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GUINEA-PIG ANTI-INSULIN SERUM

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Hyperglycaemia, probably due to acute insulin deficiency, has been provoked in the mouse (Moloney & Coval, 1955; Moloney & Goldsmith, 1957), the rat, rabbit and cat (Armin, Grant & Wright, 1960*a*, *b*) by intravenous or intraperitoneal injection of serum from guinea-pigs which have received prolonged treatment with insulin. The guinea-pig, therefore, provided a useful source of serum for the production of acute insulin deficiency in experimental animals. We now show that under certain conditions guinea-pigs yield potent anti-insulin serum which can be conveniently assayed *in vivo* in rats and which will agglutinate insulin-conjugated sheep red cells. Its insulin-neutralizing properties are probably due to antibodies which combine directly with the hormone but are not species-specific.

METHODS

Insulin. Bovine insulin (22·2 u./mg) recrystallized six times, sheep and pig insulins (20 u./mg) recrystallized once were provided by Boots Pure Drug Co. Ltd. Bovine insulin precipitated at pH 7·4 (20 u./mg) was prepared by the Wellcome Research Laboratories.

Insulin antigen. Bovine insulin (10 mg) was dissolved in a solution (10 ml.) of phenol (0·3 g/100 ml.) in water acidified with HCl to pH 2·6 approx. This solution was then emulsified in a Waring blender with an equal volume of either (*a*) liquid paraffin B.P. 7 ml. and anhydrous lanolin (Adeps lanae, B.P.) 3 ml. or (*b*) Arlacel A (Mannide mono-oleate) 1·5 ml., Bayol F (paraffin oil) 8·5 ml. and 5 mg *Mycobacterium butyricum* (killed and dried); available commercially as Bacto-Adjuvant, Complete (Freund), Difco Laboratories, Detroit. For control purposes emulsions containing no added insulin were used.

Treatment of guinea-pigs. Groups of six to twelve albino guinea-pigs were injected subcutaneously every 4 weeks with 2 ml. of freshly prepared antigen emulsion, half between the shoulders and the remainder into the flanks or inner aspects of the thighs. About 10 ml. blood was obtained by cardiac puncture under light ether anaesthesia 12–14 days after antigen injections. Sera separated from the blood drawn from animals of the same group at any one time were combined and stored at −10° C. Sera obtained from different groups of animals were usually kept separate.

Guinea-pigs weighed 300–400 g when first injected with antigen. Those in groups *E*, *G*, *K*, *L*, *M*, *P*, *R* and *S* received recrystallized bovine insulin; those in group *O* were injected with bovine insulin precipitated at pH 7·4; control animals in group *H* were injected with emulsions containing no insulin. All animals received emulsion (*a*) except in December 1959, when all but the animals in groups *L* and *M* received emulsion (*b*).

Serum injection into rats. Conscious albino Wistar rats (160–220 g) of either sex were given guinea-pig serum (0·25–1·00 ml.) by way of the tail vein by the method already described (Armin *et al.* 1960*a*). Both before and after injection they had free access to food and water. Blood samples (0·2 ml.) were obtained from the cut end of the tail after collection in watch glasses containing dried anti-coagulant (1 drop 3% potassium oxalate and 1 drop 0·3% potassium fluoride) and their sugar contents determined by a modification (Wright, 1957) of the method described by King (1951).

Assay of guinea-pig anti-insulin serum

The potencies of sera were deduced from their effects *in vivo* in rats and *in vitro* upon insulin-conjugated sheep red cells.

Assay in vivo. Three methods were adopted in which serum with or without added insulin was injected intravenously into conscious fed rats:

(*a*) Groups of four rats were injected with varying doses (0·1–1·0 ml.) of anti-insulin serum (Batch 34943) and blood-sugar estimations were carried out at 15 min intervals thereafter. The potency of the serum was determined by the method previously described (Armin *et al.* 1960*a*).

(*b*) Groups of eight animals were injected with a constant volume (0·5 ml.) of serum (Batch 34943) to which varying amounts of recrystallized bovine insulin (0–1 u.) in saline (0·25 ml.) had been added 3–12 min previously. The blood-sugar concentrations were determined at 15 min intervals for 1 hr or until the levels had returned to normal. The mean areas under the response curves and the mean changes in blood-sugar concentration produced by each mixture of serum and insulin were then calculated.

(*c*) Groups of two to four animals received a constant dose (0·5 ml.) of serum, and the blood-sugar concentrations determined at selected intervals up to 2 hr later.

It was shown previously (Armin *et al.* 1960*a*) that, irrespective of the volume of anti-insulin serum injected, the blood-sugar concentrations of conscious fed rats increase at a constant rate (mean = 3·16 ± 0·11 mg/100 ml./min) and then fall at a uniform maximum rate (mean = 5·36 ± 0·24 mg/100 ml./min). It can be calculated, therefore, that the area (A) under such a curve is given by the equation

$$A = 0{\cdot}99\,(0{\cdot}187\Delta BS + t)^2 \text{ mg/100 ml.} \times \text{min},$$

where ΔBS, measured as the blood-sugar concentration is falling, is the difference between the level (mg/100 ml.) measured t min after, and the concentration before the serum injection. Knowing the effects produced by a standardized sample of serum (Batch Y; 1·3 u./ml.; Armin *et al.* 1960*a*) it is possible to determine the approximate potency of a serum by using this equation and values of ΔBS obtained at suitably selected time intervals after its injection:

0 = inactive; no increase (< 20 mg/100 ml.) in blood-sugar concentration in 15 or more minutes;

1 = < 0·9 u./ml.; a definite increase (> 20 mg/100 ml.) in 15 or 30 min but none (< 20 mg/100 ml.) at 60 min;

2 = 0·9–1·8 u./ml.; an increase of 20–160 mg/100 ml. at 60 min but none (< 20 mg/100 ml.) at 120 min;

3 = 1·8–3·3 u./ml.; a greater increase (> 160 mg/100 ml.) at 60 min but none (< 20 mg/100 ml.) at 120 min;

4 = > 3·3 u./ml.; a large increase (> 160 mg/100 ml.) at 60 min and a definite rise (> 20 mg/100 ml.) at 120 min.

Assay in vitro. Sheep red cells were conjugated with recrystallized bovine insulin (unless otherwise stated) by the method of Arquilla & Stavitsky (1956*a*) using bis-diazotized benzidine. Guinea-pig sera were heated at 56° C for 30 min to destroy complement. Normal

rabbit serum similarly treated to destroy complement and adsorbed with an equal volume of washed sheeps' red cells was diluted 1/100 in isotonic phosphate buffer (pH 7·4). This diluted rabbit serum (NRS) was used to dilute the guinea-pig serum and to suspend the red cells.

To serially diluted guinea-pig serum (0·5 ml.; 1/20–1/5120) was added a 2% suspension of insulin-conjugated red cells (0·05 ml.), the mixture being agitated to disperse the cells and then allowed to stand at 4° C during the night. The highest guinea-pig serum dilution was then noted at which the red cells adhered as an even carpet over the bottom of the tube (Stavitsky, 1954); this is termed the *agglutinating titre* of the serum. For *control* purposes in each experiment (1) unconjugated sheep red cells were exposed to diluted (1/20) guinea-pig serum and (2) bovine insulin (0·05 mg in 0·05 ml. phosphate buffer) was added to diluted guinea-pig serum (0·5 ml.; 1/20) before exposure to the conjugated red cells.

RESULTS

Assay in vivo

Table 1 shows that the hyperglycaemic effect of 0·5 ml. anti-insulin serum (Batch 34943) is not abolished by the addition of 0·75 u. insulin, whereas all those animals injected with the mixture containing 1·0 u. insulin became hypoglycaemic within 15 min. This implies that 1·0 ml. of the serum neutralizes between 1·5 and 2·0 u. added insulin. By comparing the effects of this serum with that of a sample (Batch Y) whose potency had been established in mice, it was found by method (*a*) that 1 ml. serum

TABLE 1. Mean changes in blood-sugar concentration and mean areas under response curves produced by intravenous injection into groups of eight conscious fed rats (171–200 g) of guinea-pig anti-insulin serum (Batch 34943; 0·5 ml.) mixed 3–12 min previously with recrystallized bovine insulin (0–1·0 u.) in 0·9% saline (0·25 ml.)

	Time after injection (min)	Insulin added (u.)				
		0	0·25	0·50	0·75	1·00
Mean change in blood sugar concn. (mg/100 ml.)	15	—	+65	+60	+40	−21
	30	+93	+127	+91	+44	−25
	45	+139	+152	+67	+21	−30
	60	+168	+128	+33	+18	—
	75	+135	+71	+14	—	—
	90	+46	+27	—	—	—
	105	+9	+16	—	—	—
Mean area under response curve ± S.E. (mg/100 ml. × min × 10^{-4})		0·95 ±0·044	0·86 ±0·086	0·40 ±0·070	0·15 ±0·020	—

would neutralize 1·8 u. insulin. Also, since 0·5 ml. of the serum elevates the blood-sugar concentration by more than 160 mg/100 ml. in 1 hr (Table 1), but fails to maintain hyperglycaemia for 2 hr, 1 ml. as judged by method (*c*) neutralizes between 1·8 and 3·3 u. insulin. Thus essentially the same result is obtained whichever of these three methods of assay is used.

Assay in vitro

Table 2 shows the effects of 44 samples of guinea-pig serum upon insulin-conjugated red cells compared with the results of assays *in vivo* (method *c*). Five samples of serum from control animals (Group *H*) and one of normal guinea-pig serum failed to agglutinate or to provoke hyperglycaemia. Two sera from animals of groups *G* and *K*, following two injections of antigen, either failed to agglutinate or to produce detectable hyperglycaemia. All the remaining sera produced hyperglycaemia and agglutination at dilutions of 1/80 or more; all were obtained from insulin-treated guinea-pigs.

TABLE 2. A comparison of the titres at which 44 specimens of guinea-pig serum agglutinate sheep red cells conjugated with bovine insulin and their hyperglycaemic effects in conscious fed rats. For details of experimental methods and units of potency see text

Assay *in vivo*		Assay *in vitro*								
		Red cell agglutination titre								
Degree of activity	No. of serum samples	Nil	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
0	7	6	1	—	—	—	—	—	—	—
1	6	1	—	—	—	4	1	—	—	—
2	23	—	—	—	1	2	6	9	5	—
3	5	—	—	—	—	—	1	2	2	—
4	3	—	—	—	—	—	—	1	1	1
Totals	44	7	1	0	1	6	8	12	8	1

Species-specificity of anti-insulin serum

The addition of bovine, pig or sheep insulin to anti-insulin serum before intravenous injection significantly reduces its hyperglycaemic effect in rats (Table 3). In addition, the agglutination of sheep red cells conjugated with bovine insulin is prevented when bovine, pig or sheep insulin is first mixed with the serum (Table 4). Red cells conjugated with bovine, pig or sheep insulin are also agglutinated at comparable dilutions by anti-insulin serum (Table 4).

Effect of insulin treatment upon guinea-pigs

Over a period of 20 months, eighty-four guinea pigs were given injections of antigen for periods ranging from 8 to 19 months (Table 5); six control animals were also used (Group *H*). During this time fifty-one animals died, eighteen between the time of antigen injections and the subsequent bleeding and thirty-three after cardiac puncture. Most (22) of the deaths which followed cardiac puncture occurred within 24 hr and were due to haemorrhage into the pericardial or pleural cavities, and of those animals

TABLE 3. Mean areas under response curves produced in conscious fed rats by the intravenous injection of guinea-pig anti-insulin serum (Batch 34943; 0·5 ml.) mixed 3–12 min previously with bovine, pig and sheep insulin (0·5 u.)

Insulin added to serum	No. of rats injected	Area under response curve (mg/100 ml. × min × 10^{-4}) mean	range
Nil	8	0·95	0·76–1·15
Bovine	8	0·40	0·21–0·62
Pig	4	0·30	0·24–0·39
Sheep	4	0·34	0·21–0·43

TABLE 4. The effects of bovine, pig, and sheep insulin upon agglutination of insulin-conjugated sheep red cells; for details see text

Insulin conjugated with red cells	Insulin added to serum before exposure to red cells	Anti-insulin serum used (batch no.)	Red cell agglutination titre
Bovine	Nil	34943	
Sheep	Nil	16204	1/160–1/320
Pig	Nil	23	
Bovine	Bovine		Nil
Bovine	Sheep	34943	Nil
Bovine	Pig		Nil

TABLE 5. The responses of 9 groups of guinea-pigs treated between November 1958 and June 1960 with emulsions containing insulin. Animals in Group *H* received emulsions containing no added insulin. Animals in group *O* received bovine insulin precipitated at pH 7·4. All other groups received recrystallized bovine insulin. In December 1959 all animals except those in groups *L* and *M* were injected with emulsions containing *Mycobacterium butyricum*. The definition of serum potency (0–4) is given in the text

Group ...	*E*	*G*	*K*	*L*	*M*	*O*	*P*	*R*	*S*	*H*
First injection ...	May 1957–Sept. 1959	Dec. 1958	Jan. 1959	Mar. 1959	Apr. 1959	Aug. 1959	Oct. 1959	Nov. 1959	Nov. 1959	Oct. 1959
No. of animals used ...	11	8	8	8	6	9	10	12	12	6
1958 Nov.	2	—	—	—	—	—	—	—	—	—
Dec.	2	—	—	—	—	—	—	—	—	—
1959 Jan.	2	—	—	—	—	—	—	—	—	—
Feb.	2	1	—	—	—	—	—	—	—	—
Mar.	—	1	1	—	—	—	—	—	—	—
Apr.	4	2		—	—	—	—	—	—	—
May	—	2		—	—	—	—	—	—	—
June	—	2		2	2	—	—	—	—	—
July	4	2		2	2	—	—	—	—	—
Aug.	—	2		—	—	—	—	—	—	—
Sept.	3	2		2	2	—	—	—	—	—
Oct.	2	2		2	2	1	—	—	—	—
Nov.	4	2		2	2	1	—	—	—	—
Dec.	4	1		2		—	—	—	—	0
1960 Jan.	3	2		3		2	—	—	—	—
Feb.	—	—		3		—	—	—	—	0
Mar.	2	1		3		2	2	2	3	0
Apr.	2	2		3		2	2	4	3	0
May	—	—		2		2	2	4	4	0
June	—	—		3		2	2	4	4	0

which died later many showed evidence of similar haemorrhages. Of those which died after injections of antigen, few (2) died within 24 hr and none showed evidence of hypoglycaemia before death. It should be pointed out, however, that many of the animals which did not die from haemorrhage showed only non-specific lesions at necropsy, macroscopic evidence of infection in the lungs being found most commonly.

Table 5 shows the potencies of sera obtained at different times from these guinea-pigs. During 8 months of treatment, the control animals (Group *H*) injected with emulsions containing no added insulin never yielded serum producing hyperglycaemia in rats. After two or three injections of insulin antigen all the other groups of animals, however, produced serum with detectable activity. Thereafter each group produced serum of consistent potency, irrespective of the duration of the treatment with insulin. Thus groups *E*, *L*, *M*, *R* and *S* produced active serum (2–4) whereas groups *G*, *K*, *O* and *P* gave consistently weaker samples (1–2). The injection of emulsions containing *Mycobacterium butyricum* in December 1959 did not cause any consistent changes in the potencies of sera obtained later and no apparent advantage was gained by using insulin precipitated at pH 7·4 as the antigen (Group *O*).

DISCUSSION

The guinea-pig is the only animal which is known to produce, after treatment with insulin, serum which will provoke hyperglycaemia in other experimental animals. It seemed important, therefore, to learn the optimum conditions for the production of highly active guinea-pig anti-insulin serum. The procedure adopted was similar to that described by Moloney & Coval (1955), but our experience differs from theirs in one important respect. Moloney & Goldsmith (1957) reported that of the animals which received a comparable dose (25 u.) of insulin antigen, 58% died in hypoglycaemic convulsions after the first injection and 11% of the survivors died after the second; administration of glucose did not prevent these deaths. No deaths due to hypoglycaemia were observed in our animals at any stage in their treatment with insulin. This is possibly due to the fact that we used lanolin as an emulsifying agent, whereas Moloney & Coval (1955) used mannide mono-oleate. The majority of our deaths were directly due to haemorrhage following cardiac puncture and some were probably caused by intercurrent infections. In other respects, however, our experiences are similar to those of Moloney & Goldsmith (1957), from whose results it was concluded that serum with an insulin-neutralizing potency of about 0·8 u./ml. might be expected from most animals injected at monthly intervals with 20–50 u. insulin and bled 10–20 days later. All our animals injected with recrystallized insulin or insulin precipitated at

pH 7·4 yielded weakly active serum within 2–3 months, but later some groups produced highly active serum (> 1·8 u./ml.) whilst others yielded consistently weak specimens (< 1·8 u./ml.). The variable and unpredictable responses of these groups of guinea-pigs cannot be explained at present. No advantage was gained by incorporating *Mycobacterium butyricum* in the antigen emulsion; anti-insulin activity did not rise and chronic ulcers developed at injection sites. It was found, however, that those groups of animals which had not yielded potent serum within 5 months seldom did so later and that, with the procedure outlined above, the best yield of active serum could be assured if unresponsive animals were discarded after this time and attention concentrated on those yielding active serum. If the fatal haemorrhages produced by cardiac puncture could be avoided such animals should continue to yield active serum for long periods. However, the guinea-pig is a small animal and cannot be expected to give more than about 10 ml. blood at any one time. Another animal should therefore be sought which produces similar anti-insulin serum in larger quantities. Possibly a near relative of the guinea-pig, such as the capybara or the agouti, might prove satisfactory.

By directly neutralizing the active component of guinea-pig serum with added insulin (method *b*) it was found that the assay method previously described (method *a*; Armin *et al.* 1960*a*) gives a correct though only approximate estimate of insulin-neutralizing potency. This latter method was used to assay pooled samples of serum but it is tedious. The simpler method (*c*) described above, which requires fewer blood-sugar estimations, proved useful in determining the approximate potencies of a large number of sera samples obtained at various times from the treated guinea-pigs. The effects of these sera *in vitro* upon insulin-conjugated red cells are comparable with their hyperglycaemic actions *in vivo*; no false positive results were obtained in either system and, in general, those which proved most active *in vivo* also agglutinate at the highest dilutions. If, as is now supposed (Moloney & Coval, 1955; Wright, 1959, 1960), the active component of this serum is an insulin antibody, it seems likely that the one which neutralizes and inactivates endogenously secreted insulin in the rat must also be responsible for the agglutination of insulin-conjugated red cells.

Much evidence supports the hypothesis that insulin antibodies in guinea pig anti-insulin serum are not species-specific. Guinea-pig anti-insulin serum inhibits the effects of bovine, pig, sheep and human insulin upon glucose consumption by the isolated rat diaphragm (Wright, 1959). It also agglutinates sheep red cells conjugated with bovine, pig and sheep insulin and its action upon cells conjugated with bovine insulin is inhibited by these three insulin preparations. These observations strongly suggest

that the antibodies in guinea-pig anti-insulin serum are not species-specific and that they combine directly to inactivate the hormone. The reduction in hyperglycaemic activity produced by the addition of bovine, sheep and pig insulin could also be interpreted in the same way. However, the added insulin could exert its own biological action independently, or it could combine with injected antibody and so reduce the amount of antibody available to neutralize endogenous insulin. In either case the hyperglycaemic effect of the injected serum would be reduced and it should not necessarily be concluded therefore that any direct reaction has occurred between the injected insulin and antibody. Such a conclusion is only warranted if (1) injection of the serum alone does not produce hyperglycaemia or (2) the material injected with serum which alone provokes hyperglycaemia has no insulin-like activity *in vivo*. Moloney and his coworkers (Moloney & Coval, 1955; Moloney & Goldsmith, 1957) have shown that serum from insulin-treated guinea-pigs, rabbits, sheep and horses will neutralize the effects in mice of insulin extracted from the pancreas of the rabbit, sheep, horse, pig, ox and monkey. In the case of horse anti-insulin serum this neutralization is probably due to direct combination of insulin with the antibody in the injected serum, for this serum does not provoke hyperglycaemia in the mouse (Moloney & Goldsmith, 1957) and will form a precipitate with the hormone *in vitro* (Moloney & Aprile, 1959). Rabbit anti-insulin serum will also agglutinate insulin-conjugated red cells (Arquilla & Stavitsky, 1956*a*, *b*). Additional information such as this, and the results of the *in vitro* experiments described above, provide more conclusive evidence for a direct physical reaction between antibodies and insulin than do the results of experiments carried out *in vivo* with mixtures of anti-insulin sera and the hormone. The only criticism which has been levelled against the conclusions obtained from the experiments with insulin-conjugated red cells is that impurities in the antigen used to treat the animals could induce antibodies which react with cells conjugated with the same preparation (Lapresle & Grabar, 1957). This could hardly be true in the present instance unless, as is most unlikely, all three insulin preparations conjugated with the red cells contained the same impurity as the bovine insulin used to treat the guinea-pigs.

SUMMARY

1. The insulin-neutralizing potency of guinea-pig anti-insulin serum can be estimated from the effects it produces on intravenous injection either alone or in conjunction with insulin into fed conscious rats.

2. Active serum also agglutinates sheep red cells conjugated with insulin by means of bis-diazotized benzidine.

3. The antibodies which guinea-pig anti-insulin serum is thought to contain are not species-specific; evidence obtained from experiments *in vitro* suggests that they combine directly with the hormone.

4. On treatment with insulin suspended in an emulsion guinea-pigs yield active serum within 3 months. Within 5–6 months it is possible to select those which will produce very active anti-insulin serum. The major cause of death amongst treated animals was haemorrhage due to cardiac puncture but none died from hypoglycaemia.

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