar oxidation in the peripheral tissues generally are stimulated [Best *et al.* 1926]. Marks [1936] found, with eviscerated cats receiving glucose intravenously, that pre-treatment with suitable anterior pituitary extracts inhibited the action of insulin in increasing the muscle glycogen level, while Himsworth & Scott [1938, 2] found that the accelerating action of insulin on the spontaneous fall of the blood sugar level which normally occurs in hepatectomized rabbits, is lacking if the rabbits are treated with a preparation of the glycotropic substance before hepatectomy. We may therefore assume that, in intact rabbits, treatment with the glycotropic substance inhibits the increase in the peripheral absorption of sugar which normally follows the administration of insulin. The complete abolition of insulin hypoglycaemia in the pituitary-treated animals shown in Fig. 2 is explicable on the basis that the action of insulin in inhibiting the production of sugar in the liver has also been abolished under the influence of the pituitary extract. The form of curve illustrated in Fig. 1 is more difficult to explain. The simplest assumption is that in the livers of these animals the glycogenolytic action of adrenaline, secreted in response to the fall of blood sugar, has been greatly exaggerated by treatment with the pituitary extract, so that the

initial fall of blood sugar causes an excessive outpouring of sugar from the liver. However, as we have seen above, there is no reason to suppose that the glycogenolytic action of adrenaline in the liver is greatly increased under the influence of our pituitary extracts. It is possible, of course, that in the liver the effectiveness of glycogenolytic nervous stimuli is increased under the influence of the glycotropic substance, and that such stimuli normally play a part in raising the blood sugar to its normal level after insulin hypoglycaemia. Definite evidence on this point is lacking, however, but it may be mentioned that Cori & Cori [1927] have found that the fall of liver glycogen which normally follows the injection of a small dose of insulin into a fasting mouse is just as marked in adrenalectomized animals as it is in normal mice. This suggests the possibility that a stimulus other than that of adrenaline secreted from the adrenal glands is acting on the liver during insulin hypoglycaemia.

Rabbits treated with pituitary extracts containing the glycotropic substance have more liver and muscle glycogen at the end of a short period of fasting than do normal animals. One would expect that the presence of a large amount of glycogen in the liver would modify the extent of the fall of blood sugar level after insulin administration, but the result illustrated in Fig. 4 shows that raising the liver glycogen by this means to over 75 % above its normal fasting value had no very great influence on the hypoglycaemic response to the injection of 2 units of crystalline insulin. This suggests that the liver glycogen level cannot by itself be the controlling factor in the production of insensitivity to the hypoglycaemic action of insulin by treatment with our pituitary extracts. We must conclude, therefore, that until further results are available, the simplest assumption is that of a direct antagonism, both in the liver and peripherally, between the action of the glycotropic substance and that of insulin.

SUMMARY

1. The glycotropic (anti-insulin) principle of the pituitary gland is not identical with any of the following pituitary substances: prolactin (i.e. the pituitary substance capable of stimulating growth of the pigeon crop gland), thyrotropic hormone, gonadotropic hormone, oxytocic and vasopressor substances.

2. Preparations of the glycotropic substance containing no detectable prolactin, and preparations of prolactin containing no detectable glycotropic substance, have been obtained.

3. In rabbits which are rendered insensitive to the hypoglycaemic action of 2 units of insulin by treatment with the glycotropic substance, the hyperglycaemic action of subcutaneously injected adrenaline is substantially increased, but the effect of intravenously infused adrenaline is not significantly affected. In such rabbits sugar tolerance is slightly diminished.

4. The glycotropic substance apparently does not antagonize the hypoglycaemic action of insulin by facilitating the glycogenolytic action in the liver of adrenaline secreted by the adrenal glands. It may directly antagonize the action of insulin both in the liver and in the peripheral tissues.

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