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ANTIBODIES TO INSULIN

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There is a question which might be asked even at this late date and which is suggested by the subject for discussion; and the question is this: are antibodies induced by insulin? It is true that an affirmative answer has already been given to the question; still there are some reasons why it might be re-examined. For example, there is good evidence to indicate that certain *in vitro* reactions which have been ascribed to insulin-anti-insulin are due instead to antigen other than insulin which may contaminate crystalline insulin, and corresponding antibody.[5] There is also the matter of inactivation of insulin by serum from insulin-resistant animals. One might ask, is it possible that such neutralization is effected, not by antibody but by destruction of insulin as, for example, by an enzyme?

I shall have something to say about these questions and I hope to show that insulin can indeed induce in animals a corresponding antibody; and in addition I shall present results which have to do with different types of anti-insulins and with insulins which differ in respect of neutralization.

By antibodies to insulin I mean antibodies which neutralize the physiological action of insulin. This is the type of antibody which is encountered with certain diabetics, fortunately rarely, and which is responsible in these for the ineffectiveness of insulin to control the disease.

Resistance to insulin, thanks to antibody formation, can also be induced in animals as was first shown by Lowell and Franklin[6] with the rabbit.

In our work we induced antibody formation in animals of a number of species by the injection of crystalline ox or pig insulin in Freund's adjuvant.

The presence of antibodies in the sera of animals and of humans was detected by a method first used by Banting *et al.*,[1] namely inhibition by antisera of insulin-induced hypoglycaemic convulsions in mice. In detail, starved mice were injected with serum or

dilutions of serum mixed with an amount of insulin which, when injected with saline or with normal serum, caused convulsions in 90–100 per cent of the mice.[7] Table I gives results of a typical test.

Three groups of mice were injected as shown. Each mouse received the same amount of insulin, namely 0.05 unit. Each mouse

TABLE I

MOUSE NEUTRALIZATION TESTS

Mixture injected per mouse			*Number mice injected*	*Number convulsions*	*Comment*
Units insulin		*Volume and dilutions of serum*			
0.05	+	0.25 ml. undil.	6	0	Insulin neutralized
0.05	+	0.25 ml. 1/2 dil.	6	6	Insulin not neutralized
0.05	+	0.25 ml. 1/4 dil.	6	6	Insulin not neutralized

received the same volume of serum or of diluted serum, viz. 0.25 ml. The mice which received undiluted serum showed no convulsions, all other mice showed typical convulsions. That is, 1 ml. of undiluted serum was capable of neutralizing 0.2 unit of insulin but was not capable of neutralizing 0.4 unit.

TABLE II

NEUTRALIZATION OF INSULIN WITH ANTISERA

Anti-insulin (origin)		*Insulin (extracted from pancreas of)*
Guinea-pig Rabbit Sheep Horse Human	Neutralizes	Pig (Swine) Ox Rabbit Sheep Horse Monkey (macaca rhesus) Chicken Fish (Ling) Whale Human

Table II gives a summary of some neutralization results. Anti-insulin had been induced in the guinea-pig, the rabbit, the sheep and the horse by a series of injections of crystalline ox or pig insulin in Freund's adjuvant. The human serum was from a diabetic who had developed resistance to insulin.

The neutralizing capacity of each serum was tested against each of the extracted insulins shown in the second column, by the convulsion-inhibition test. It was found that each antiserum neutralized each of the insulins shown. That is, antiserum from the guinea-pig neutralized not only pig or ox insulin which had been used to induce anti-insulin but also insulin from the rabbit, sheep, horse, monkey, chicken, fish (ling), whale and human; and so with antiserum from the rabbit and the sheep and the horse and the human. And note that antiserum from the rabbit neutralized insulin extracted from rabbit pancreas, and similarly antiserum from the sheep, the horse and the human neutralized insulin extracted from the pancreas of the sheep, the horse and the human, respectively.

There were good reasons for the assumption that neutralization of insulin by antiserum was an antigen-antibody phenomenon. For example, just as with antibodies in general, anti-insulin had been induced by a series of injections of a protein, in this case insulin; neutralizing activity was associated with the globulin fraction of the antiserum; and finally insulin could be recovered from a neutral insulin-anti-insulin mixture by extraction with acid alcohol, thus indicating that inactivation was not due to destruction.

Since it appeared probable that one had to do here with an antigen-antibody system, the possibility of *in vitro* reaction was explored and in particular the possibility of precipitation or flocculation in mixtures of insulin and anti-insulin. In all preliminary observations which were made using anti-insulin derived from the guinea-pig, rabbit, sheep and horse, precipitation was not observed. Finally, however, in mixtures of insulin and certain specimens of serum from a resistant horse, typical flocculation occurred.

A summary of the course of immunization of the horse which yielded flocculating serum is given in Table III.

Lots A, B, C and D consisted of relatively large amounts of serum, 1 litre or more. Flocculation occurred with mixtures of insulin and serum of lots C and D; but not with serum of lots A and B.

I shall give very briefly results on flocculation from a paper which has been submitted for publication.[8]

Results in Table IV have to do with the determination of the equivalence zone of insulin-anti-insulin.

Each flocculation mixture consisted of a constant amount of flocculating serum (0.2 ml.) plus the amount of insulin shown in the first column in a total volume of 0.7 ml. pH 7.4. Equivalence zone

was determined by standard procedure, namely: two sets of mixtures as in the first two columns were prepared. After flocculation had taken place all mixtures were centrifuged and supernatants poured off. To one set of supernatants a constant amount of anti-insulin as shown was added and to the other set a constant amount

TABLE III

RESPONSE OF HORSE NO. 2589 TO INJECTIONS OF INSULIN

Time (days)	*Units ox insulin injected*	*Potency of serum (units insulin neutralized per ml. serum)*	
0	6250		
28	6250	0.4	
33		0.2	
47			
49	6250	0.8	Lot A
61		0.8	Lot B
70	6250	0.8	
85		0.8	
92	6250	0.8	Lot C
105		0.8	
112	6250	0.8	Lot D
134			

of insulin. Flocculation results with these are shown in the last two columns. The equivalence zone is that in which there is excess of neither insulin nor anti-insulin.

From washed floccules of the equivalence zone we were able to recover insulin and anti-insulin in good yields. The recovered insulin and anti-insulin were assayed by mouse convulsion technique

TABLE IV

EQUIVALENCE ZONE OF INSULIN-ANTI-INSULIN

Units ox insulin in 0.5 ml.	*Antiserum 'C' ml.*	*Flocculation*	*Flocculation in supernatants*	
			Plus 0.025 ml. antiserum 'C'	*Plus 0.25 units insulin*
0.46	0.2	+	—	+
0.58	0.2	+	—	+
0.71	0.2	+	—	tr
0.89	0.2	+1st	—	—
1.0	0.2	+1st	+	—
1.2	0.2	+	+	—
1.4	0.2	+	+	—
1.7	0.2	+	+	—
2.1				

and not by flocculation. Here then is evidence that typical antigen-antibody flocculation may occur with insulin and anti-insulin.

This conclusion is supported by other evidence.

Table V shows graphically the result of an agar diffusion test.

TABLE VI

EFFECT OF ANTI-INSULIN ON ENDOGENOUS INSULIN

Anti-insulin (origin)		*Endogenous insulin*
Guinea-pig	Neutralizes	Mouse
Horse	Does not neutralize	Mouse
Human	Does not neutralize	Mouse
Mouse, Guinea-pig, Rabbit, Sheep, Horse, Human	Endogenous insulin of anti-insulin producer not neutralized	

The insulin and anti-insulin solutions were equal in volume and were in equivalence amounts. The position and curvature of the line of precipitation is consistent with a smaller molecular species, insulin, diffusing towards a larger molecular species, anti-insulin (horse globulin).

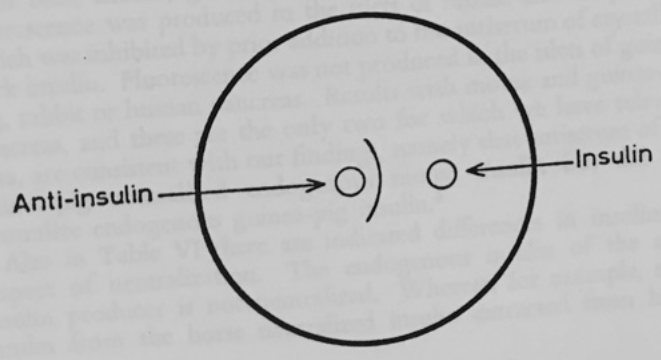

FIG. 1

There is a further piece of evidence which indirectly supports the conclusion that flocculation is due to insulin and anti-insulin, and which depends on the following considerations: Boyd and Hooker[2] derived a formula for the relation between the ratio of antibody to antigen in floccules at the equivalence point and the molecular weight of antigen.

The ratio of anti-insulin to insulin in the equivalence zone was calculated from total nitrogen of floccules and an estimate of the amount of insulin precipitated.

When this ratio was used in the formula of Boyd and Hooker a value for the molecular weight of insulin of 24,000 to 36,000 was obtained. This value is in the order of magnitude of the molecular weight of insulin in aqueous solution, pH 7 to 8.

There are different types of neutralizing antibodies. These, for example, can be flocculating or non-flocculating. Indeed the flocculating serum with which we worked contained in addition to flocculating antibodies non-flocculating antibodies, as we were able to show by fractionation with ammonium sulphate. Other differences in antisera are shown in Table VI. Here are three neutralizing antisera, viz. guinea-pig, horse and human, all of which were produced by injection of crystalline ox or pig insulin. Only the antiserum from the guinea-pig neutralized mouse-endogenous insulin as indicated by the production of signs of diabetes in the mouse. In this connection some work of Lacy and Davies[4] is of interest. These investigators labelled with fluorescein isocyanate a globulin fraction of guinea-pig serum containing anti-insulin. The labelled anti-insulin globulin was used to stain frozen sections of pancreas from beef, mouse, guinea-pig, rabbit and man. A yellow-green fluorescence was produced in the islets of mouse and ox pancreas which was inhibited by prior addition to the antiserum of crystalline pork insulin. Fluorescence was not produced in the islets of guinea-pig, rabbit or human pancreas. Results with mouse and guinea-pig pancreas, and these are the only two for which we have relevant data, are consistent with our findings, namely that antiserum of the guinea-pig neutralized endogenous mouse insulin but did not neutralize endogenous guinea-pig insulin.[4]

Also in Table VI there are indicated differences in insulins in respect of neutralization. The endogenous insulin of the anti-insulin producer is not neutralized. Whereas, for example, anti-insulin from the horse neutralized insulin extracted from horse

pancreas, it did not neutralize the endogenous insulin of the horse-producing anti-insulin since the animal was healthy and showed no signs of diabetes, and so for the antisera of the rabbit and the sheep and even the human. This latter was a severe diabetic woman whose diabetes could not be controlled by insulin nor by an oral preparation. The disease, however, was controlled by a low caloric diet and on this regimen the patient successfully withstood the amputation of a leg which had to be done because of a gangrenous foot.[3]

Table VII is also concerned with differences in insulins. This table gives a summary of properties of guinea-pig insulin.

TABLE VII

GUINEA-PIG INSULIN

1. Mixture of guinea-pig and ox insulins may be separated by partition chromatography.
2. Guinea-pig insulin not neutralized by anti-insulin (mouse convulsion test).
3. Guinea-pig insulin lowers blood sugar of mouse injected with anti-insulin from guinea-pig.
4. Guinea-pig insulin *does not* lower blood sugar of anti-insulin-producing guinea-pig.

Since guinea-pig insulin is not effective in lowering the blood sugar of a guinea-pig in which antibodies have been induced by ox or pig insulin, it is altogether improbable that it would be useful for the treatment of a diabetic who has developed antibody-type resistance to injected insulin.

Table VIII shows a comparison of neutralization of ox and cod insulins.

TABLE VIII

COMPARISON OF COD AND OX INSULINS

Anti-insulin (horse)	*Fraction convulsing*	*Anti-insulin (horse)*	*Fraction convulsing*
	Ox insulin (0.05 u. per mouse)		*Cod insulin (approx. 0.03 u. per mouse)*
Undil.	0/12	Undil.	2/12
1/2	0/12	1/2	7/12
1/4	1/12	1/4	8/12
1/8	12/12	1/8	7/12
Saline	12/12	Saline	8/12

These are different. Evidence from flocculation and neutralization[8] indicates that soluble ox insulin-anti-insulin complex is dissociated. Results here indicate that soluble cod insulin-anti-insulin complex has a larger dissociation constant than ox insulin-anti-insulin.

It would be interesting to know why endogenous insulins are not neutralized by antibodies which neutralize insulin extracted from pancreas of the same species. And it would certainly be desirable to have available an insulin effective in insulin-resistant diabetics, that is to say, an insulin which is not neutralized by antibodies induced in the human by ox or pig insulin.

Summary

By the injection of ox or pig insulin into animals it is possible to induce the formation of antisera which are capable of neutralizing the physiological effect of insulins extracted from the pancreas of a number of species of animals. Such sera in mixture with insulin may or may not exhibit the phenomenon of flocculation. A study of flocculation and neutralization with an anti-insulin serum from a horse showed clearly that neutralization of insulin by the serum was an antigen-antibody reaction. It is highly probable that neutralization of insulin by non-flocculating antisera is also an antigen-antibody reaction.

Antibodies to insulin may differ not only in respect of flocculation and non-flocculation but also in respect of insulins neutralized. For example, antiserum induced in the guinea-pig by the injection of ox or pig insulin was capable of neutralizing endogenous mouse insulin, whereas anti-insulins induced in the horse, human and mouse were not.

Extracted insulins from the pancreas of the ox, pig, rabbit, sheep, horse, monkey, chicken, fish (ling) and man were neutralized by antisera induced by ox or pig insulin in the guinea-pig, rabbit, sheep, horse and man. Extracted insulin from guinea-pig pancreas was not neutralized, as shown by inhibition of convulsions and by blood-sugar test in mice; it was, however, neutralized in the guinea-pig carrying antibodies actively induced by ox or pig insulin. Insulin extracted from cod pancreas is incompletely neutralized, an effect which depends probably on mass action.

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