LXIX. STUDIES ON THE FRACTIONATION OF DIABETOGENIC EXTRACTS OF THE ANTERIOR PITUITARY GLAND

BY FRANK GEORGE YOUNG¹

*From the Department of Biochemistry, University College, London, and the
National Institute for Medical Research, N.W. 3*

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IN a series of short communications the author has presented evidence to show that a factor in anterior pituitary extracts, often associated with prolactin, can abolish the hypoglycaemic action of insulin in intact rabbits [Young, 1936, 1, 2; 1937, 1, 2]. The name "glycotropic factor" [Young, 1936, 2] was suggested as a convenient provisional designation for this substance, not because it is believed that this factor is different from all the known pituitary hormones, but because, until the question of whether or not this substance antagonizes all the known actions of insulin is settled, the description "anti-insulin" or "contra-insulin" substance is unsatisfactory. Evidence has been brought forward to show that the glycotropic factor is not identical with the gonadotropic or thyrotropic hormones or with prolactin [Young, 1937, 1], although closely associated with preparations of the latter, but the question of whether or not it is identical with any of the other hormones of the anterior pituitary gland must at present remain open. More recently other investigators have found that the injection of preparations of prolactin may influence the blood sugar level of injected animals [Long, 1937, 2; Riddle, *et al.* 1937]. Indeed the latter authors suggest that prolactin has a diabetogenic action. On the other hand, Nelson *et al.* [1935] found that injections of prolactin did not induce the appearance of diabetic symptoms in monkeys or rabbits.

The author has stressed the fact that although two injections of prolactin can, under suitable circumstances, completely abolish any hypoglycaemic response to the subsequent intravenous administration of 2 units of insulin in the intact rabbit, the blood sugar level of the injected animal is not materially affected thereby, marked hyperglycaemia being absent [Young, 1936, 1, 2; 1937, 2]. The present investigation is concerned only with the diabetogenic action of pituitary extracts, and the question of the non-hyperglycaemic insulin resistance in prolactin-injected rabbits will be discussed in a subsequent communication. The fact that prolactin administration can induce insulin resistance in rabbits, together with the observation that prolactin injections maintain a high glycogen level in fasting rabbits [Young, 1937, 1] suggested the necessity of investigating thoroughly the possible diabetogenic action, in this species, of prolonged daily prolactin injections. However, in the preceding paper it has been shown that the rabbit is not a satisfactory animal for the demonstration of diabetogenic activity, although the dog is suitable in this respect. It was therefore of importance to determine whether or not prolactin is diabetogenic in the dog. The investigations described in the preceding paper showed that preparation

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and conservation of a crude extract at 0° are essential for the demonstration of diabetogenic activity, so that it was necessary to investigate the possible diabetogenic properties of prolactin prepared and stored at 0°. The corresponding "non-prolactin" fractions were also tested.

In the preceding paper experiments were described in which large doses of a crude extract of commercial dried gland failed to induce diabetes on administration to dogs. As these crude extracts were rich in thyrotropic activity it seemed improbable that the thyrotropic hormone *per se* could be diabetogenic. Nevertheless, it seemed advisable to exclude the thyrotropic hormone completely by testing a partially purified preparation of thyrotropic hormone, free from prolactin.

In connexion with another investigation, young rhesus monkeys were given daily injections of ordinary prolactin for long periods. Opportunity was taken to determine any effect of these injections on the blood sugar level.

Methods

The general methods are those of the preceding paper.

Preparation of extracts. The preparations of prolactin used in the present investigation all possessed glycotropic activity, and the term "prolactin" is used to designate a substance having the ability to cause growth of the pigeon crop-gland [Riddle & Braucher, 1931] and the power to abolish, in suitable conditions in the rabbit, the hypoglycaemic action of injected insulin [Young, 1936, 1, 2]. Although prolactin free from glycotropic activity was not used in the present investigation, an extract containing glycotropic activity but no demonstrable prolactin was tested.

(1) "*Ordinary prolactin*", i.e. *prolactin extracted from commercial (B.D.H. Ltd.) dried gland and fractionated at room temperature.* Ordinary prolactin was obtained from acetone-dried ox anterior lobe by a modification of the aqueous alcohol extraction method of Bates & Riddle [1935]. The modification consisted in the substitution of the fractionation with 70% alcohol in the presence of NaCl by a large number of precipitations from water at pH 5.5. After 6–12 such reprecipitations the prolactin fraction was found to be free from Na_2SO_4 , and to contain no detectable gonadotropic or thyrotropic activity as determined by the rabbit ovulation and guinea-pig thyroid hyperplasia tests, respectively. This prolactin fraction will be described as "ordinary prolactin". The fraction soluble at pH 3–4 in the presence of H_2SO_4 and Na_2SO_4 , which contained the thyrotropic and gonadotropic hormones, was dialysed (Visking cellophane) against distilled water at 2° until free from SO_4^{--} . The precipitate, which formed during dialysis, was spun off, and the soluble material precipitated with alcohol and dried. This fraction constituted "ordinary non-prolactin". A number of preparations of prolactin were obtained from dried gland by the simple pH 5.5 precipitation method. The experimental results with material prepared in this manner were similar to those with "ordinary prolactin", although it was not possible to free the prolactin fraction from thyrotropic and gonadotropic activities by this procedure.

(2) "*Prolactin-C*", i.e. *prolactin extracted at 0° from fresh tissue and fractionated at low temperature.* The fresh ox glands were collected and dissected as described in the previous paper. The minced, frozen tissue was ground with sand and 3 ml./g. of cold saline in the cold store; $N/5$ NaOH was stirred in until the pH was about 8.5 (faint green with thymol blue) and the mixture left overnight in the cold store. All subsequent procedures were carried out at a temperature as

close as possible to 0°. The next morning the pH was again adjusted to 8.5 if necessary and the mixture centrifuged in chilled pots for 10 min. at 3000 r.p.m. The thick extract was decanted and the residue discarded. The pH of the extract was adjusted to 5.5 (faint pink with chlorophenol red) with *N*/5 HCl and the mixture again centrifuged. The clear, supernatant fluid was then pipetted off and the precipitate stirred at pH 8.5 with a volume of cold saline equal to that originally used for extraction. After 30 min. stirring, the pH was adjusted to 5.5 and the mixture again centrifuged. Four precipitations of the material insoluble at pH 5.5 (prolactin) were effected in this way. The final prolactin precipitate was stirred with cold saline at pH 8.0, the total volume being equal to that of the saline used originally for extraction. This constituted the "prolactin-C" preparation. A clear solution at pH 8 was not obtained owing to the presence of much colloidal matter. The clear supernatant liquid, from the first precipitation of the prolactin at pH 5.5, was adjusted to a volume equal to the final volume of the prolactin fraction by addition of a part of the supernatant liquid from the second prolactin precipitation, and the pH adjusted to 8.0. This constituted the "non-prolactin-C" fraction. Both the prolactin and the non-prolactin fractions were stored at 0° and used within 4 days of preparation.

The amounts of "prolactin-C" and "non-prolactin C" are expressed in terms of the number of g. of fresh gland used to provide the given amount of extract. As with the crude saline extract of fresh gland, an extract of *X* g. of fresh tissue (abbreviated for convenience to *X* g. equivalent of extract) does not contain all the extractable material in *X* g. of gland owing to the undetermined loss in the discarded residues.

For the purposes of rough comparison it may be stated that 1 mg. of ordinary prolactin is equivalent to about 60 mg. of fresh gland.

Biological tests. Prolactin was assayed by the pigeon crop-gland method as described by Rowlands [1937]. Thyrotropic hormone was estimated by the method of Rowlands & Parkes [1934]. Gonadotropic activity of ox pituitary extracts was assayed by ability to cause ovulation in the oestrous rabbit. Glycotropic activity was determined on rabbits by a modification of the method previously described [Young, 1936, 1].

RESULTS

(I) *General biological assays*

The results of assays for prolactin (Table I), thyrotropic hormone (Table II) and gonadotropic hormone (Table III) on typical preparations used in the present investigation, are given in the following tables.

Table I. *Prolactin assays by the method of Rowlands [1937]*

Substance	Amount injected daily	Increase in crop- gland per 300 g. pigeon—av. of 10 birds g.
"Ordinary prolactin"	1 mg.	2.7
"Ordinary non-prolactin"	20 mg.	0
"Prolactin-C"	0.033 g. equivalent of fresh tissue	1.4
"Non-prolactin-C"	0.50 g. equivalent of fresh tissue	0

Table II. *Thyrotropic hormone assays by the method of Rowlands & Parkes [1934]*

Substance	Amount injected daily	Increase in thyroid weight—av. of 10 cavies mg.
"Ordinary prolactin"	20 mg.	- 3.5
"Ordinary non-prolactin"	1 mg.	+ 32.5
"Prolactin-C"	0.17 g. equivalent of fresh tissue	+ 5.0
"Non-prolactin-C"	0.17 g. equivalent of fresh tissue	+ 43.0

Table III. *Gonadotropic hormone assays*

Substance	Approximate dose for ovulation in 50% of oestrous rabbits
"Ordinary prolactin"	> 100 mg.
"Ordinary non-prolactin"	0.6 mg.
"Prolactin-C"	7 g. equivalent
"Non-prolactin-C"	0.17 g. equivalent

Glycotropic activity was determined as follows. A rabbit was deprived of food at 5 o'clock in the evening and given an injection of the substance under investigation, a similar injection being given at 9.30 a.m. on the following morning. At 2 p.m. 2 units of crystalline insulin were given intravenously and the blood sugar level determined at frequent intervals during the following 4 hr. A typical result is given in Fig. 1. In this instance two injections, each of

Fig. 1. Effect of two injections of prolactin on the blood sugar response to 2 units of crystalline insulin administered intravenously to the rabbit. Curve (a). Response before prolactin injections. Curve (b). Response after prolactin injections. The arrow indicates the time of insulin injection. The second prolactin administration had been made $4\frac{1}{2}$ hr. before the insulin injection.

125 mg. of ordinary prolactin, completely abolished the hypoglycaemic effect of 2 units of intravenously administered crystalline insulin, although the initial blood sugar level was not affected. The approximate minimum amounts of different substances required, in similar tests, to abolish completely, or almost completely, the hypoglycaemic action of 2 units of crystalline insulin, are given in Table IV.

Table IV. *Glycotropic factor assays*

Substance	Minimum dose required for each of two injections to abolish insulin hypoglycaemia in the above test
"Ordinary prolactin"	80-150 mg.
"Ordinary non-prolactin"*	100-180 mg.
"Prolactin-C"	About 2 g. equivalent of fresh tissue
"Non-prolactin-C"*	About 3 g. equivalent of fresh tissue

* If the anterior lobe tissue extracted contained any posterior lobe principles, these would be present in this fraction. If present in sufficient amount they would render impossible a test of the glycotropic activity of this fraction by the above method.

The presence of glycotropic activity in the "ordinary prolactin", which contains no detectable gonadotropic and thyrotropic hormones, and in the "non-prolactin fraction" which contains no detectable prolactin, shows that the glycotropic factor cannot be identical with the gonadotropic or thyrotropic substances, or with prolactin.

(II) *The possible diabetogenic action of prolactin in the rabbit*

Rabbits frequently excrete sugar when injected daily for a long period with large doses of ordinary prolactin. Fig. 2 shows the result of a typical experiment

Fig. 2. The daily sugar excretion of rabbits receiving daily injections of 100 mg. of "ordinary prolactin".

in which 6 rabbits (3 ♂ and 3 ♀) were each injected daily with 100 mg. of ordinary prolactin. Although as much as 6.2 g. of sugar were excreted during 24 hr. in one instance, ketonuria and polyuria were absent at all times. Two rabbits did not excrete sugar at any time during an experiment lasting 117 days. Three pregnant rabbits also excreted sugar during the course of similar daily prolactin injections (Fig. 3). It will be seen that there is no obvious relationship between the time of littering and the time of onset of glycosuria. In all these experiments milk appeared in the mammary glands of the adult female rabbits within 1 week of the initiation of daily prolactin administrations.

When "prolactin-C" was administered daily to rabbits for a long time glycosuria occurred once in seven experiments, with a maximum value of 1.9 g./day. This result differs from that for ordinary prolactin, and resembles that for control experiments (preceding paper).

Although the administration of a suitable preparation of prolactin to a rabbit may result in the abolition of the hypoglycaemic action of injected insulin without causing a hyperglycaemia during the course of the experiment, if ordinary prolactin is used in an experiment of this kind, glycosuria may appear

Fig. 3. The daily sugar excretion of pregnant rabbits receiving daily injections of 100 mg. of "ordinary prolactin". The arrows indicate the day of littering, the figures on the arrows giving the number of healthy young in the litters.

2 days or more after the last injection of prolactin. It has never been observed when "prolactin-C" is used. A similar sugar excretion was observed in control experiments in which an extract of rat liver was injected instead of prolactin.

Experiments in which rabbits were given daily injections of "ordinary non-prolactin" and "non-prolactin-C" gave results similar to those for control experiments (preceding paper).

(III) *Experiments with dogs*

Of four dogs given daily intraperitoneal injections of 400–1000 mg. of ordinary prolactin, none became diabetic. One animal excreted 6.8 g. of sugar on the 4th day of injection, but no other instance of glycosuria was observed in these experiments. Ketonuria and marked polyuria were absent, although a definite increase in urine excretion (to about 800 ml. daily) occurred in some instances. No case of lactation was observed in these male dogs, and in general the injection of large doses of prolactin had little observable effect on them.

Two dogs which received daily intraperitoneal injections of "ordinary non-prolactin", the daily dose rising from 100 to 300 mg., did not exhibit glycosuria at any time, the only observable effect of the injections being a slight ketonuria and polyuria (1000–1400 ml./day). Loss of body weight was not observed. Both animals subsequently became diabetic on receiving daily doses of crude anterior lobe extract containing less than 1/7th of the amount of thyrotropic hormone which had previously been ineffective.

Neither of two dogs injected with "prolactin-C" exhibited glycosuria although the dose injected daily was increased to the equivalent of 25 g. of fresh gland. Fig. 4 illustrates one of these experiments. The figures on the arrows indicate the weight of fresh gland extracted to provide the daily dose of "prolactin-C". At the arrow marked X the daily injection of "prolactin-C" equivalent to 25 g. of fresh gland was supplemented by the addition of "non-prolactin-C", also equivalent to 25 g. of fresh gland. Glycosuria appeared on the 4th day of injection of this mixture and rose to 65.4 g./day on the 7th day. A substantial ketonuria was also exhibited. The glycosuria and ketonuria disappeared on the 9th day of injection. This experiment suggested that "non-prolactin-C"

alone might be effective, and this was found to be so. Fig. 5 illustrates one of the two similar experiments carried out in this connexion. In this instance the dog used was a litter-mate of that providing the results for Fig. 3. The effective dose of "non-prolactin-C" is greater than might be expected from the amount of fresh gland used in its preparation, but this is probably due to the unavoidable

Fig. 4. Bitch receiving daily injections of "prolactin-C". The figures on the arrows give the weight, in g., of fresh ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. On the day indicated by the arrow carrying X the daily injection of "prolactin-C" equivalent to 25 g. of fresh gland was supplemented by the addition of "non-prolactin-C", also equivalent to 25 g. of fresh gland. The animal used in this experiment was the litter-mate of that used in the experiment illustrated in Fig. 5.

Fig. 5. Bitch receiving daily injections of "non-prolactin-C". The figures on the arrows give the weight, in g., of fresh ox anterior lobe used to prepare the amount of extract injected daily, from the day indicated by the arrow, onwards. Injections ceased on the day indicated by the arrow carrying 0. The animal used in this experiment was the litter-mate of that used in the experiment illustrated in Fig. 4.

losses which occur in the preparation of this fraction. The last injection of "non-prolactin-C" (Fig. 5) was given on the 35th day of the experiment, at which time the sugar excretion was 113 g./day. The urine became sugar-free on the following day and ketonuria disappeared 2 days later.

(IV) *Experiments with monkeys*

Four young female monkeys were injected daily with 60–200 mg. of ordinary prolactin, for periods ranging up to 9 months. No significant variation was found in blood sugar determinations made at weekly intervals, and at no time were any symptoms of diabetes mellitus observed in these injected animals.

DISCUSSION

The experiments with dogs indicate that prolactin *per se* is not diabetogenic in the intact animal, and further that diabetogenic preparations may be obtained which contain no detectable prolactin, as assayed by the pigeon crop-gland method. This is perhaps surprising in view of the marked glycotropic activity of the prolactin preparations used. Moreover, prolactin, prepared at low temperature and under conditions which permit the preparation of potent diabetogenic fractions, failed to produce a diabetic condition when given in large doses to dogs.

With rabbits, prolonged administration of prolactin induced, in many instances, a delayed glycosuria, ordinary prolactin being more effective than prolactin-C in this respect. This glycosuria was not accompanied by ketonuria or polyuria, and a condition of diabetes could not be accurately diagnosed. The rabbit apparently differs from the dog in being more prone to exhibit a transient glycosuria in response to injections of prolactin, a difference similar to that found with non-specific, control injections (preceding paper). In a glycotropic test (see above) the blood sugar level of a rabbit receiving injections of prolactin is not markedly affected, although the hypoglycaemic response to injected insulin may be completely abolished. Under the conditions of this test the liver glycogen content of the experimental rabbits is much greater than that of control animals [Young, 1937, 2], and the abolition of the hypoglycaemic action of insulin is probably associated with a rapid and effective response of the hepatic glycogenolytic mechanism to a suitable stimulus. It is found that rabbits which have become insulin-resistant following administration of glycotropic factor respond to a very slight emotional stimulus by a substantial rise of blood sugar. It is therefore not surprising that instances of hyperglycaemia and glycosuria are observed in rabbits after a number of daily injections of prolactin, although it must be emphasized that, in the absence of ketonuria, a diabetogenic action of prolactin is not thus demonstrated. The need for greater precision in the use of the word "diabetogenic" has already been stressed in the preceding paper. What was there said of crude pituitary extracts applies equally to partially purified preparations, viz. that the use of the description "diabetogenic" should be confined to extracts the daily injection of which induces, after a latent period of some days, a state of hyperglycaemia, glycosuria, ketonuria, polyuria and polydipsia. The fact that daily prolactin injections induce, in some rabbits, nothing more than a transitory hyperglycaemia and glycosuria is not, *per se*, evidence of a diabetogenic action of the substance. The negative results with dogs receiving prolactin injections, and the positive finding with prolactin-free preparations, show that prolactin is not the diabetogenic substance of the anterior lobe.

Riddle *et al.* [1937] have recently found that, in pigeons, daily injections of prolactin for 1–8 days increase the blood sugar level by 7–33% and conclude "thus in pigeons the diabetogenic response is found to be a response to prolactin". Again it must be emphasized that a slightly raised blood sugar level

does not constitute a diabetic condition, and that in prolactin-injected animals a slight emotional stimulus, such as that associated with the taking of blood samples, is very prone to provoke hyperglycaemia.

As prolactin possessing marked glycotropic activity is not truly diabetogenic in the normal rabbit or in the normal dog, it is clear that the diabetogenic factor and the glycotropic substance are not identical. Nevertheless, all the diabetogenic preparations used in the present investigation possessed marked glycotropic activity, and it seems probable that the diabetogenic factor comprehends the glycotropic substance together with another factor or factors.

The foregoing discussion applies only to intact animals. Recently, Long [1937, 1, 2] has found that the sugar excretion, but not the ketonuria, of a hypophysectomized-depancreatized cat is increased by the administration of prolactin-adrenotropic preparations of the pituitary gland. This author [1937, 1] has made the tentative suggestion that the glycosuric substance of the anterior pituitary gland is either prolactin or the adrenotropic hormone. Long [1937, 2] suggests that the diabetogenic factor may be composed of two constituents: one, which is heat-stable, being able alone to precipitate glycosuria but not ketonuria in the hypophysectomized-depancreatized animal, while the presence of the other, which is heat-labile, as well as that of the first, is necessary to produce glycosuria and ketonuria in the intact animal. The relevant results of the present investigation are in entire agreement with Long's view. It seems probable that Long's heat-stable factor which is unable to induce glycosuria and ketonuria in normal animals, is our glycotropic factor. If this is so, then Long's suggestion may be extended to include the supposition that the heat-stable constituent of the diabetogenic complex is not prolactin but a factor which can produce a condition of insulin resistance in the intact animal without any marked effect on the blood sugar level (glycotropic factor).

In the experiments with "ordinary non-prolactin" the amount of thyrotropically active substance injected daily substantially exceeded that given in experiments with "non-prolactin-C". As "ordinary non-prolactin" lacks diabetogenic activity, the observed diabetogenic activity of the "non-prolactin-C" fraction could not have been due merely to its thyrotropic activity.¹ It seems improbable that a combination of the glycotropic factor and the thyrotropic hormone alone is diabetogenic, as this combination was present in the "ordinary non-prolactin" fraction as well as in the extract of commercial, acetone-dried gland, which, in the preceding paper, was found to be ineffective. These considerations do not rule out the possibility that the thyrotropic hormone is an essential constituent of the diabetogenic complex [cf. Barnes & Regan, 1933], but do suggest that the presence of at least one other substance is necessary for the exhibition of diabetogenic activity. The possibility that another factor is the ketogenic substance of the anterior lobe [Burn & Ling, 1930] is an attractive one, and is at present under investigation.

SUMMARY

1. The daily injection of an alkaline extract of fresh ox anterior pituitary gland, prepared at low temperature, from which the prolactin has been removed, induces the symptoms of diabetes in dogs.

¹ The thyrotropic activity considered here is that which induces hyperplasia of the thyroid gland [cf. Rowlands & Parkes, 1934]. The assumption is usually made that the factor inducing this response is identical with that causing increased secretion of the thyroid gland, but the possibility that more than one factor is concerned cannot be ruled out.

2. The daily injection of large doses of prolactin does not produce diabetes in dogs, even when the prolactin is prepared from fresh glands at a low temperature, under conditions which permit the preparation of active diabetogenic preparations.

3. The conclusion is drawn that neither prolactin nor the glycotropic ("anti-insulin") factor closely associated with, but distinct from it, is diabetogenic in intact dogs.

4. The administration of prolactin to rabbits or monkeys does not induce a diabetic condition, although rabbits may exhibit a transitory glycosuria. This transitory glycosuria cannot correctly be described as a diabetic condition since it is not accompanied by ketonuria or polyuria.

5. The daily administration of large amounts of partially purified thyrotropic hormone to dogs failed to induce a diabetic condition.

6. The diabetogenic factor of the anterior pituitary gland is probably complex. The glycotropic and thyrotropic factors are possibly constituents of this complex, but prolactin is not.

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