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Effects of various drugs on insulin antibodies

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Summary

1. Insulin antibodies were induced in young guinea-pigs of both sexes weighing 300–400 g and housed in a room maintained at 28° C ± 2° C, by subcutaneous injection of 2 ml of freshly prepared insulin antigen emulsion between the shoulders once every month.

2. To estimate the titre of serum antibody the serum was incubated with a known concentration of insulin for 90 min at 37° C and the insulin not bound to antibody was estimated by the rat hemidiaphragm method.

3. No significant ($P>0.5$) development of insulin antibody could be detected in the serum samples collected 1 month after the first and 15 days after the second monthly injections in groups of ten male guinea-pigs and six female guinea-pigs. However, the titre of insulin antibody in the serum of these groups of guinea-pigs 15 days after the third monthly injection of insulin antigen emulsion was significantly ($P<0.01$) raised. There was no further increase in the titre of insulin antibody in the sera 15 days after the fourth and fifth monthly injections of insulin antigen emulsion. Thus the peak titre was reached 15 days after the third monthly injection of the antigen.

4. Two groups of ten male guinea-pigs each received testosterone propionate or diethylstilboestrol daily for 1 week after each monthly injection of insulin antigen emulsion. Two other groups of six female guinea-pigs each received testosterone propionate or diethylstilboestrol in a similar manner. One more group of ten female guinea-pigs received both sex hormones for 1 week after each monthly injection of insulin antigen emulsion. Testosterone facilitated the induction of insulin antibody in the serum of males but did not affect the antibody titre in the female guinea-pigs. Diethylstilboestrol facilitated the induction of insulin antibody in the serum of groups of either sex, the peak titre being attained after the second monthly injection of insulin antigen emulsion. The response of the females which received both sex hormones was similar to that of females which received diethylstilboestrol alone.

5. Fifteen days after the third monthly injection of insulin antigen emulsion a group of ten guinea-pigs received hydrocortisone subcutaneously each day for 1 month. The serum antibody titre was estimated at the end of the drug treatment, and was significantly ($P<0.01$) reduced.

6. Fifteen days after the third monthly injection of insulin antigen emulsion three different groups of five–six guinea-pigs each received tolbutamide, chlorpropamide or phenformin orally every day for a month. Antibody titres of the serum were estimated at the end of this period ; there was no significant ($P>0.05$) reduction in groups receiving chlorpropamide or phenformin, but

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the serum antibody titre of the group receiving tolbutamide was significantly ($P<0{\cdot}01$) raised.

7. Fifteen days after the third monthly injection of insulin antigen emulsion different groups of five–six guinea-pigs received one of the following: 5-bromouracil, 5-fluorouracil, 6-mercaptopurine, methotrexate, 6-azauridine, busulphan, chlorambucil, cyclophosphamide, actinomycin-D or mytomycin-C intraperitoneally each day for 5 days. The serum antibody titre of all the groups of guinea-pigs was significantly ($P<0{\cdot}01$) reduced. On the other hand the serum antibody titre of a control group of six guinea-pigs receiving normal saline intraperitoneally each day for 5 days was not significantly ($P>0{\cdot}5$) affected.

Introduction

Although there are several clinical reports implicating insulin antibodies in the development of insulin resistance (Colwell & Weiger, 1956 ; Friedlander & Bryant, 1959), sufficient attention has not been paid to insulin antibodies. In this study insulin antibodies were produced in guinea-pigs, because of the ease with which this can be done (Morse, 1959). Glucocorticoids are beneficial in insulin resistance (Oakley, Field, Sowton, Rigby & Cunliffe, 1959) ; oestrogens have immunopotentiating action (Nicol & Ware, 1960 ; Charles & Nicol, 1961) ; and the literature on the immunosuppressive action of anticancer drugs is accumulating (Hitchings & Elion, 1963 ; Schwartz, 1965 ; Gabrielsen & Good, 1967 ; Cooper & Amiel, 1968). Therefore, the effect of hydrocortisone, sex steroids and anticancer agents on the insulin antibody titre of the serum in guinea-pigs was examined. Oral antidiabetic agents were also included in the study for a possible mediation of their action through reduction of the titre of insulin antibody, particularly since Yalow & Berson (1961) have reported that some patients with insulin resistance may benefit perceptibly from the addition of one of the oral hypoglycaemic agents such as tolbutamide or phenethylbiguanide.

Methods

Induction of insulin antibody

Insulin antibody was induced in young guinea-pigs of either sex, weighing 300–400 g, housed in a room maintained at 26° C $\pm$ 2° C with an air conditioner. Diet consisted of banana, carrot, green leafy vegetables and grain.

The insulin antibody was induced in a manner essentially similar to that described by Armin, Grant & Wright (1960). Two millilitres of freshly prepared insulin antigen emulsion were injected subcutaneously between the shoulders once every month. To prevent the effects of hypoglycaemia all injected guinea-pigs were given 5% glucose solution in place of drinking water during the first 24 h of injection of antigen emulsion. Despite this some animals did develop hypoglycaemic coma 8 h after injection. They were given 15 ml of 5% glucose solution by stomach tube. The mortality rate due to the first injection of insulin antigen emulsion was approximately 20%.

Handling of serum

About 6 ml of blood were withdrawn from each animal under light ether anaesthesia by cardiac puncture. The mortality rate due to direct cardiac puncture was

nearly 10%. The serum was separated and the serum glucose concentration estimated. The serum was kept at 4° C until required. Extra glucose was added to the serum to make a final glucose concentration of 300 mg%. The serum was diluted 1 : 10 immediately before use and the diluted serum was handled in the same way as standard insulin solution. For studying the temporal development of insulin antibody the titres were estimated 1 month after the first injection of insulin antigen and then 15 days after the subsequent injections.

Assay of antibody

To estimate antibody in the serum, the diluted serum was first incubated with a known concentration of insulin, and the excess insulin was estimated by the rat hemidiaphragm method described by Randle (1954). The solution used for incubating the rat hemidiaphragm consisted of phosphate buffer medium (Stadie & Zapp, 1947) prepared as follows: 25% NaCl (1·8 ml) and 5% $MgCl_2$, H_2O (1·0 ml) were mixed and made up to 50 ml with distilled water ; 5% Na_2 HPO_4, H_2O (10·0 ml) and 5% NaH_2PO_4, $2H_2O$ (2·8 ml) were mixed and made up to 50 ml with distilled water. At the time of estimation of antibody titre, the two solutions were mixed together. The pH of this solution was 6·8. Oxygen was bubbled through 50 ml of this buffer medium for 15 min, then 150 mg of glucose was added. The medium was used for incubating the rat hemidiaphragm and also for diluting the insulin solution. Five times recrystallized insulin (23·4 U/mg) was dissolved in distilled water previously adjusted to pH 2·8 with glacial acetic acid, to give a final concentration of 40 U/ml. This stock solution was kept in a refrigerator until required. Standard insulin solution (1 mU/ml) was prepared by diluting stock insulin solution with the glucose buffer medium.

Rat hemidiaphragms removed from male albino rats weighing 120–150 g were incubated in the flasks of a Warburg respirometer. As a rule six of the eight flasks contained 0·2 ml of diluted serum (1 : 10) and 1·8 ml of standard insulin solution (1 mU/ml) each. One contained 2 ml of glucose buffer medium and one contained 2 ml of insulin solution. The Warburg flasks were incubated for 90 min at 37° C ± 0·2° C and shaken at a rate of 90 oscillations per minute. After 90 min one hemidiaphragm was added to each flask after gentle blotting and the flasks were again incubated for a further 90 min as before. Since there were eight flasks, hemidiaphragms from four rats were incubated simultaneously. It was thus possible to assay three different samples/dilutions of serum in one lot. At the end of the second incubation period, the residual glucose was estimated in each flask by the method of Folin & Malmros (1929). The hemidiaphragms were removed, washed in distilled water, dried in a hot air oven at 105° C for 2 h and weighed. The final glucose uptake over the incubation period was calculated in mg per 10 mg dry weight of hemidiaphragm for 100 ml of glucose buffer medium. The residual insulin (not taken up by the antibody) was computed on the basis of increase in the uptake of glucose (10 mg dry weight of hemidiaphragm/100 ml glucose buffer medium). Since 1 mol of insulin would be expected to combine with 1 mol of antibody, 1 mU/ml of insulin nullified by the serum was considered as equivalent to the presence of 1 mU/ml antibody in the serum.

Complement consumption test

A rapid qualitative way of testing the antibody titre of biological materials is the complement consumption test. The principle is that all normal, fresh, unheated

sera contain a non-specific thermolabile factor known as complement. The C_1 component of complement binds to antibody when the latter is attached to antigen. Lysis of antibody sensitized sheep erythrocytes added to the system is a measure of the free complement in the system. Most of the serum samples were also assayed for the antibody titre by this test. The procedure followed was essentially that described by Pav, Jezkova & Skrha (1963).

Drugs

Details of drug administration are given under **Results** at appropriate places. Recrystallized beef insulin (23·4 U/g) was used throughout.

Statistical methods

All the statistical calculations were carried out as described by Finney (1952).

Handling of glassware

All glassware was kept immersed in a solution of chromic acid overnight followed by repeated rinsing first with tap water and then with glass distilled water. The glassware was then rinsed with 5% silicone emulsion and dried in hot air oven at 105° C for 2 hours. This procedure was repeated every fortnight.

Results

Standardization of the method for the assay of insulin antibody in the serum of guinea-pigs treated with insulin antigen

The effect of various concentrations of insulin (0·01, 0·1, 1 and 10 mU/ml) on the amount of glucose utilized ((mg glucose uptake/10 mg dry weight of hemidiaphragm)/100 ml of glucose buffer medium) by the hemidiaphragm was investigated. When insulin was incubated with 0·2 ml of serum from guinea-pigs treated with insulin antigen the dose-response curve was shifted to the right. Three different dilutions of serum (collected 15 days after the third monthly injection of antigen) that is, 1:10, 1:100 and 1:1,000 were used. The shift was parallel with dilutions of 1:1,000 and 1:100 but not parallel with 1:10 dilution (Fig. 1). Addition of 1:1,000 standard anti-insulin serum (Burroughs Wellcome Research) also produced a parallel shift of the dose-response curve to the right (Fig. 1). It can be seen from Fig. 1 that the titre of antibody in standard anti-insulin serum was roughly ten times higher than that induced in the sera of guinea-pigs in this investigation.

Insulin antibody titre in the serum of guinea-pigs given insulin antigen emulsion

Serum of twelve normal female guinea-pigs was tested for a possible non-specific nullification of the action of insulin on the glucose uptake of the hemidiaphragm. Serum samples from three of the twelve guinea-pigs could produce some nullification of the action of insulin whereas those from the nine other guinea-pigs could not. The mean value for the entire group was 330 ± 640 μU/ml.

A group of seven male guinea-pigs was treated with the emulsion without insulin for 3 consecutive months and the serum was tested 15 days after the third injection of emulsion for a possible non-specific nullification of the action of insulin on the

glucose uptake of the hemidiaphragm. Serum samples from three of the seven guinea-pigs did not antagonize the uptake of glucose stimulated by insulin while samples from the other four produced some nullification of the action of insulin. The mean value for the entire group was 280 ± 500 μU/ml.

The above two means did not differ significantly ($P > 0{\cdot}3$) from each other and, therefore, for subsequent comparisons the mean value for the normal female guinea-pigs was used.

One month after the first injection of antigen emulsion the mean titre of serum insulin antibody was 310 ± 440 μU/ml in ten male guinea-pigs and 620 ± 576 μU/ml in six females. The values were not significantly different ($P > 0{\cdot}5$) from the value obtained for normal female guinea-pigs indicating that no induction of antibody had occurred after the first injection of insulin emulsion. Fifteen days after the second injection of insulin emulsion the mean serum antibody titre in male guinea-pigs was 440 ± 720 μU/ml; in the females it was 900 ± 400 μU/ml. The values were not significantly higher ($P > 0{\cdot}5$) than the value obtained for normal female guinea-pigs. No significant production of antibody was thus demonstrable 15 days after the second injection of insulin antigen emulsion. Fifteen days after the third injection of antigen the mean serum antibody titre in male guinea-pigs was $7{,}500 \pm 500$ μU/ml while in females it was $8{,}330 \pm 630$ μU/ml, and was very significantly higher than the mean titres 15 days after the second monthly injection ($P < 0{\cdot}01$). Mean serum

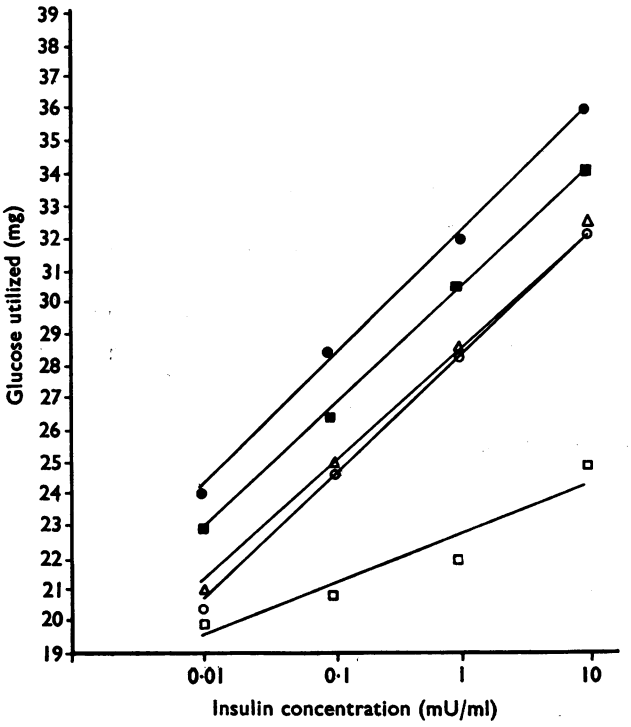

FIG. 1. Concentration/response curves for the amount of glucose utilized by the rat hemidiaphragm ((mg/10 mg dry weight of hemidiaphragm)/100 ml of glucose buffer medium) with insulin alone (●——●) and in the presence of anti-insulin serum 1:1,000 (■——■); 1:100 (△——△) and 1:10 (□——□) and standard anti-insulin serum 1:1,000 (○——○). Abscissa: concentration of insulin. Ordinate: ((mg glucose utilized/10 mg dry weight of hemidiaphragm)/100 ml of glucose buffer medium). The lines are the calculated regression lines and each point represents the mean of four observations.

antibody titres 15 days after the fourth and fifth monthly injections were not significantly different from the mean titres after the third monthly injection ($P>0{\cdot}5$ and $P>0{\cdot}1$ respectively). These results (Fig. 2) indicate that the first significant increases in the activity and the peak activity are seen at the same time, that is 15 days after the third monthly injection of antigen, and that the subsequent monthly injections of insulin antigen do not elicit a further rise in the antibody titres in the serum.

Effect of sex hormones on the induction of insulin antibody

Effects of testosterone propionate ((0·25 mg/g-pig)/day) subcutaneously and diethylstilboestrol ((0·5 mg/g-pig)/day) subcutaneously on the induction of insulin antibody were studied in both the sexes. In female guinea-pigs the combined effect of the two (injected simultaneously) on the induction of insulin antibody was also studied. The results are summarized in Table 1.

Testosterone facilitated the induction of insulin antibody in the serum in the male group but did not affect the antibody titre in the females. Diethylstilboestrol facilitated the induction of insulin antibody in the serum of both sexes. The peak insulin antibody titre in the serum in these animals was attained after the second monthly injection of insulin antigen emulsion.

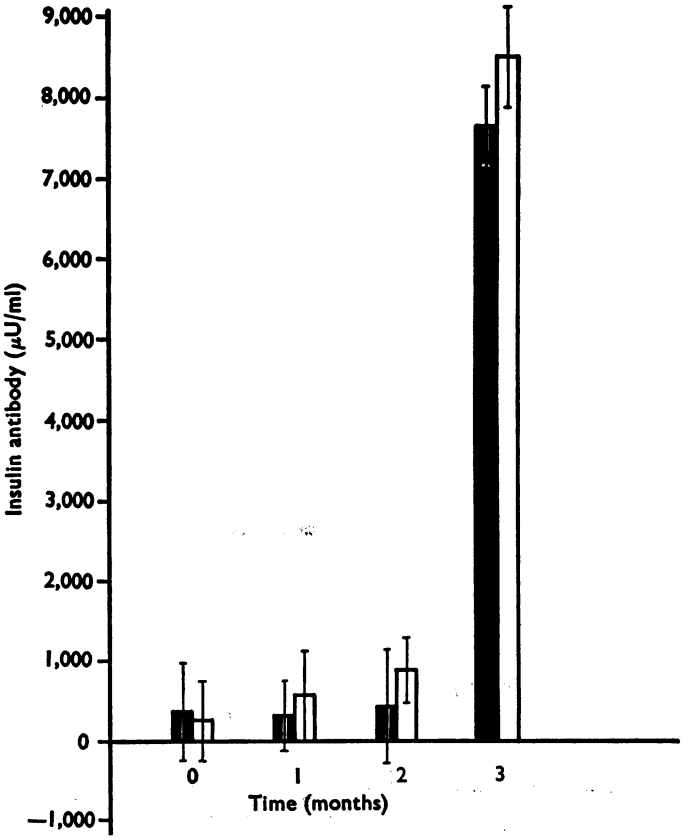

FIG. 2. Serum insulin antibody titre (μU/ml) in male (■) and female (□) guinea-pigs 1 month after the first and 15 days after the second and third monthly injections of insulin antigen emulsion. Abscissa: time in months. Ordinate: μU/ml insulin antibody. Vertical bars indicate the standard deviation.

The response of the group of female guinea-pigs receiving both the sex hormones was similar to that of the group of females receiving diethylstilboestrol alone.

Hydrocortisone

Fifteen days after the third monthly injection of insulin antigen emulsion, a group of ten guinea-pigs was given hydrocortisone (2·0 mg/kg) subcutaneously each day for a month. There was a significant ($P<0·01$) reduction in the antibody titre in the serum of these animals (Table 2).

Oral antidiabetic agents

Fifteen days after the third monthly injection of insulin antigen emulsion three different groups of five to six guinea-pigs each received tolbutamide (50 mg/kg), chlorpropamide (25 mg/kg) or phenformin (12·5 mg/kg) orally each day for a month. There was no significant ($P>0·05$) reduction in the antibody titre in the serum in the groups receiving chlorpropamide or phenformin. The antibody titre in the serum in the group receiving tolbutamide was significantly ($P<0·05$) raised (Table 2).

TABLE 1. *Effect of sex hormones on the mean insulin antibody titre in the serum of guinea-pigs* (μU/ml±S.D.)

Treatment	Sex	Before injection of antigen	30 days after first injection of antigen	15 days after second injection of antigen	15 days after third injection of antigen
Normal (control)	Male $n=10$	330±640	310±440	440±720	7500±500
	Female $n=6$	280±500	620±576	900±400	8330±630
Testosterone propionate (0·25 mg/guinea-pig)*	Male $n=10$	– –	585±520	‡ 1300±560	† 9250±660
	Female $n=6$	– –	420±370	1140±470	7320±540
Diethylstilboestrol (0·5 mg/guinea-pig)*	Male $n=10$	– –	† 3900±1240	† 9300±600	† 9300±600
	Female $n=6$	– –	† 4750±750	† 9300±600	† 9400±410
Testosterone propionate (0·25 mg/guinea-pig) and diethylstilboestrol (0·5 mg/guinea-pig)*	Female $n=10$	– –	† 5000±910	† 9300±600	† 9400±410

†$P<0·01$; ‡$P=0·05$; *n*, Number of animals. *Given subcutaneously in 0·5 ml normal saline daily for 1 week after each injection of insulin antigen emulsion.

TABLE 2. *Effect of hydrocortisone and oral antidiabetic agents on the mean insulin antibody titre in the serum of guinea-pigs* ($\mu U/ml$±S.D.)

Treatment	Before treatment	After treatment
Normal saline (control) $n=6$	9200±500	9200±550
Hydrocortisone* (2 mg/kg) $n=10$	8300±510	† 1150±740
Normal saline (control) $n=6$	8330±720	† 9500±350
Tolbutamide (50 mg/kg)* $n=5$	7000±310	† 8600±540
Chlorpropamide (25 mg/kg)* $n=5$	8000±700	8600±540
Phenformin (12·5 mg/kg)* $n=6$	8300±510	9000±380

†$P<0·01$, ‡$P<0·05$ ' after treatment ' compared with ' before treatment '; *n*, Number of animals.
* Daily for one month starting 15 days after the third monthly injection of insulin antigen emulsion. Antibody titre in the serum was estimated immediately after the termination of drug treatment. Hydrocortisone was given subcutaneously in 0·5 ml normal saline and tolbutamide, chlorpropamide and phenformin were suspended with gum acacia and given orally in a volume of 10 ml in normal saline.

TABLE 3. *Effect of anticancer agent on the mean insulin antibody titre in the serum of guinea-pigs* ($\mu U/ml \pm$S.D.)

Treatment	Before treatment	After treatment
Normal saline (control) $n=6$	9000±700	9000±700
5-Bromouracil (50 mg/kg)* $n=6$	8500±540	4000±870 †
5-Fluorouracil (10 mg/kg)* $n=6$	9000±700	4250±800 †
6-Mercaptopurine (3 mg/kg)* $n=6$	7500±400	1500±420 †
Methotrexate (0·1 mg/kg)* $n=6$	9000±670	1650±480 †
6-Azauridine (0·5 g/kg)* $n=5$	9400±410	1600±410 †
Bulsulphan (20 mg/kg)* $n=5$	9400±410	2400±410 †
Chlorambucil (5 mg/kg)* $n=5$	8200±460	2200±560 †
Cyclophosphamide (8 mg/kg)* $n=5$	9000±640	2500±1150 †
Actinomycin-D (15 mg/kg)* $n=6$	9250±660	2160±760 †
Mytomycin-C (1 mg/kg)* $n=6$	8500±460	2000±610 †

† $P<0.01$. n, Number of animals. * Given intraperitoneally in 0·5 ml normal saline daily for 5 days starting 15 days after the third monthly injection of insulin antigen emulsion. Antibody titre in the serum was estimated immediately after the termination of drug treatment.

TABLE 4. *Comparison of data (obtained by both rat hemidiaphragm method and complement consumption test) on the modification by drugs of insulin antibody titre in the serum of guinea-pigs*

	Before treatment		After treatment	
Treatment	Rat hemidiaphragm method. Mean (μU/ml±S.D.) insulin antibody	Complement consumption test*	Rat hemidiaphragm method. Mean (μU/ml±S.D.) insulin antibody	Complement consumption test*
5-Fluorouracil $n=6$	9,000±700	++++	4,250±800 †	++
6-Mercaptopurine $n=5$	7,500±400	++++	1,500±420 †	+
Methotrexate $n=6$	9,000±670	++++	1,650±480 †	+
6-Azauridine $n=5$	9,400±960	++++	1,600±410 †	+
Busulphan $n=5$	9,400±410	++++	2,400±410 †	+
Chlorambucil $n=5$	8,200±460	++++	2,200±560 †	+
Cyclophosphamide $n=5$	9,000±640	++++	2,500±1,150 †	+
Actinomycin-D $n=6$	9,250±660	++++	2,160±760 †	+
Mytomycin-C $n=5$	8,500±460	++++	2,000±610 †	+
Phenformin $n=6$	8,300±510	++++	9,000±380	++++

* The first test tube with complete haemolysis served for evaluation of results. If the results of the first test tube with complete haemolysis and those of the series containing antigen did not differ, the test was negative. If the difference was by more than two test tubes the result was recorded as + by three test tubes as ++ by four test tubes as +++ and by five or more test tubes as ++++. † $P<0.01$. n, Number of animals.

Anticancer agents

Fifteen days after the third monthly injection of insulin antigen emulsion different groups of five to six guinea-pigs were treated as shown in Table 3 with 5-bromouracil, 5-fluorouracil, 6-mercaptopurine, methotrexate, 6-azauridine, busulphan, chlorambucil, cyclophosphamide, actinomycin-D or mytomycin-C daily for 5 days. Antibody titre in the serum of all the groups of guinea-pigs was significantly reduced ($P<0.01$) (Table 3).

Complement consumption test

In some experiments, concomitantly with the assay of antibody by the principle of nullification of the action of insulin in stimulating the uptake of glucose by the rat diaphragm, assay was also done by the complement consumption test. The data obtained by the two methods are given in Table 4. As can be seen the results obtained by both the methods are in good agreement with each other.

Discussion

Insulin activity can be assayed in several ways. Those methods depending on blood-sugar responses of intact animals are tedious ; besides, the preparative procedures are lengthy and there is high mortality in experimental animals during the performance of the test. The radioimmunoassay method, though highly specific, could not be used in our laboratory because of the lack of counting facilities. Further, the presence of insulin antibody in the plasma interferes with the assay procedure and leads to faulty results.

Wright (1959) used the rat hemidiaphragm method for the assay of anti-insulin serum. He studied glucose uptake by the hemidiaphragm incubated in the presence of insulin and anti-insulin serum, and observed that serum obtained from guinea-pigs sensitized to bovine insulin inhibited the increase normally produced by bovine insulin upon glucose uptake of the isolated rat hemidiaphragm. Serum from normal guinea-pigs had no such action. This inhibition was not species specific as serum from guinea-pigs sensitized to insulin from one animal source (cattle) inhibited the effects produced by insulin obtained from other animals (pig, sheep and man).

In this investigation, insulin activity was assayed by the rat hemidiaphragm method, since this method also requires the use of small amounts of anti-insulin serum for the determination of insulin antibody titres. Moreover, plasma insulin concentrations both in non-diabetic and diabetic subjects reported from this laboratory by the rat diaphragm method (Parikh, Dave, Shah, Patel, Parikh & Gulati, 1970) are in accord with the concentrations obtained by other workers (Wright, 1957 ; Randle, 1954 ; Bornstein & Lawrence, 1951 ; Vallance-Owen, Hurlock & Please, 1955).

There was no hyperglycaemia in guinea-pigs treated with insulin emulsion even though all showed a good titre of insulin antibody. The failure of the anti-insulin serum to provoke a hyperglycaemia in the guinea-pigs was not unexpected. Moloney & Coval (1955) have shown that serum from guinea-pigs treated with bovine or pig insulins neutralizes the hypoglycaemic action in mice of bovine, pig, sheep and rabbit insulins but not that of insulin from the guinea-pig. Such insulin treated guinea-pigs remain normoglycaemic and tolerate convulsive doses of pig insulin. They concluded, therefore, that the guinea-pig secretes insulin which is

immunologically distinct from the hormones secreted by the pig, sheep, rabbit and cow. Harris, Sanger & Naughton (1956) have found that bovine, pig, sheep and horse insulins differ slightly from one another in chemical composition, whilst minor immunological differences have been demonstrated by Berson & Yalow (1959). It seems likely that chemical structure is related to immunological behaviour. This suggests that the structure of guinea-pig insulin differs markedly from that of other insulins. Indeed, Goldsmith & Moloney (1957) have already shown that guinea-pig insulin has at least one physical property which distinguishes it from bovine insulin. This phenomenon suggests a new problem in the investigation of chemical structures of different insulins. It would be of interest, therefore, to extend the range of animals injected with guinea-pig anti-insulin serum.

At physiological pH, insulin exists chiefly as a multivalent hexamer to which a single insulin antibody molecule combines. In this investigation insulin is considered as univalent; therefore, all the calculations pertaining to the binding of insulin antibody to insulin are based on the univalent characteristic of the hormone.

It is well documented that the production of antibody takes place following repeated injections of antigen, the first injection of antigen acting as a sensitizing dose. Our data revealed a similar pattern of antibody formation. The first injection of insulin antigen acted as a sensitizing dose; no antibody was detected in plasma 1 month after the first injection. The production of antibody had just started 15 days after the second dose. The third injection of insulin acted as a booster dose since the antibody titre had reached its peak 15 days after this injection. With subsequent injections of insulin antigen the antibody titre remained high. Monthly injections of emulsion without insulin for 3 months did not lead to the formation of antibody.

In this study quantitative estimation of antibody titre in the serum was carried out by incubating it with a known concentration of insulin and estimating the excess insulin by the rat hemidiaphragm method. To keep a check on this method a more rapid qualitative test based on the principle of complement consumption was also performed in some of the experiments. The results with the two methods were in good agreement. Similarly results of antibody titres in the serum by the two methods before and after drug treatment were in agreement.

In male guinea-pigs treated with testosterone the antibody titre in the serum was significantly raised 15 days after the second monthly injection of insulin antigen. The titre 15 days after the third monthly injection was also significantly higher than that in control guinea-pigs. But in testosterone treated females the insulin antibody titre in the serum remained lower than in untreated female guinea-pigs and testosterone treated males.

Administration of diethylstilboestrol for 7 days immediately after the first injection of insulin antigen led to a significant rise of antibody titre in the serum 15 days later. The peak titre was attained earlier, that is after the second monthly injection of antigen. Subsequent injections of antigen did not produce any further rise. This indicates that diethylstilboestrol has a definite stimulatory effect on the production of insulin antibody. The potentiating action of diethylstilboestrol in the two sexes was more or less equal. When testosterone and diethylstilboestrol were injected together in female guinea-pigs along with insulin antigen the rate of production of insulin antibody was similar to the group given diethylstilboestrol alone. The results confirm the views of other workers that oestrogens facilitate the induction

of antibody (Weinstein, 1939; Von Haam & Rosenfeld, 1942; Charles & Nicol, 1961).

Sex hormones are quite commonly used as therapeutic measures in many clinical conditions. Our findings suggest that caution should be observed in the routine use of these hormones in diabetic subjects who are dependent on insulin, since insulin resistance may develop faster in these subjects.

In guinea-pigs treated with hydrocortisone the insulin antibody titre in the serum was significantly reduced. There is a positive role of corticosteroids in the treatment of insulin resistant diabetes even though it is still not possible to assign a definite mechanism for their action. Without attempting a detailed analysis of the effect of corticosteroids on antibody responses, it seems safe to say that they do not interfere with the union of antigen and antibody but rather they act to suppress the reaction of the tissue to the presence of these substances in a combined state. Free antigen, when presented repeatedly to the reticuloendothelial system, leads to the production of larger amounts of antibody. Corticosteroids depress the reticuloendothelial system. On the basis of this it may be concluded that adrenal steroids may inhibit synthesis of antibody following specific sensitization and interfere with the anamnestic production of antibodies. It is tempting to speculate that endogenous adrenal glucocorticortecoids suppress the sensitizing action of antigens. The temporal facilitation of antibody production observed with the sex steroids could then be assumed to be due to an antagonism between the sex steroids and hydrocortisone at the level of antibody forming cells. However, explanation for the discrepant effects of testosterone in male and female guinea-pigs must await further work.

All the three groups of anticancer agents, that is antimetabolites, alkylating agents and anticancer antibiotics, showed significant depressant effect on the insulin antibody titre. These drugs inhibit protein synthesis and, as antibody production involves protein synthesis, it is possible that their action can be attributed to this mechanism.

Insulin was generously supplied by Boots Pure Drug Co., Bombay; tolbutamide by Hoechst Pharmaceutical Ltd., Bombay; chlorpropamide by Pfizer Private Ltd., Bombay; phenformin by U.S. Vitamin & Pharmaceutical Corp. (India) Ltd., Bombay; 5-bromouracil by Sigma Chemical Co., St. Louis; 5-fluorouracil by Prof. V. V. Modi, Department of Bacteriology, Faculty of Science, Baroda; 6-mercaptopurine, chlorambucil, busulphan and anti-insulin serum by Burroughs Wellcome & Co., London; methotrexate by Lederle Laboratories Division, New York; cyclophosphamide by Khandelwal Laboratories Private Ltd., Bombay; 6-azauridine by SPOFA United Pharmaceutical Works, Praha; actinomycin-D by Merck, Sharp & Dohme Research Laboratory, Rahway; and mytomycin-C by National Cancer Institute, Bethesda.

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