THE DEVELOPMENT OF METHODS FOR THE PURIFICATION OF INSULIN AND GLUCAGON ANTIBODIES AND SOME OF THEIR BIOCHEMICAL AND IMMUNOCHEMICAL APPLICATIONS AND PROPERTIES.

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C O N T E N T S.

A B S T R A C T.

1. Radioimmunoassays for insulin and glucagon were developed, using ethanol precipitation to separate bound hormone from free. A study of the optimum assay conditions was made in which the components of the assay buffer, the assay temperature, pH, and length of incubation were varied.

2. The sensitivity of the glucagon assay was shown to be limited by the affinity of the antisera raised. In an attempt to raise antibodies of higher affinity, a study of the conditions of immunisation was undertaken, in which the nature of the immunogen, the adjuvant in which it was administered, and the species of animal immunised was varied. Rabbits were found to give the best immune response.

3. Techniques were developed to separate high affinity antibodies from low affinity sera using specific immunoadsorbents of glucagon and insulin covalently coupled to cellulose derivatives. The resulting purified antibodies, although of higher affinity than the sera from which they were derived, were not of sufficient affinity to give assays of greatly improved sensitivity.

4. Using immunoadsorbents, antibodies were concentrated from low titre sera and were intravenously injected into rats in doses sufficient to neutralise the pancreatic content of each hormone. There was no change in blood glucose following injection of glucagon antibodies, in contrast to the marked hyperglycemia after injection of insulin antibodies.

Glucagon antibodies were apparently neutralised at a much slower rate than insulin antibodies, as measured by their rate of disappearance from the serum.

5. The pancreatic glucagon contents of genetically obese, hyperglycemic mice, as measured by immunoassay, were found to be elevated to approximately twice the levels of lean control mice, whereas insulin levels were increased five to ten times.

6. In similar measurements on pancreases from Streptozotocin-diabetic rats, the glucagon levels were not significantly different from levels in control animals.

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GENERAL INTRODUCTION.

A. THE ASSAY OF GLUCAGON.

The initial objectives of this work were to develop a sensitive immunoassay for glucagon which could be used to study factors affecting the secretion of the hormone from isolated islets of Langerhans, and to measure plasma glucagon levels.

It can be calculated from data in the literature (Buchanan, Vance and Williams, 1969; Edwards, Howell and Taylor, 1969, 1970; Chesney and Schofield, 1969) that in order to measure the amount of glucagon released into 1 ml. of incubation medium from one islet from a mouse, rat or guinea pig, it is necessary to have an assay capable of measuring concentrations as low as 200 pg./ml.

Basal plasma glucagon levels, measured by radioimmunoassay, are also in the order of 200 pg./ml. (Hazzard, Crockford, Buchanan, Vance, Chen and Williams, 1968; Katsilambros, Rahman, Hinz, Fubganger, Schroder, Staub and Pfeiffer, 1970; Aguilar-Parada, Eisentraut and Unger, 1969), although at an early stage in the development of radioimmunoassay, higher levels were quoted.

Before the advent of radioimmunoassay techniques, there was no glucagon assay sufficiently sensitive to measure low concentrations in the range required. *In vivo* bioassays, based on the hyperglycemic response to intravenous glucagon (e.g. Tarding, Nielsen, Keiser-Nielsen and Nielsen, 1969) were not suitable for measuring less than 0.5 µg. The most sensitive *in vitro* bioassay, based on the activation of cat liver adenyl cyclase (Makman and Sutherland, 1964) could only measure down to 5 ng./ml. Subsequently, however, a bioassay based on the stimulation of lipolysis in

isolated chicken fat cells has been described (Langslow and Hales, 1970) which has been used to measure plasma glucagon levels, quoted to be 200-300 pg./ml.

At the time when the present work was begun, the immunoassay of glucagon had been described by several investigators (see Table 3), some of whom had reported assays of sufficient sensitivity for these proposed studies. However, immunoassays carried out at the beginning of this study were found to be much less sensitive than many of the assays reported in the literature, and could not be used to measure concentrations of glucagon less than 5 ng./ml. It was not therefore possible to carry out the initial aims of the present work, and an investigation was undertaken into ways of improving the sensitivity of the immunoassay.

The basis of a radioimmunoassay is the competition of labelled and unlabelled antigen for binding to a specific antibody. The binding of labelled hormone is quantitatively inhibited according to the concentration of unlabelled antigen present, enabling the concentration of unlabelled antigen in unknown samples to be determined.

In setting up a radioimmunoassay for a peptide hormone there are problems connected with both the labelling of the hormone, and the separation of bound hormone from free. These have been extensively reported in the literature for a large range of different immunoassay systems and are considered in detail elsewhere. In this study glucagon was labelled by the standard iodination technique of Hunter and Greenwood (1962) using ^{125}I, and bound hormone was separated from free by a modification of the method of Heding (1966) first described for the assay of insulin, in which antibody-bound hormone was precipitated with ethanol.

The major problem preventing the development of a sensitive glucagon immunoassay was that of raising antiserum of adequate affinity. It seems likely that the production of high affinity antiserum is largely a matter of chance, and that there are no immunisation techniques which enable high affinity antibodies to be produced consistently. This view has been advanced by experienced investigators in the field of radioimmunoassay. Thus Unger and Eisentraut (1969) state that "proof that the immunisation technique is a critical determinant to production of useful (glucagon) antiserum is not yet convincing".

Attempts were made to circumvent the problem of the low affinity of antibodies to glucagon by:

1. An investigation of ways in which the assay conditions could be improved to give higher binding affinity with existing sera:
2. Immunisation of a large number of animals and a variety of different species using several immunogens and immunisation procedures.
3. The selective purification of antibodies of high affinity from sera of low average affinity.

1. Immunoassay conditions

Other investigators have attempted to improve the affinity of glucagon antibodies by changing the assay conditions, although most of these studies have been limited to measuring the optimum pH of the assay, and relatively few experiments have been reported in which other variables have been studied. Even the studies in which the pH of the assay has been investigated have led to different claims for optimum conditions. Experiments were therefore carried out in which the effects of temperature, the constituents of the assay buffer, and other factors were varied.

2. Immunisation.

There are many immunisation methods which have been used to produce glucagon antisera with high specificity or affinity. A review of the literature appears in Table 3. Very few investigators have quoted the range of affinities and titres of all the sera that were produced by the particular immunisation technique which was adopted, although this information is necessary before a valid comparison of immunisation methods can be made.

The immunisation techniques applied in the present study involved the comparison of free glucagon and glucagon conjugated to protein as immunogens in rabbits, and both titre and affinity of each antiserum were measured.

In addition to investigating the production of antibodies in rabbits and guinea pigs, a pilot study to investigate the production of glucagon antibodies in chickens and goats was also carried out.

3. Selective purification of high affinity antibodies.

Studies were carried out to establish whether there was any variation in affinity between different classes of glucagon antibodies separated by gel filtration.

In other studies, attempts were made to selectively purify high affinity antibodies from low affinity sera by the use of immunoadsorbents prepared by coupling glucagon covalently to cellulose derivatives. A large excess of antiserum was incubated with immunoadsorbent in the expectation that only antibodies of relatively high affinity would be bound, and subsequently eluted, from the immunoadsorbent.

Attempts to purify high affinity glucagon antibodies have not been previously reported, although a wide variety of different immunoadsorbents have been described for recovering antibodies to other antigens by adsorption followed by acid elution (Gyenes, Rose and Sehon, 1958; Webb and Lapresle, 1961; Robbins, Haimovich and Sela, 1967; Avrameas and Ternynck, 1967; and Miles and Hales, 1968 a, b, 1969).

B. THE INTRAVENOUS ADMINISTRATION OF GLUCAGON ANTIBODIES TO RATS.

Understanding of the metabolic effects of insulin has been advanced by the use of insulin antibodies to induce acute insulin 'deficiency'. For this reason it was considered of interest to investigate whether metabolic effects of glucagon deficiency could be induced by the injection of glucagon antibodies.

Glucagon acts by stimulating both glycogenolysis and gluconeogenesis in the liver, and it might be expected that the administration of glucagon antibodies to starved, or partly starved, rats in which blood glucose is mainly originating from the liver, would lead to decreased glucose release, and to a lowering of blood glucose levels.

In these studies immunoadsorbents were used to concentrate antibodies from low affinity sera, and the antibodies were injected intravenously into rats. At the time when this work was begun, there were no detailed reports of similar investigations in the literature.

C. THE MEASUREMENT OF PANCREATIC GLUCAGON

Although the α_2 cells of the islets of Langerhans store sufficient glucagon to be measured by radioimmunoassays of low sensitivity, there is very little published information on the levels of glucagon present in the pancreas.

In the present study pancreatic glucagon levels were measured in mice and rats under different conditions. If a sufficiently sensitive glucagon immunoassay had been available it would have been of considerable interest to have applied the assay to the measurements of serum levels of the hormone also, as these provide more direct information about the rate of glucagon secretion.

1. Measurements in genetically-obese mice

Genetically obese mice have elevated blood glucose levels and increased levels of both serum and pancreatic insulin. Obese mice were available in the Department during this work, and it was therefore of interest to investigate whether pancreatic glucagon levels were also raised in comparison with lean animals.

2. Measurements in Streptozotocin-diabetic rats

Studies on animals made diabetic with Streptozotocin have been carried out over many years in the Department. This drug has been shown to cause complete destruction of the β cells of the islets of Langerhans, but there is some dispute as to whether α_2 cells are damaged also. Streptozotocin-diabetic rats have been shown to have elevated blood glucagon levels (Katsilambros, Rahman, Hinz, Fubganger, Schroder, Staub, and Pfeiffer, 1970) and it was decided to investigate whether there was an accompanying change in the level of pancreatic glucagon.

D. EQUIVALENT STUDIES WITH INSULIN

Throughout the course of this work many of the techniques that were ultimately used with glucagon were first developed with insulin. The main reason for developing methods using this hormone was the availability of both insulin and high titre, high affinity anti-insulin serum, and the fact that iodinated insulin could be bought commercially. The results obtained with insulin and insulin antibodies were of interest in themselves, and experiments with both hormones are therefore described throughout this thesis.

CHAPTER 1.

THE RADIOIMMUNOASSAY OF INSULIN AND GLUCAGON.

A. INTRODUCTION.

1. Iodination.

The methods used to iodinate glucagon were adapted from those of Hunter and Greenwood (1962) for the iodination of growth hormone. It was proposed to use ^{125}I as tracer in place of ^{131}I, used by these authors, because ^{125}I has three times the counting efficiency of ^{131}I (Freelander, 1969), although both isotopes are of similar specific activity as supplied commercially. ^{125}I has a longer half life than ^{131}I, so an additional advantage is that there is a longer period in which the specific activity of the iodinated product is high enough for it to be used in radioimmunoassay.

Several authors, e.g. Izzo, Roncone, Izzo and Bale (1964), Ooms and Arquilla (1966) and Yalow and Berson (1969a), have shown that insulin iodinated to one atom per molecule is less easily displaced by unlabelled insulin from complex with antibody than insulin with four atoms per molecule. By analogy, it was decided to iodinate to an average of one iodine atom per molecule of glucagon, giving one quarter the maximum possible labelling.

Yalow and Berson (1969a)have described a method for purifying both iodinated insulin and glucagon by binding undamaged hormone to a dry cellulose column. Unreacted iodine and damaged fragments are washed through, and undamaged iodinated hormone is eluted from the column with iodoacetamide-treated plasma.

In this work it was proposed to separate iodinated glucagon from damaged fragments and free iodine by gel-filtering the iodination products with G.25 Sephadex. Separating by means of Sephadex has the advantage of giving complete recovery on elution. As a consequence it is possible to calculate the final concentration of

glucagon in each fraction, making the assumption that the specific activity of glucagon in different parts of the hormone peak is the same. It is important to know the concentration of iodinated glucagon because this figure is used to calculate the association constant of glucagon with antibody from immunoassay curves.

2. Separation techniques.

Most immunoassays that have been developed involve a stage at which each of a series of samples of antibody-bound hormone is separated from free hormone. This must be achieved without disturbing the equilibrium of the reaction.

The various techniques for doing this can be largely classified, according to the principle of the method of separation used, into two groups:
1. Methods in which the separation is achieved by adsorbing free hormone to a material with much higher affinity for free than for bound hormone.
2. Methods in which the total globulin of the incubation mixtures is separated from free hormone by precipitation.

Methods that fall into the class of group 1. are those which use paper chromatography, cellulose, charcoal or anion exchange resins to bind free hormone, and were among the first to be applied to the measurement of normal insulin concentrations in serum.

In 1959 Yalow and Berson developed an assay in which incubation mixtures were applied to one end of cellulose strips. Free insulin was adsorbed at the site of application, while antibody-bound insulin was separated from the area of application by electrophoresis in an open system allowing solvent flow.

Similar techniques have been used by many workers since this initial report. There have been several

modifications of the original method, including the use of chromatography with solvent flow rather than electrophoresis, and the use of ion exchange paper as a replacement for filter paper for the chromatographic separation.

Methods similar to the chromatographic separation of the early insulin assays are still widely used for glucagon assays, both in serum and pancreatic extracts, e.g. Unger, Eisentraut, McCall and Maddison (1961), Grodsky, Hayashida, Peng and Geschwind(1961) (these authors also used a salt precipitation technique to separate bound and free glucagon), Assan, Rosselin, Drouet, Dolais and Tchobroutsky (1965), Samols, Tyler, Magyesi and Marks (1966), Lawrence (1966), Unger, Ketterer and Eisentraut (1966), Worobec, Locke, Hall, Ertl, Ernst and Deininger (1967), Schopman, Hakeng and Steendijk (1967), Orskov, Thomsen and Yde (1968), Unger and Eisentraut (1969) and Assan, Rosselin, Tchobroutsky and Freychet (1969).

The disadvantage of both electrophoretic and chromatographic separations is that they involve the tedious application of a large number of different incubation mixtures to paper strips. Orskov (1967) and Orskov, Thompsen and Yde (1968) have introduced a chromatographic method that doesnot involve the application of samples in the same way. Filter paper is placed into each incubation mixture at the bottom of a tube. The mixture evaporates from the top of the paper which acts as a wick. Antibody-bound hormone is carried to the top, and free hormone remains bound at the bottom of the paper which is cut in two and counted.

As a natural extension to the concept of using the binding properties of polypeptide hormone to cellulose as a separation technique, Wright, Makulu, Malaisse,

Roberts and Yu (1968) have developed an assay in which free insulin is bound to M.N. 300 cellulose powder as a 10% slurry in buffer, leaving antibody-bound insulin in solution.

Nonaka and Foa (1969) have applied this method to the separation of bound and free glucagon in the assay of glucagon released from incubated pancreatic islets.

Attempts to exploit the characteristic property of polypeptide hormones of binding to particulate materials have not been confined to the use of cellulose. For example, finely divided charcoal has also been used to separate both free insulin and free glucagon from bound hormone by adsorption. The method was originally described by Herbert, Lau, Gottlieb and Bleicher (1965) for the assay of insulin, and was subsequently modified by Stocks, Pearson and Odom (1968) and applied to the assay of glucagon by Aguilar-Parada, Eisentraut and Unger (1969), and Leclercq-Meyer, Mailhe and Malaisse,(1970). In all these methods, Norite A charcoal was first coated with Dextran 80 and was then used as a specific adsorbant for the free hormone, the function of Dextran 80 being to inhibit the binding of high molecular weight molecules such as gamma-globulin.

Rosselin, Assan, Yalow and Berson (1966)have developed similar immunoassay techniques using silica and silicates to bind free insulin, human growth hormone, human parathyroid hormone and ACTH.

Anion exchange resins have also been used to specifically bind free insulin by Meade and Klitgaard (1962), Meade and Kleist (1967) and Young and George (1965). Young and Kraegen(1968)have further developed this method and have used it for the simultaneous assay of insulin and glucagon, using the hydroxide form of Amberlite G. 400 to bind both. However, the blood concentrations of glucagon reported by these authors were higher than those of most other workers (ca.

50 ng./ml.), suggesting that this method was not reliable when applied to assay of serum glucagon.

The obvious disadvantage of any adsorption method of separating proteins such as polypeptide hormones from globulins that is not entirely specific is that there are a large number of possible materials in serum that are potentially capable of interfering or competing. For example, there is often a non-specific effect of serum proteins. Thus Stocks, Pearson and Odom (1968) have shown that plasma markedly inhibits the adsorption of insulin to charcoal and found it necessary to add charcoal-adsorbed plasma to standard curve samples in order for it to be possible to relate the counts bound by experimental samples to the standard curve.

Methods that are classified in group 2, i.e. methods in which antibody is precipitated in the separation procedure, have been widely applied to the measurement of polypeptide hormones. Of these, methods in which antibody to the hormone is precipitated with species-specific antiserum are of especial importance, and were first used to assay insulin by Hales and Randle (1963), and Morgan and Lazarow (1963).

As originally described, radioactive insulin, standard or unknown unlabelled insulin, and guinea pig anti-insulin serum were incubated until the reaction was essentially complete, and anti-guinea pig gamma-globulin (raised in rabbits) was then added in amounts that caused complete precipitation of all insulin antibodies. The precipitate was then separated by Millipore filtration (Hales and Randle) or by centrifugation (Morgan and Lazarow).

These methods were substantially less laborious than earlier chromatographic and electrophoretic techniques, and represented an important advance in methodology. There can, however, be difficulties connected with the second antibody precipitation step.

For example, it has been shown that there is a tendency for the globulins of the sera of some species to complex with the already formed second antibody precipitate to give soluble complexes, giving falsely high values for serum insulin measurements in these species. There is evidence that complement may also have a similar effect by solubilising double antibody precipitates (Brunfeldt and Jorgensen, 1967).

The double antibody method has been adapted to measure insulin and growth hormone simultaneously using ^{125}I and ^{131}I as tracers by Morgan (1966), and to the assay of glucagon by Hazzard, Crockford, Buchanan, Vance, Chen and Williams (1968) and Shima and Foa (1968).

One of the original methods developed for insulin assay (Grodsky and Forsham, 1960) involved the use of 17% sodium sulphite at 4^{o} or sodium sulphate at 25^{o} to precipitate bound insulin. Several investigators have subsequently developed techniques for the measurement of insulin and glucagon based on the principle of this assay, i.e. the use of solvents or salts to completely precipitate gamma-globulin, leaving free antigen unprecipitated in solution.

Baum, Brown and Crabtree (1964) have published insulin assay results using a salt precipitation technique, and Probst and Colwell (1966) have assayed low concentrations of glucagon using ammonium sulphate precipitation. Solvent precipitation has been used by several investigators. Gordis (1960), Moloney and Aprile (1960), Heding (1966) and Welborn, Richards and Russell Frazer (1967) have all devised methods for precipitating insulin antibodies. These vary from one to the other in several respects, such as the pH, temperature and salt concentrations of the assay. The separation technique of Heding (1966) has been recently applied by her to the measurement of glucagon (Heding, 1971), and also used to measure glucagon (in a modified form) by Edwards, Howell and Taylor (1970). Similar techniques have been

used to measure thyroid stimulating hormone (Odell, Wilber and Paul, 1965), luteinizing hormone (Wilson and Hunter, 1966), and growth hormone (Tomoda and Hreshchyshyn, 1968). In each of these latter assays 55% ethanol combined with a high salt concentration (either 5% NaCl or 10% $NH_4^+CH_3COO^-$) was used to obtain precipitation.

Several other antibody precipitation techniques have been developed, but not subsequently applied widely to the assay of hormones. Thus Beck, Zaharko, Roberts, McNeil, King and Blankenbaker (1964) used the phenomenon that insulin antibodies protect insulin against proteolytic breakdown by supernatant extracts of liver homogenates as the basis for an assay. Iodinated insulin, insulin unknowns or standards, and insulin antiserum were incubated together. At the end of the incubation period 'insulinase' was added and the hormone-precipitated radioactivity measured.

This method might be expected to suffer from the possible drawback that if the 'insulinase' is not specific, but has peptidase activity against most proteins, the extent of insulinase degredation will vary with the protein concentration of the incubation medium. The authors admit that the protecting effect of antiserum was only partial.

Horiuchi, Kakinuma and Iriyama (1966) have devised a procedure in which antibody bound and free insulin were separated by gel-filtration on G. 100 Sephadex. A similar technique was devised by Genuth, Frohman and Lebovitz (1965) using G. 75 Sephadex. This separation must take some considerable time during which there may be dissociation of the insulin antibody complex leading to 'tailing' of the antibody elution peak. This was in fact observable from the published elution profiles.

A further disadvantage of Sephadex filtration is that it is tedious, as a large number of Sephadex columns need to be set up, with many fractions assayed from each. Only a method that can separate bound and free hormone in a much shorter time than can be achieved with Sephadex filtration is suitable for use as a separation technique.

An important recent advance in technique is the use of preprecipitated antibodies prepared in an immunologically active, but insoluble form. In this way the necessity to precipitate antibody after reaction with free and labelled hormone is avoided. Thus insoluble antibodies have been prepared by coupling immune gamma-globulin to G. 25 superfine Sephadex by means of cyanogen bromide (Wide, 1969), by cross-linking immune serum with ethyl chloroformate (Donini and Donini, 1969), and by non-covalently coating the inside of plastic tubes and discs with antibodies in such a way that they remained bound throughout an immuno-assay (Catt, 1969).

These techniques have mainly been used to measure gonadotrophins, although each is of general application, and therefore of potential use for glucagon assay also.

In addition to the methods of assay which have been discussed above, there are others which cannot be classified in the same way that are not based on the competition of radioactive and cold polypeptide hormone for antibody.

Arquilla and Stavitski (1956) and Miles and Hales (1968a and b) have both developed insulin assays in which

the theoretical basis is the competition of antibody for free and bound polypeptide hormone, rather than hormone for antibody.

The method developed by the earlier authors was the first ever devised for the assay of insulin using immunological techniques. Insulin was covalently bound to sheep erythrocytes, which could then be agglutinated by insulin antiserum. This agglutination was suppressed to an increasing extent by increasing concentrations of free insulin, which acted as a competitor for antibody binding. The assay was not unfortunately of great sensitivity.

The assay of Miles and Hales (1968a, b and 1969) was based on the competition of iodinated antibody with free (i.e. cold standard or unknown) insulin and insulin covalently bound to cellulose. The amount of iodinated antibody bound to the cellulose-insulin 'adsorbent' was measured as a function of the concentration of cold insulin standards.

3. Theoretical aspects of immunoassay.

a) Affinity.

All immunoassay curves have shapes which deviate from those expected if the assays were obeying a simple dilution law, because the hormones react with specific antibody reversibly:

$$Ab + Ag \underset{}{\overset{K_{ab}}{\rightleftharpoons}} (AgAb)$$

Reversibility can be expressed by the equilibrium constant K_{ab}, henceforth referred to as the 'affinity constant' of the reaction, and calculated in units of L/M in this work. Affinity constants obtained from radioimmunoassay data are of value because they give information about the sensitivity of immunoassays carried out with different sera or under different conditions.

Berson and Yalow (1959) have derived equations for calculating equilibrium constants from radioimmunoassay data. The simplest of these equations is:

$$B/F = K_{ab}(Ab^{O} - B)$$

where:

B = concentration of total bound antigen at equilibrium (measured in this work in molar units)

F = concentration of total free antigen at equilibrium (measured in the same units)

Ab^{O} = concentration of total antibody combining sites in the assay mixture.

This equation was derived by making the following asuumptions.

1. A univalent antigen (Ag) reacts reversibly with a single order of antibody combining sites (Ab) to give a complex (AgAb).

2. The equilibrium constant of the antigen-antibody reaction is the same for all antibody molecules in the reaction mixture.
3. The antigen exists in one molecular form in solution.
4. Iodinated antigen has the same equilibrium constant for binding as cold antigen.
5. The antigen-antibody reaction has reached complete equilibrium by the time free and bound antigen are separated, and the separation procedure does not change the proportion of bound to free hormone.

The equilibrium constant can be measured therefore by plotting B/F versus B. The slope of the resulting curve, which should be a straight line, is $-K_{ab}$.

The present author has derived equations to allow values for B/F and B to be calculated from the counts precipitated in each set of tubes of an ethanol precipitation immunoassay:

$$B = \left(\left(\frac{Y - A}{X - P}\right)(AG^{*}) + (ag)\right)\left(\frac{Z - A}{Y - A}\right)$$

and $$B/F = \left(\frac{Z - A}{Y - Z}\right)$$

where: X is the activity added to each tube,
Y is the maximum activity which can bind to the antibody,
Z is the activity of the sample,
A is the activity bound in the absence of antibody i.e. the non-specific binding,
P is the background activity,
(AG^{*}) is the final estimated total concentration of radioactive antigen, (uncorrected for damage),
(ag) is the final concentration of cold antigen.

In deriving these latter equations, the additional assumption was made that non-specific binding is caused by the precipitation of iodinated antigen that is no longer cross-reactive. It has not been estab-

lished that either this or other assumptions made in deriving the equations presented above are valid for either glucagon or insulin assay. However, according to Ekins and Newman (1969), most B/F versus B plots are linear over a considerable range of values of B and only become curved at relatively high concentrations of hormone. Equilibrium constants can therefore be derived from the linear points of these plots. It is not possible to give a precise significance to the values obtained, as these are derived by assuming that all specific antibodies in the serum used for the assays are of the same equilibrium constant. Sera are composed of specific antibodies with a range of equilibrium constants so that the values of K_{ab} must be 'average' values.

b) Initial slope and assay sensitivity.

During this work various attempts were made to improve assay sensitivity by altering the conditions of assay, e.g. by changing the antiserum, buffer constituents, pH, incubation time, temperature of assay, or iodinated hormone fraction used. It was, therefore, necessary to compare assay curves differing in one of these respects quantitatively, to determine whether the changed assay resulted in improvement.

Midgley, Niswender and Rebar (1969) have defined sensitivity in terms which can be applied to immunoassays, as the smallest amount of unlabelled hormone that can be distinguished from zero.

Ekins and Newman (1970) suggest a quantitative definition of sensitivity as:

$$\frac{\text{the standard error of the zero response}}{\text{the slope of the assay curve at zero cold hormone conc.}}$$

The 'zero response' for an ethanol precipitation assay is the counts precipitated in the absence of unlabelled hormone.

Sensitivity, therefore, depends upon two factors, the error in the determination of the counts bound in the absence of cold hormone, and the initial slope of the assay curve. Thus a direct comparison of the quantitative values obtained for the sensitivities of two assay curves, calculated from the equation of Ekins and Newman (1970) does not enable one to distinguish whether any difference that may be found is due to a difference in initial slope (and is, therefore, intrinsic to the assay conditions or serum used), or results from a difference in standard error.

The standard error of the zero response has two additive components - the 'counting error' and the 'experimental error'. The counting error has an accurately defined value, and can be adjusted by altering the time for which the samples are counted. The experimental error can also be modified because it varies with factors which are intrinsic to the assay method, such as the number of replicates, the equipment used for pipetting, and the skill of the experimenter.

Therefore, if two assays are being compared which are only expected to differ in intrinsic factors such as the initial slope (for example, when two different sera are being compared, and the counts bound in the absence of cold hormone have been adjusted to be the same for each), it is of greater value to compare the initial slopes directly, rather than to compare sensitivities. An additional reason for comparing slopes rather than sensitivities in this type of experiment is that the measurement of standard error is not sufficiently accurate. Apparent differences may therefore be simply due to error in measurement of the error, not to real differences in methodology.

Another method of comparing two assays is to compare the equilibrium constants derived from assay data. This can also provide useful information about assay sensitivity.

The initial slope of an ethanol precipitation assay is determined mainly by the concentration of antibody, the concentration of iodinated hormone, and the affinity constant of the serum. The concentrations of antibody and iodinated hormone (but not of course the equilibrium constant) can by modified experimentally to give maximum sensitivity. Ekins and Newman (1969, 1970) have derived various equations enabling the optimum antibody and iodinated hormone concentrations to be calculated from a knowledge of the equilibrium constant. According to Ekins and Newman (1970) the optimum sensitivity derived in this way (S_{max}) is inversely proportional to the equilibrium constant of the antiserum used:

$$S_{max} = \mathcal{E}/K_{ab}$$

where $\mathcal{E}$ is the relative experimental error of the counts bound without added unlabelled hormone.

The ratio of the equilibrium constants of two different assays is therefore equal to the reciprocal of the ratio of the maximum sensitivities, and a comparison of the equilibrium constants obtained from two assays performed under different conditions gives a direct comparison of the optimum sensitivities that can be obtained. The conditions can be compared even if the assays were performed with different batches of iodinated hormone, different concentrations of antiserum and at different times.

Hence equilibrium constants are extremely useful for relating assays which have not been performed under conditions in which initial slopes can be meaningfully compared, and have, therefore, been frequently calculated from immunoassay data in this work. It is recognised, however, that assays that would be expected to give mainly a difference in standard error of the zero response cannot be compared by these means. Thus equilibrium constants would not give any indication of the relative sensitivities of assays differing for example in the method of separating bound from free hormone.

Such variabilities were not, however, investigated during this work.

c) Precision.

A measure of the precision $\triangle h$, at any given point of an assay curve is given (according to Ekins and Newman, 1970) by the standard deviation of replicate estimates of the concentration of an unknown sample.

These authors have shown that:

$$\triangle h = \frac{\triangle R_h}{dR/dh}$$

where $\triangle R_h$ is the standard deviation of the counts of replicates of the unknown sample,

dR/dh is the slope of the assay curve (measured as change in counts per unit change in hormone concentration) at the corresponding point on the curve.

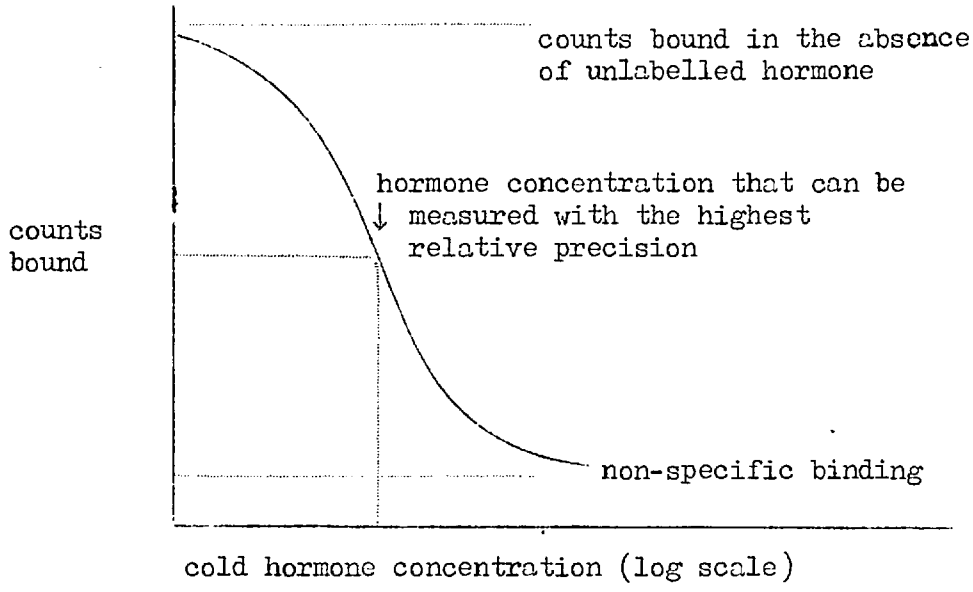
During this work it was consistently found that there was no tendency for the standard deviation of the replicate counts of each point of a given standard curve to increase or decrease with the amount of cold hormone assayed. There was variation in the standard deviation of different sets of replicates, but this variation did not show any obvious trend.

If it is assumed that all of the variation in standard deviation of different sets of duplicates is caused by error in the measurement of the standard deviation, it follows that the true value of $\triangle R_h$ is the same for every point of a given curve.

Therefore the precision at a given point of an assay will be inversely proportional to the slope of the standard curve at that point.

If a standard curve is plotted as counts bound

versus log. of the cold hormone concentration, as has been done with some of the experimental assay curves illustrated below, the effect is to make relative differences in concentration equal. Such curves are sigmoidal in shape, and display an almost perfect point symmetry, approaching the counts bound in the absence of cold hormone, and the non-specific binding, asymptotically:



The slope of the curve plotted in this way is maximal at a cold hormone concentration corresponding to the counts half way between the counts bound in the absence of unlabelled hormone, and the non-specific binding. This is the concentration which can be measured most accurately in relative terms, as the change in counts for a given percentage increase in concentration of cold hormone is larger than at any other part of the curve. It represents the ideal concentration to which all experimental samples should be diluted before assay.

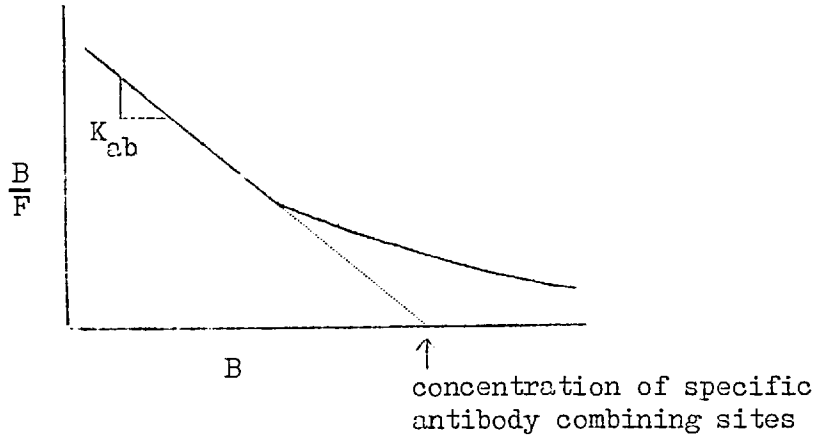
It is the opinion of the author that the sensitivity of an assay (as defined by Midgley et al., 1969) is not the only criterion as to whether an assay

is suitable for measuring a given concentration of hormone, and that the concentration of highest relative precision should also be taken into account. In this work, values falling outside the range of one fifth to five times this concentration (i.e. values at the extreme ends of an assay curve), have been ignored.

d) Measurement of antibody.

i. Absolute measurement.

The antibody content of a serum can be obtained from B/F versus B plots (Berson and Yalow, 1959). The value of B corresponding to B/F = 0 from the extrapolated linear part of the plot is equal to the concentration of specific antibody binding sites of binding constant similar to the value obtained from the slope:



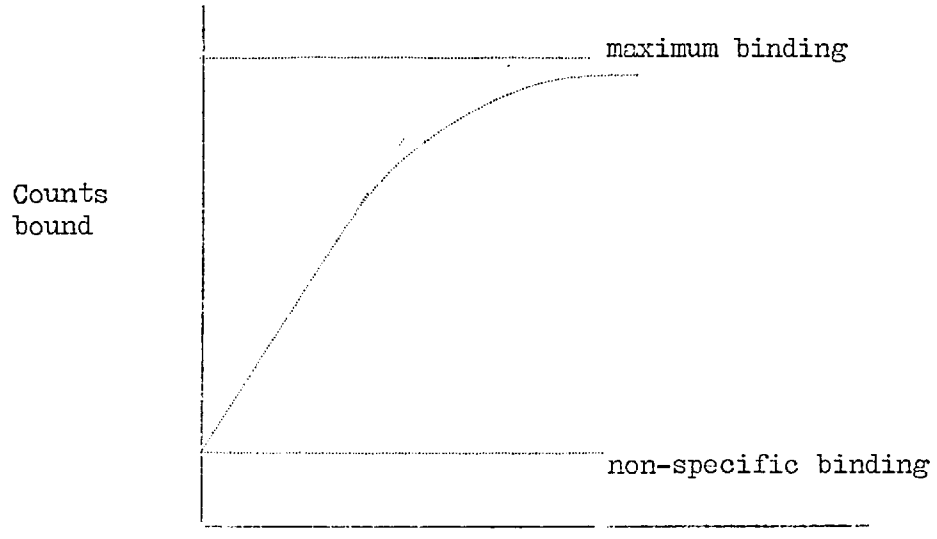
ii. Measurement relative to a serum chosen for comparison.

Performing a complete immunoassay for each of a large number of serum samples is tedious, and a method was developed which enabled the approximate relative antibody content of several sera, or serum fractions, to be measured quickly.

The principle of the method was to compare the iodinated hormone bound by several two-fold dilutions of each of the 'unknown' sera or fractions to be

assayed with the amount bound by a wide range of dilutions of a serum chosen for comparison.

Identical volumes of different dilutions of the 'comparative' serum and 'unknown' sera or fractions were each incubated with the same volume of a fixed concentration of iodinated hormone, and the mixtures were ethanol precipitated and counted. There was always an increase in the counts bound with increase in serum concentration which was linear at low concentrations, but flattened off at high concentrations, until there was no further increase:



Concentration of serum (µl./ml. buffer)- linear scale

Plots of the type illustrated above were used to derive values for the concentration of the 'comparative' serum giving the same precipitated counts as each of the dilutions of the 'unknown' sera or fractions assayed. Several values for the apparent relative antibody concentrations of each of the 'unknown' samples were obtained in this way, one for each dilution incubated.

This technique is only valid if the two sera being compared have the same equilibrium constant. Many of the antibody assays were of fractions from experiments

in which immunoadsorbents were used. In these experiments the serum from which the fractions were obtained was always chosen as the comparison serum. There was usually little difference in equilibrium constant between the serum and the fractions derived from it, so the method can be claimed to be accurate under these conditions.

No such claim could be made when the method was used to compare sera from different animals. In this case its valuewas that it enabled sera with very low equilibrium constants or titres to be quickly differentiated from those of potential use in immuno-assay.

The lower the equilibrium constant, the lower the counts at which the assay curve would be expected to depart from linearity. Therefore, if the antibody content of a serum measured by this method is plotted against the concentration of serum assayed, the resulting curve will have a positive slope if the serum is of higher equilibrium constant than the comparative serum, and a negative slope if the equilibrium constant is lower.

B. PREPARATIONS AND METHODS.

This section presents details of methods used during this work for the assay of insulin and glucagon and antibodies. Some of these were simple adaptations of existing methods for other hormones (e.g. the iodination precedure). Other aspects of the assay, such as the technique for separating iodinated glucagon from free iodine, the composition and pH of the immunoassay buffer, and length of assay, were developed during the course of the work.

1. Materials for immunoassay.

a) Immunoassay buffer.

Radioactive hormone, standard hormone, and antiserum, were each diluted in a buffer containing:

0.04 M veronal-acetic acid buffer, pH 8.6
0.04 M sodium chloride
0.025% (w/v) sodium thiomersalate
0.1% (w/v) bovine plasma albumin, fraction V
100 K.I.U./ml. Trasylol

This buffer is referred to subsequently as veronal assay buffer.

Thiomersalate was added to prevent bacterial growth, and albumin to inhibit the binding of glucagon to glassware. NaCl was included as ethanol precipitation has been shown to be incomplete unless monovalent anions are present in the buffer at a concentration greater than 20 mM (Heding, 1966), and Trasylol was added to the assay buffer to inhibit serum peptidases which degrade glucagon (Hazzard et al., 1968; Eisentraut, Whissen and Unger, 1968). The ethanol precipitation assay method is not suitable for measuring glucagon in the presence of large quantities

of protein, so smaller amounts of Trasylol were added than would be included for measurements in undiluted serum.

b) Standard solutions of insulin and glucagon.

Standard solutions of insulin and glucagon were prepared at concentrations of 200 ng./ml. or 250 ng./ml., by weighing 1 mg. or more to an accuracy of 1 µg., dissolving the hormone in 0.01 M HCl (in the case of insulin) or 0.1 M glycine buffer pH 9.0 (in the case of glucagon), and diluting the resulting solution with veronal assay buffer. Aliquots were frozen in small quantities in stoppered tubes at -25°. Fresh standards were prepared approximately every six months, although they did not appear to deteriorate over this period.

c) Iodinated glucagon.

Glucagon was iodinated by the Chloramine T method of Hunter and Greenwood (1962) developed for the iodination of growth hormone. 0.25 M phosphate buffer, pH 7.4 (20 µl.), was added to a fresh sample of ca. 1 mC of carrier-free ^{125}I in neutralised NaOH (15 µl.), followed by glucagon dissolved in 0.01 M HCl (20 µl.). (The concentrations of glucagon was adjusted to give equal concentrations of total iodine and glucagon in the iodination mixture).

Chloramine T (3.5 mg./ml.)in phosphate buffer (20 µl.) was then added, and after 10 seconds reaction, sodium metabisulphite (2.4 mg./ml.) in phosphate buffer (100 µl.) to stop the reaction.

The total iodination mixture was then transferred to a 20 ml. column of medium or fine G. 25 Sephadex, pre-equilibrated with 0.01M veronal/acetic buffer pH

8.6, containing 1% (w/v) albumin.

Iodinated glucagon was eluted with the same buffer in slightly less than half the column volume, and iodine bound to damaged glucagon fragments, and free iodide after about 1 column volume. Column fractions were collected in 1 ml. quantities in plastic tubes, and the fractions containing glucagon were each diluted to 10 ml. with a solution of Trasylol (100 K.I.U./ml.) in water.

Iodinations were usually of a high yield (up to 80%) giving a specific activity of about 200 C/g., corresponding to a labelling of half to one iodine atom per glucagon molecule.

2. Methods.

a) Pipetting.

Solutions were pipetted with a 100 µl. Hamilton microsyringe.

b) The assay of insulin antibodies.

Serial dilutions of the serum or sample to be assayed (75 µl.) were incubated in triplicate (usually) with 2.5 ng./ml. ^{125}I insulin (75 µl.). After incubating overnight at 4°, the tubes were ethanol precipitated (see below) and the precipitate counted.

c) The assay of insulin.

Antiserum solution (50 µl.) diluted to bind about 60% of the maximum counts that could be precipitated in

the antibody assay described above, was incubated with unknown or standard solutions of cold insulin (50 µl.) and 2.5 ng./ml. of ^{125}I insulin (50 µl.) for one to two days at 4°. The tubes were then ethanol precipitated and counted.

d) Assay of glucagon and glucagon antibodies.

The assays were performed exactly as described for insulin and insulin antibodies. However, because the rate of reaction was slower, and the incubation time was increased for glucagon assay from one tothree days. Iodinated glucagon was usually pipetted at a concentration of 1 to 2 ng./ml.

e) Ethanol precipitation.

Absolute ethanol (0.65 ml.), precooled to 4°, was added to each tube (containing 150 µl. of incubation reagents) by means of a multiple pipetting syringe to give a final concentration of 81.25% v/v ethanol. The tubes were mixed and then centrifuged for 30 minutes at 6,000 g in an angle-head centrifuge precooled to 0°, emptied by pouring, everted and left to drain.

The precipitates contained all the antibodies in the original mixture.

f) Counting.

In most experiments, the sample tubes were counted in a 'Tracerlab' autogamma crystal scintillation counter to a minimum of 10,000 counts. However, some experiments were performed before an automatic counter was acquired by the Department. The samples in these

experiments were counted manually in an 'Echo' scintillation counter to about 3,000 counts each, and the assays only performed with replicates of three, giving less accurate results than achieved with automatic counting for a longer period. Where relevant, therefore, mention is made of the machine used to count samples.

C. THE ASSAY OF ANTIBODIES.

Figs. 1 and 2 illustrate typical assay curves for insulin and glucagon antibodies respectively. These are of a characteristic sigmoid shape when plotted with the concentration of antiserum on a log. scale. Such curves were frequently used to measure the concentration of antibodies in unknown dilutions of fractions of the anitserum used for assay, and were also used to obtain an approximate assessment of the affinity and antibody content of unknown sera.

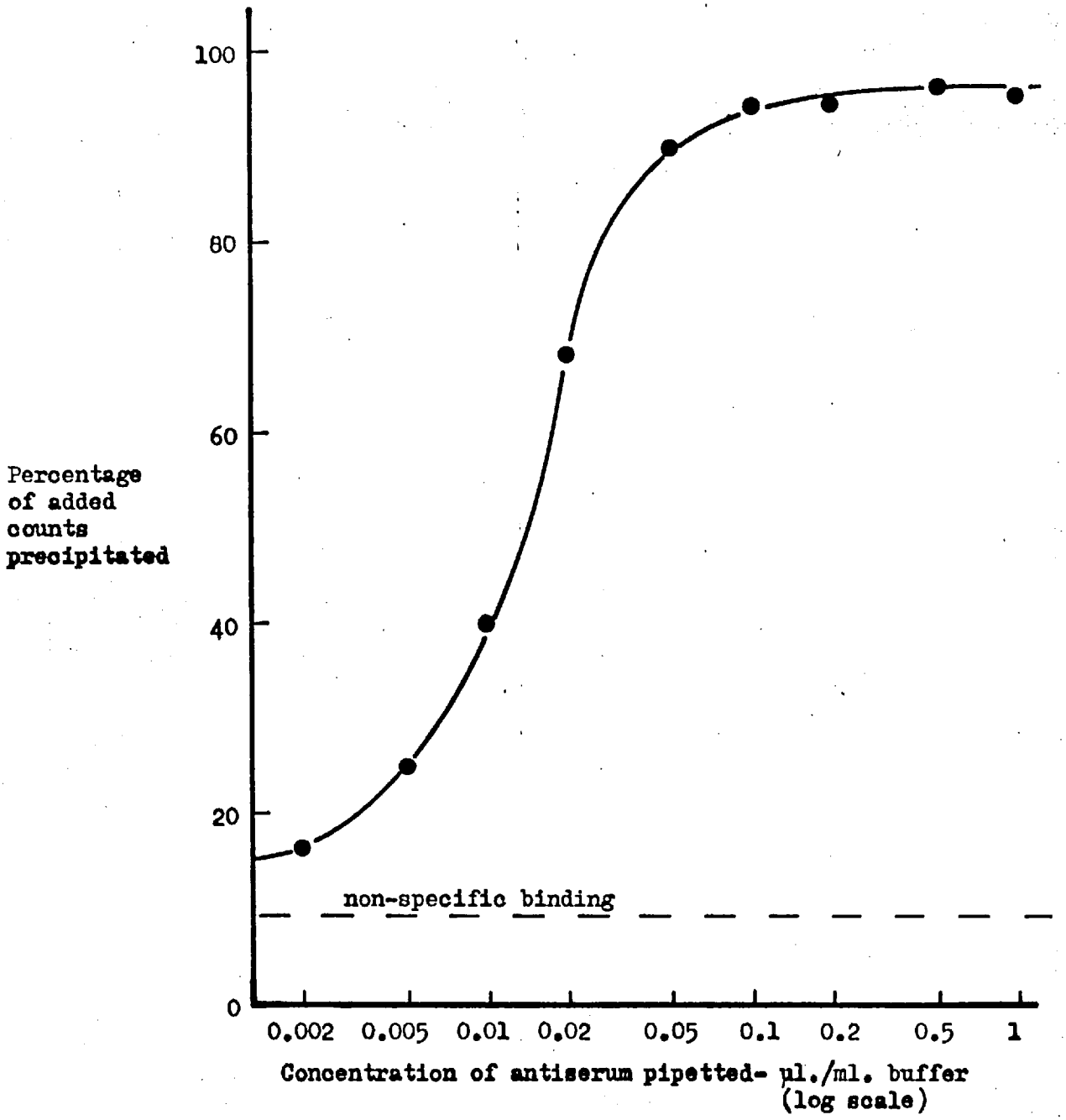


Fig. 1. A TYPICAL INSULIN ANTIBODY ASSAY CURVE.

Equal volumes (75 µl.) of a range of approximately two-fold dilutions in veronal assay buffer of guinea pig anti-insulin serum 8 were incubated with the same volume of a 2.5 ng./ml. solution of ^{125}I-insulin in veronal assay buffer at 4°, for 12 hours. The tubes were then ethanol precipitated, and counted in an automatic counter. Replicates of four tubes were used.

The assay curve presented in this Figure was used to obtain the results of Table 25 .

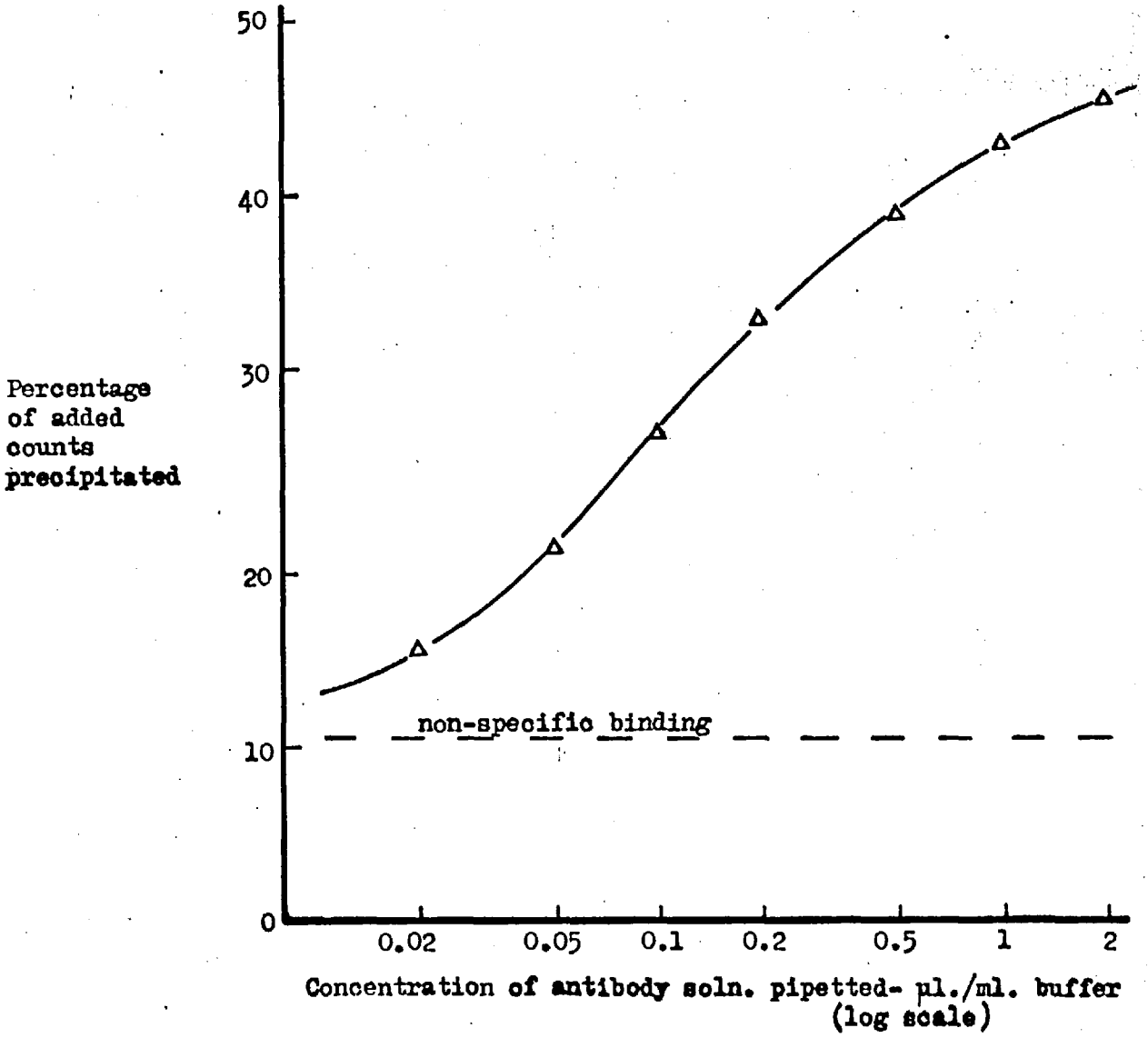


Fig. 2. A TYPICAL GLUCAGON ANTIBODY ASSAY CURVE.

Equal volumes (75 µl.) of a range of approximately two-fold dilutions in veronal assay buffer of a solution of purified, concentrated, glucagon antibodies were incubated with the same volume of a 1.0 ng./ml. solution of ^{125}I-glucagon in veronal assay buffer at 4°, for 36 hours. The tubes were then ethanol precipitated, and counted in an automatic counter. Replicates of four tubes were used.

The assay curve presented in this Figure was used to obtain the results of Table 23 .

D. DATA FROM IMMUNOASSAY CURVES.

In this section, immunoassay curves for insulin and glucagon are presented to illustrate the various ways in which the affinity of the sera and the concentration of reagents affect the range and sensitivity of the assay curves that were obtained.

Fig. 3 presents an insulin assay curve using a serum with a typical equilibrium constant and antibody content (serum 8). In addition a theoretical curve that would be obtained if a dilution principle were being obeyed (i.e. if the equilibrium constant were infinity), is plotted on the same graph. It is adjusted to give the same counts bound at zero cold hormone concentration as the experimental curve.

The data for the experimental curve is also plotted as B/F versus B in Fig. 4. This plot is linear at low insulin concentrations, becoming curved above 2 ng./ml.

Fig. 5 presents two glucagon immunoassay curves using serum 4B, which was of higher affinity than any other serum produced during this work. Both assays were performed simultaneously, and differed only in the concentration of serum used in each. The corresponding B/F versus B plots are found in Fig. 6.

Various parameters can be obtained from the insulin and glucagon curves and these are presented in Table 1. It is clear that the difference in equilibrium constants of insulin and glucagon for their respective antibodies (about 17 fold) is reflected in a difference in these parameters. The concentration of hormone that could be measured with greatest precision was quite similar to that calculated for a curve with infinite equilibrium constant for the insulin assay (1.8 times the theoretical value). However, the corresponding

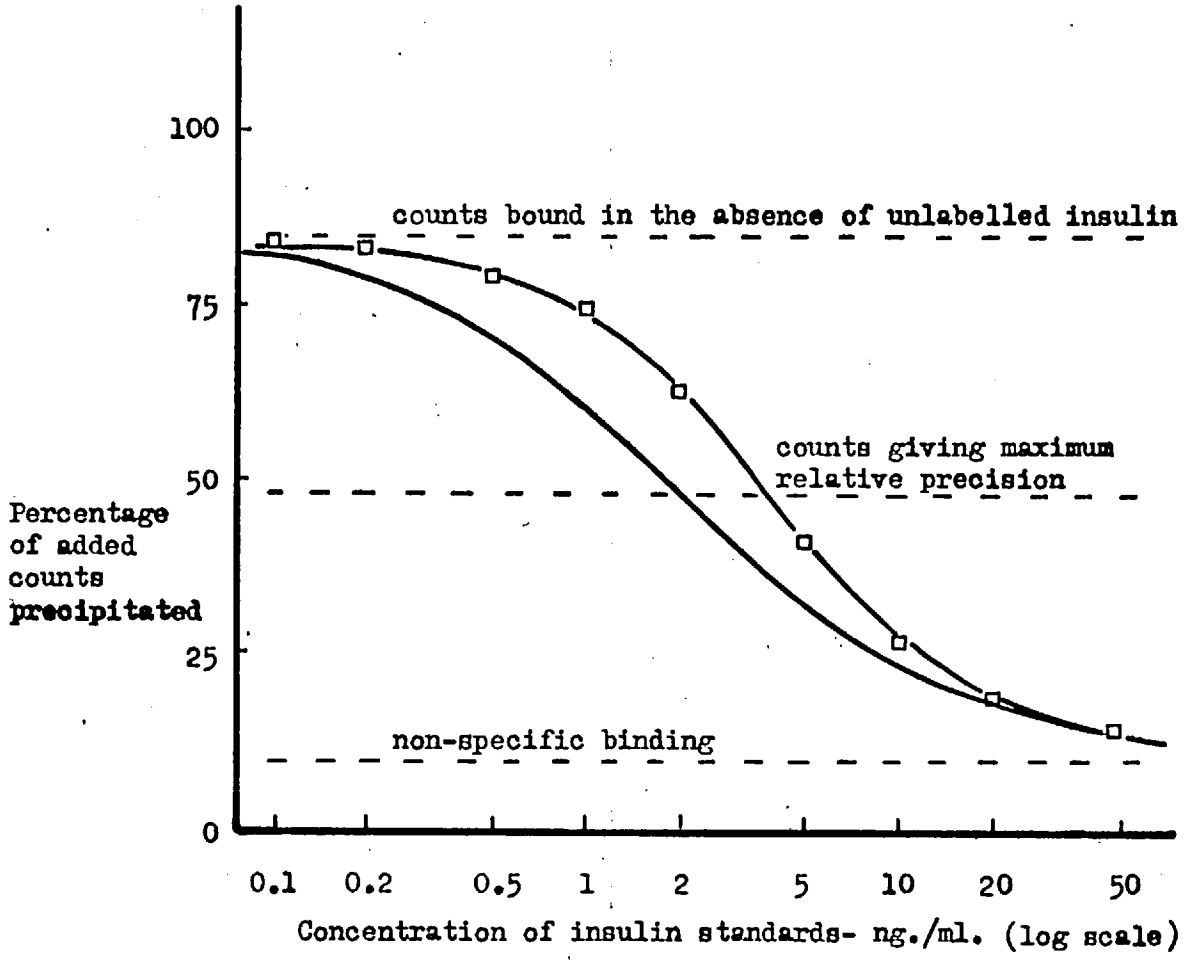


Fig. 3. A COMPARISON OF AN EXPERIMENTAL INSULIN IMMUNOASSAY CURVE WITH A THEORETICAL CURVE FOR A REACTION OF INFINITE AFFINITY.

^{125}I-insulin (50 µl: 2.5 ng./ml.) was incubated at 4° (in replicates of five) with each of a series of insulin standard solutions (50 µl.), in the presence of serum 8 diluted 1 in 30,000 (50 µl.). After 2 days, cooled ethanol (0.65 ml.) was added, the tubes were centrifuged at 3,500 g, and the drained precipitates were counted in an automatic counter. 99% of the added counts was precipitated by an excess of antiserum.

-□-□- experimental curve
—— theoretical curve

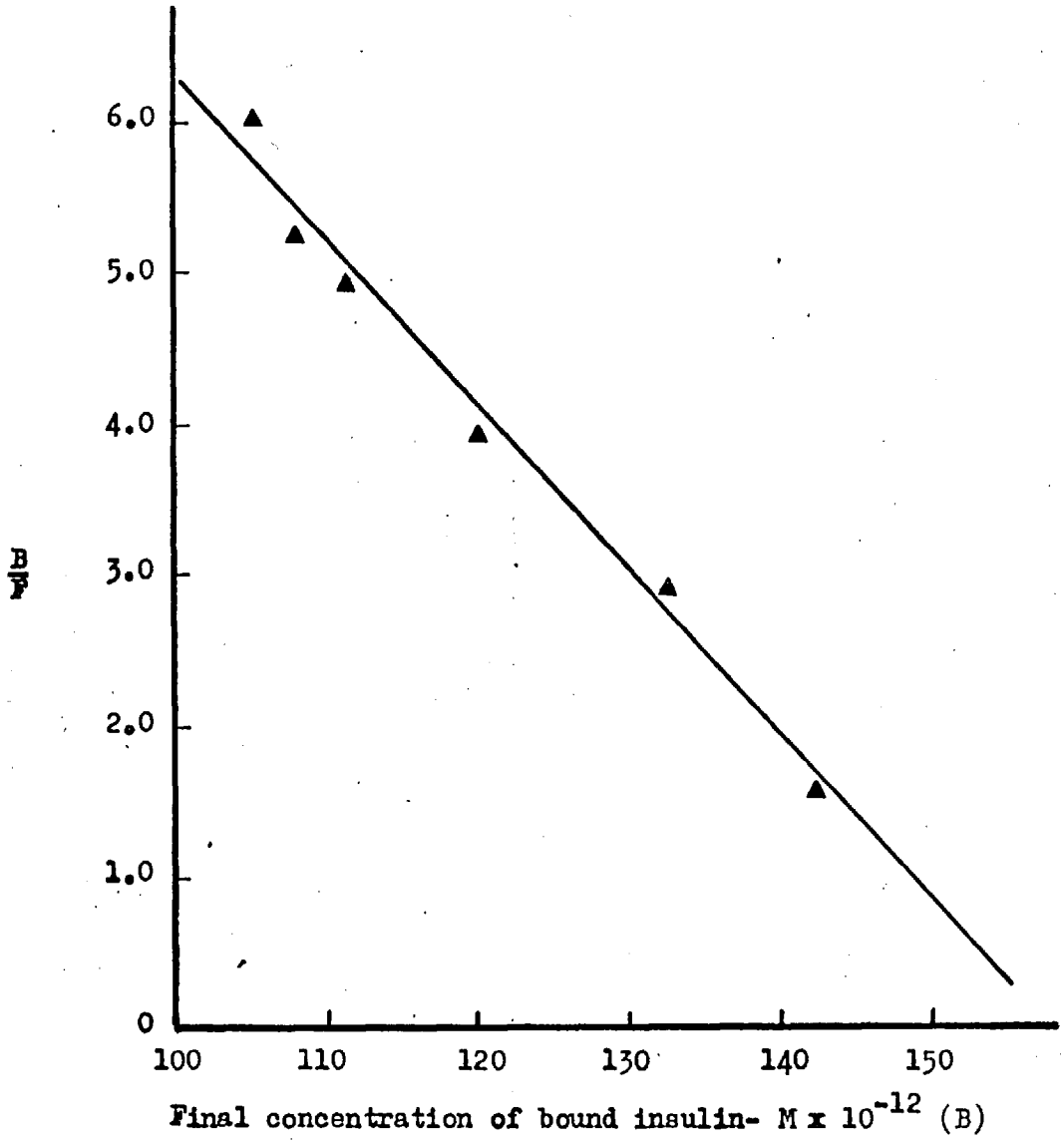


Fig. 4 A PLOT OF THE RATIO OF BOUND TO FREE INSULIN (B/F) AS A FUNCTION OF THE FINAL CONCENTRATION OF BOUND INSULIN (B) FOR AN INSULIN ASSAY.

The values for B/F and B are calculated from experimental data also presented in the previous Figure (Fig. 3)

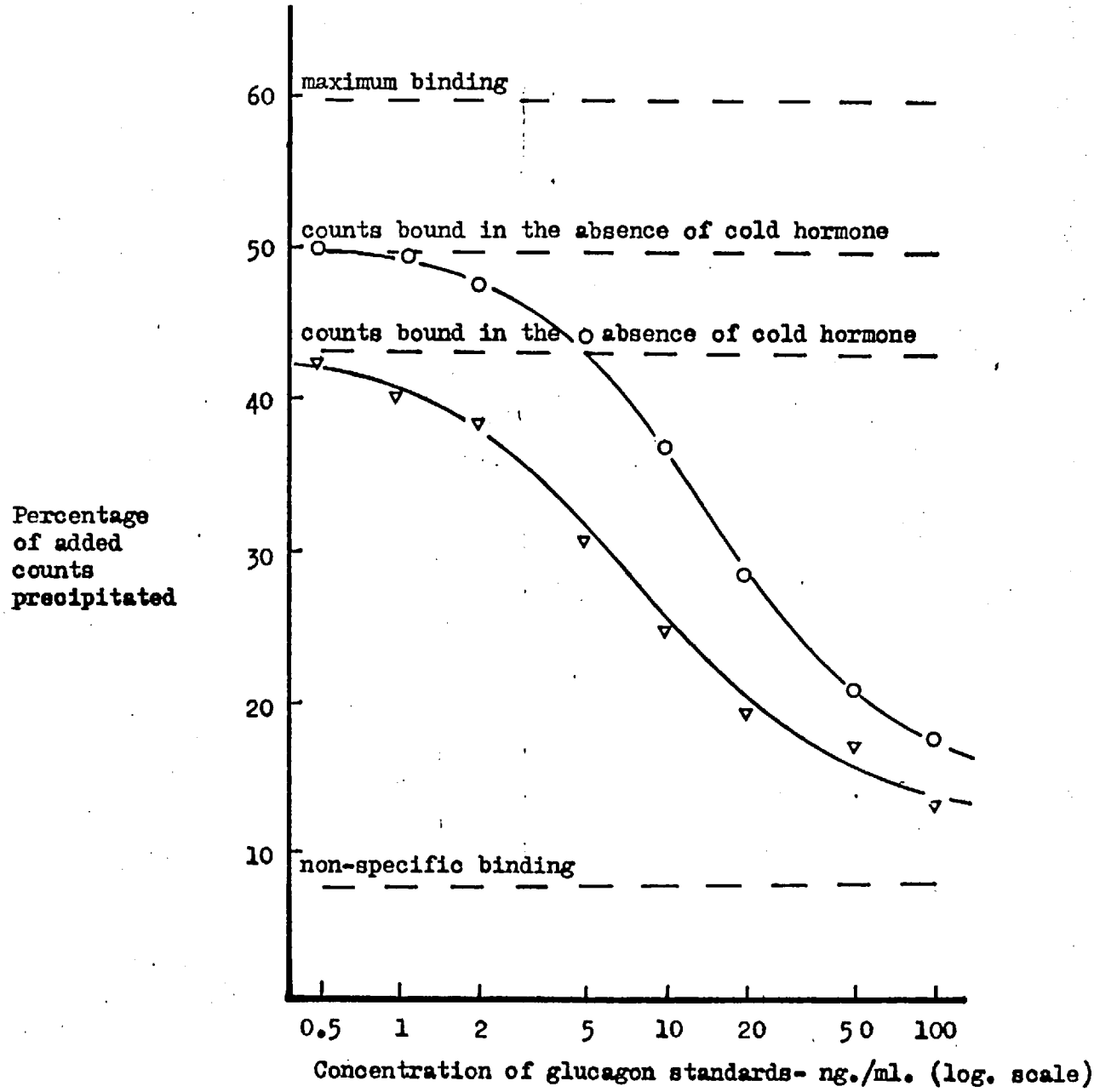


Fig. 5. A COMPARISON OF TWO GLUCAGON ASSAYS USING DIFFERENT CONCENTRATIONS OF THE SAME ANTISERUM.

^{125}I-glucagon (50 µl: concentration, uncorrected for damage, 1.17 ng./ml., in veronal assay buffer) was incubated in replicates of five, with a range of standard glucagon solutions (50 µl.), and with rabbit antiserum 4B, diluted 1 in 200 (—O—O—) or 1 in 500 (—▽—▽—), at 4°, for three days. Cooled ethanol (0.5 ml.) was then added to each tube, and the tubes were immediately centrifuged. The drained precipitates were counted in an automatic counter.

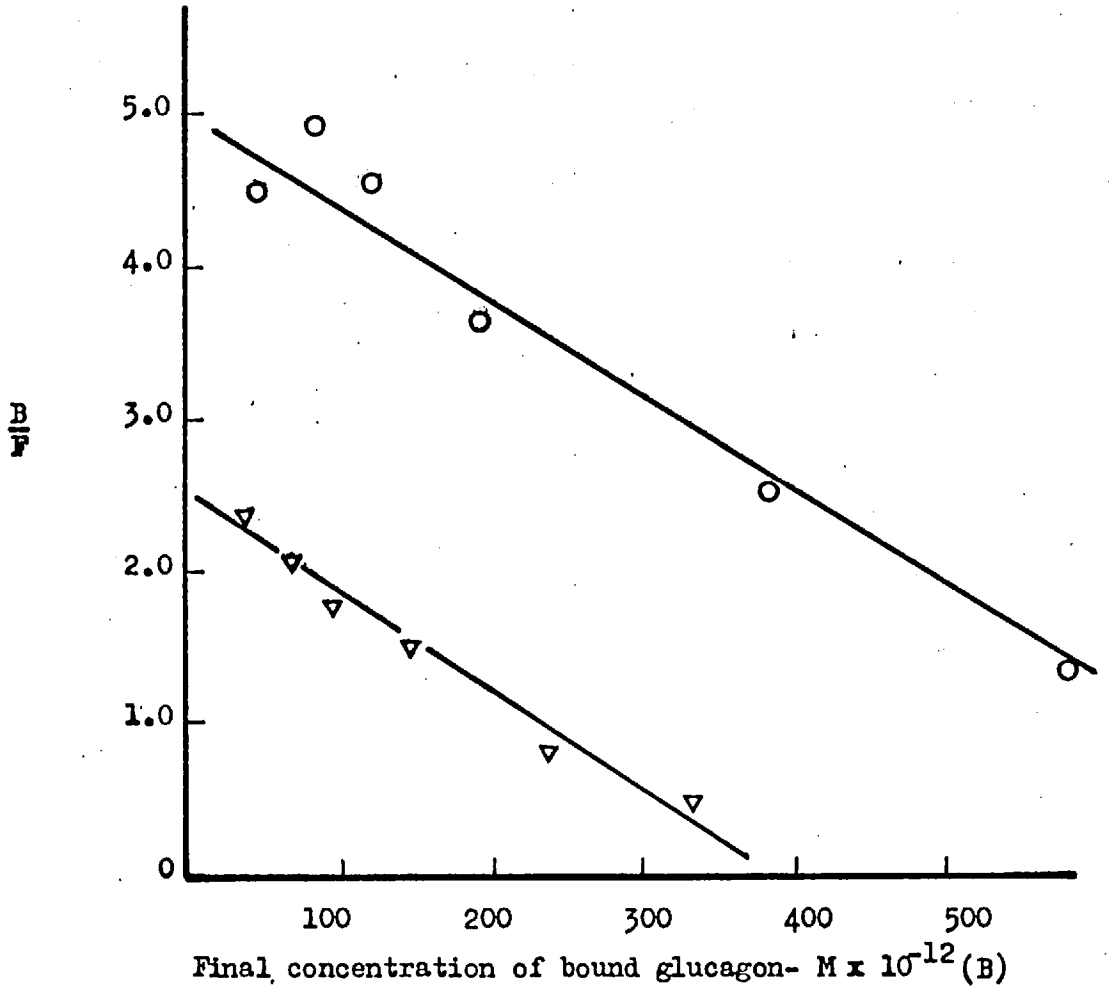


Fig. 6. A PLOT OF THE RATIO OF BOUND TO FREE GLUCAGON (B/F) AS A FUNCTION OF THE FINAL CONCENTRATION OF BOUND GLUCAGON (B) FOR TWO GLUCAGON ASSAYS.

The values for B/F and B are calculated from experimental data also presented in the previous Figure (Fig. 5). The two assays were identical, except that serum 4B was used at different dilutions (1 in 200-O-O-O-and 1 in 500-▽-▽-▽-) in each.

Table 1. DATA DERIVED FROM INSULIN AND GLUCAGON IMMUNOASSAY CURVES.

	Insulin assay	Glucagon assays (a)	(b)
Equilibrium constant ($1./M \times 10^9$)	111.7	6.29	6.77
Titre (µg. antigen neutralised per ml. serum)	84.8	1.68	1.92
Conc. hormone that can be measured with greatest precision (ng./ml.)	3.97	19.3	8.6
Concentration that would be measured with greatest precision if $K_{ab}=\infty$	2.22	0.60	0.60
Initial slope (% change in counts bound per 1.0 ng. change in standard unlabelled hormone concentration)	11.05	2.72	6.09
Initial slope if $K_{ab}=\infty$. (Same units as above)	37.12	127.1	109.3
Standard error of measurement of the replicates in the absence of cold hormone (% of counts bound)	0.29	0.88	0.85
Sensitivity (ng./ml.)	0.026	0.32	0.14

Glucagon curves (a) and (b) are presented in Fig. 5. In curve (a), antiserum 4B was diluted 1 in 200, whereas for curve B, the same serum was diluted 1 in 500. The insulin immunoassay curve is found in Fig. 3.

figure for glucagon assay curves (a) and (b) was 32 and 14 times the theoretical value respectively. Similarly the insulin assay curve had an initial slope of 30% of the equivalent theoretical curve, but the corresponding ratios for the glucagon assays were 2% and 5%.

It is difficult to compare the insulin and glucagon assay curves directly, because they were performed with different concentrations of reagents. The concentrations used in the insulin assay were far from ideal for maximum sensitivity using the criteria of Ekins & Newman (1969), whereas those for the glucagon assay (b) were near optimum. However, the initial slope of the insulin assay was higher than that of the glucagon assay, especially if the slopes are compared on an equivalent molar basis.

The insulin and glucagon assay curves illustrated were atypical of those usually obtained because they were much more sensitive. The high sensitivity was not caused by abnormally high initial slopes, but because assays were deliberately selected in which the replicates for each point gave uniformly low standard deviations.

It is interesting to note that the insulin and glucagon sera not only differed in binding affinity for antigen, but also differed in titre. The insulin antiserum had 27 times as much specific antibody as the glucagon antiserum.

It was stated in the introduction that there was no tendency for the standard deviation of the replicate counts of each point of a given standard curve to increase or decrease with the amount of cold hormone assayed. This was established from a comparison of the standard deviations of counts bound at low concentrations of cold hormone with the standard

deviations of counts bound at high concentrations of cold hormone for each of a series of eleven assays, including the six glucagon assay curves of Fig. 23.

By way of illustration, the five points at lowest concentration of the assay curve of Fig. 3 (average counts bound = 35,351) had an average standard deviation of 290 $\pm$ 115 (S.D.) counts, while the five points at highest cold insulin concentration (average counts bound = 12,993) had an average standard deviation of 205 $\pm$ 114 counts.

Neither in this assay, nor in any of the other two analysed using the Student's t test was there a significant difference in the standard deviations of points grouped in this way, showing that there was no discernable trend in standard deviations.

E. ATTEMPTS TO IMPROVE THE IMMUNOASSAY BY ALTERING THE CONDITIONS OF INCUBATION.

1. Components of the assay buffer.

a) A comparison of glucagon immunoassay in phosphate buffer and veronal buffer.

The buffer added to the medium of the first insulin and glucagon assays in this work was phosphate pH 7.4, as this was added to the original medium for the ethanol precipitation assay of insulin by Heding (1966).

The buffer was changed to veronal because there was less non-specific binding (6.7%) in this than in phosphate buffer (18%) as can be seen from Fig. 7, which presents the results of simultaneous assays in the two media. Except for this difference in non-specific binding, the curves were otherwise similar in the two buffers. It will be noted that the two media used in this experiment differed in pH as well as in the nature of the buffer used. It was not clear which of these factors was responsible for the difference in non-specific binding.

b) A comparison of glucagon immunoassays at different pH values.

Glucagon immunoassays were performed at different pH values over the range 6.5 to 9.0, in tris-buffered assay media. It was not possible to assay over this pH range in veronal buffer, as diethyl barbituric acid precipitates at pH values below 8.0. The results (Fig. 8) suggest that there was little effect of pH on the initial slopes of the assay curves, but that there was a small effect

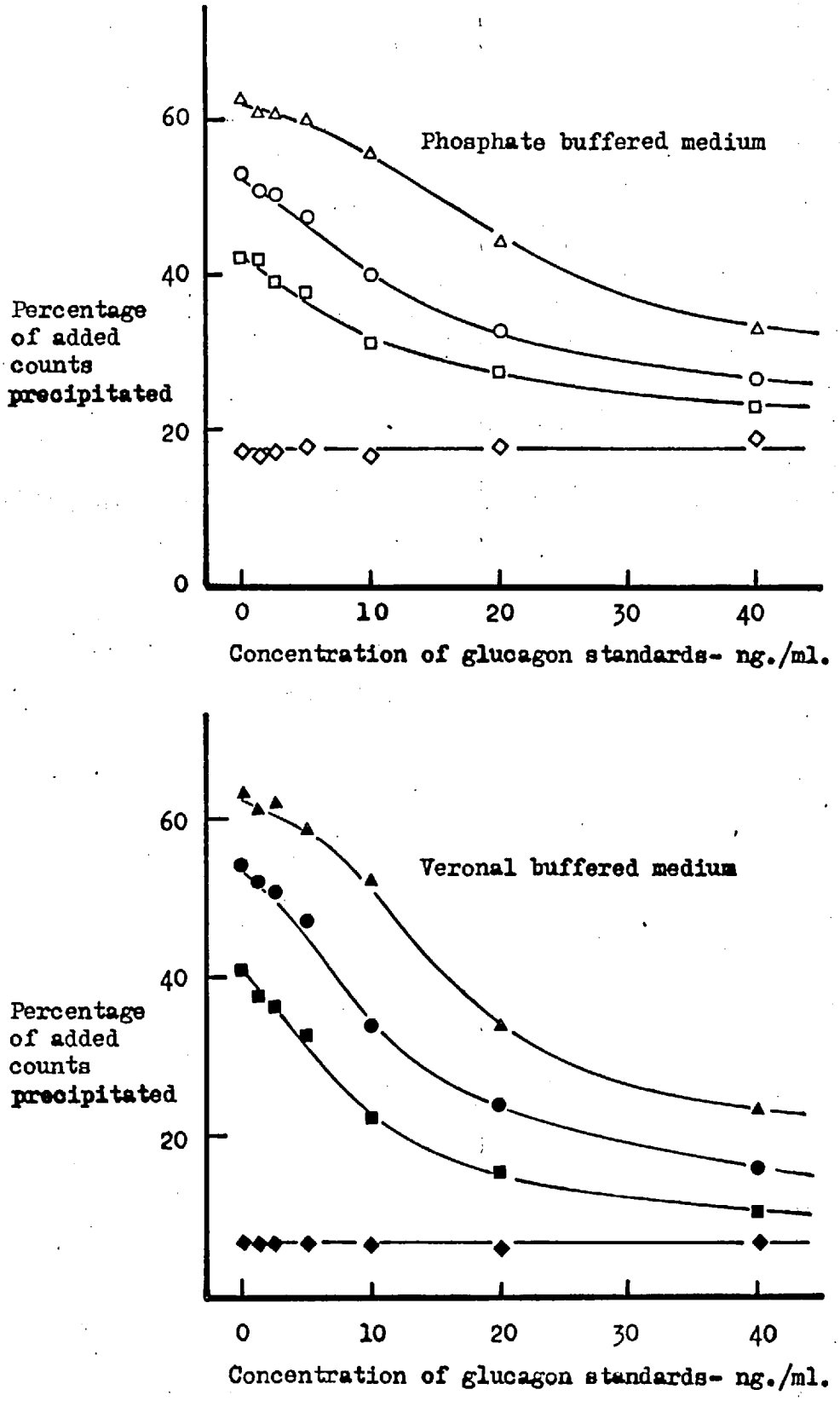


Fig. 7. A COMPARISON OF GLUCAGON ASSAY IN PHOSPHATE BUFFER AND VERONAL BUFFER.

Immunoassays were carried out in two different media similar to veronal assay medium, except that one was buffered with 0.04 M veronal, pH 8.2, and the other with 0.04 M phosphate, pH 7.4. The incubations were carried out at three different dilutions of rabbit antiserum 4B, 1 in 200 (△–△,▲–▲), 1 in 400 (○–○,●–●) and 1 in 800 (□–□,■–■), and in the absence of antiserum (◇–◇,◆–◆). ^{125}I-glucagon was pipetted at a concentration of 1.7 ng./ml, and replicates of three were used. After three days incubation, the tubes were ethanol precipitated, and the precipitates counted in a manual counter.

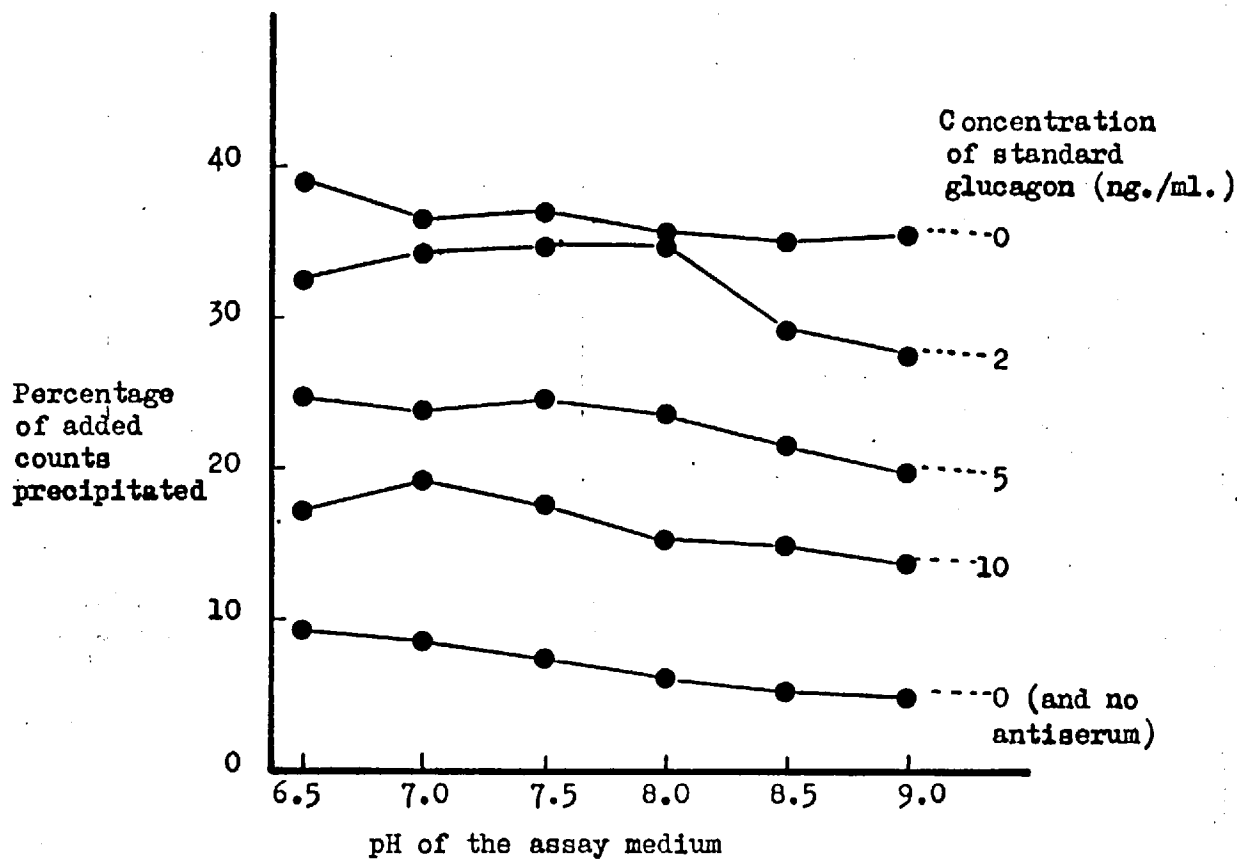


Fig. 8. THE INFLUENCE OF pH ON THE REACTION OF GLUCAGON WITH ANTISERUM.

Immunoassays were performed at a series of different pH values in media buffered with tris-HCl (0.04M), but otherwise identical to veronal assay buffer. ^{125}I-glucagon was pipetted at a concentration of 1.7 ng./ml., at each pH, and the replicates of three tubes were incubated for two days before ethanol-precipitation, and were counted in a manual counter.

The curves presented above illustrate the extent of binding at different unlabelled glucagon concentrations as a function of pH.

of pH on the non-specific binding. This was higher at pH values from 6.5 to 7.0 than over the pH range 8.5 to 9.0 to an extent (66%) that was statistically significant at the 0.1% level. The results of this experiment suggest that the difference in non-specific binding in phosphate and veronal buffer was greater than any effect expected solely from a difference in pH.

Fig. 9 presents the assay curve in tris buffer at pH 8.5 from the pH variation experiment discussed above, and an assay in veronal buffer, pH 8.17, performed at the same time, and under the same conditions. The two curves were almost identical, making it likely that the effect of pH on immunoassay in veronal buffer would be very similar to the effect on immunoassay in tris.

c) The effect of different concentrations of proteins.

Fig. 10 illustrates the effect of different concentrations of albumin and horse serum on the non-specific binding of an insulin assay. At the lowest concentration of protein, about 13% of the added iodinated insulin was precipitated by ethanol. With increasing protein concentration, there was a further linear increase in the non-specific binding.

An experiment showing the effect of different concentrations of albumin on a glucagon assay is presented in Fig. 11. The results are similar to those of Fig. 10 and also suggest that the non-specific binding increased at high protein concentrations linearly in glucagon assays as in insulin assays.

These results are in accord with those of Welborn,

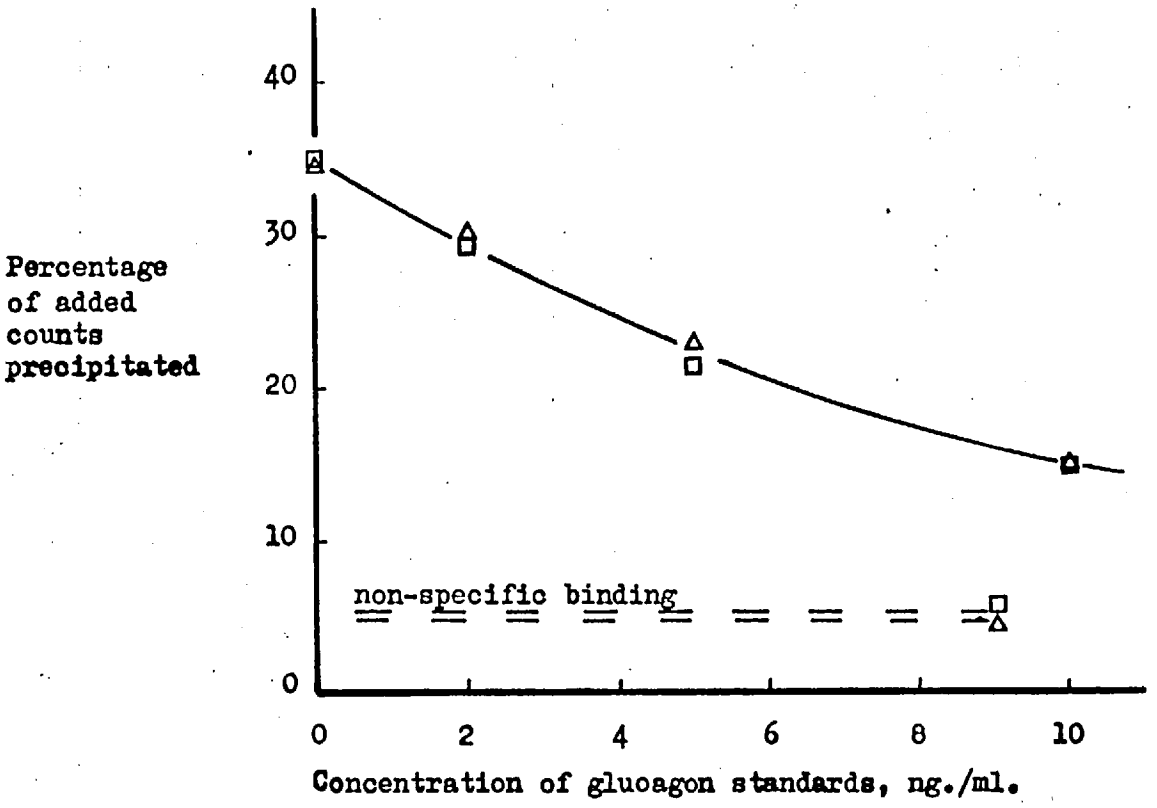


Fig. 9. A COMPARISON OF GLUCAGON IMMUNOASSAY IN MEDIA BUFFERED WITH VERONAL-ACETATE AND TRIS-HCl.

Assays were performed in media buffered with 0.04 M veronal-acetate, pH 8.2 (-Δ–Δ-), or 0.04 M. tris-HCl, pH 8.5 (-□–□-), but otherwise identical to veronal assay buffer. In both assays, replicates of three were used, rabbit antiserum 4B was diluted 1 in 400, iodinated glucagon was pipetted a concentration of 1.7 ng./ml, and the total incubation time was two days. At the end of this period, the tubes were ethanol-precipitated, and the precipitates were counted in a manual counter.

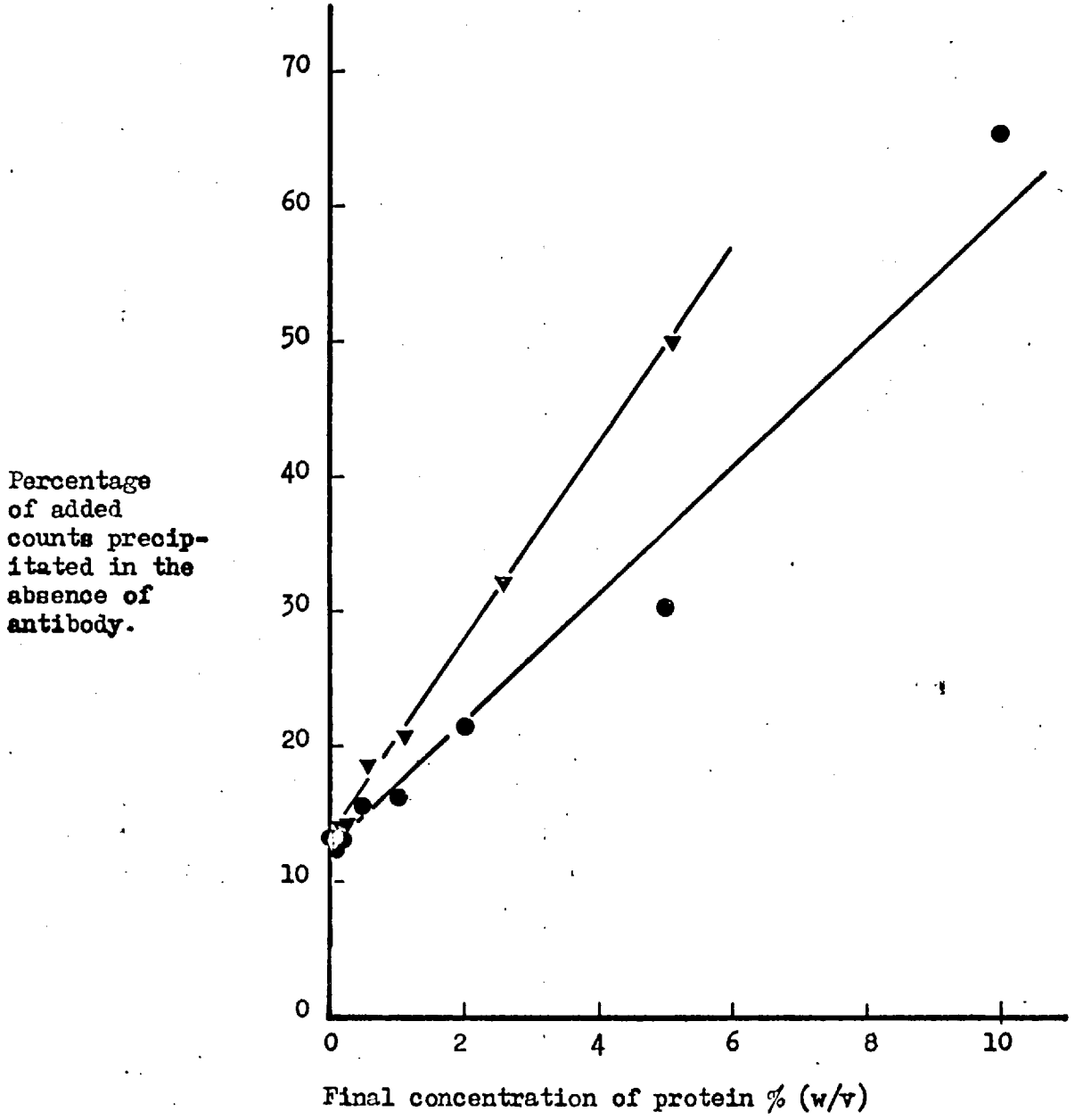


Fig. 10. THE EFFECT OF DIFFERENT CONCENTRATIONS OF HORSE-SERUM AND BOVINE SERUM ALBUMIN ON THE NON-SPECIFIC BINDING OF AN INSULIN ASSAY.

Dilutions of horse serum in veronal assay buffer from undiluted to 1 in 100, and albumin solutions in veronal assay buffer from 0 to 19.9% were mixed in 75 µl. volumes in replicates of three with ^{125}I-insulin in veronal assay buffer (75 µl.: 2.5 ng./ml.) containing albumin (0.1%, i.e. the usual concentration). After cooling the tubes to 4°, the contents were ethanol-precipitated, and the precipitates counted in an automatic counter. The horse serum was assayed to determine the protein content by the biuret method.

(-●-●-) albumin-containing buffers
(-▼-▼-) horse serum-containing buffers

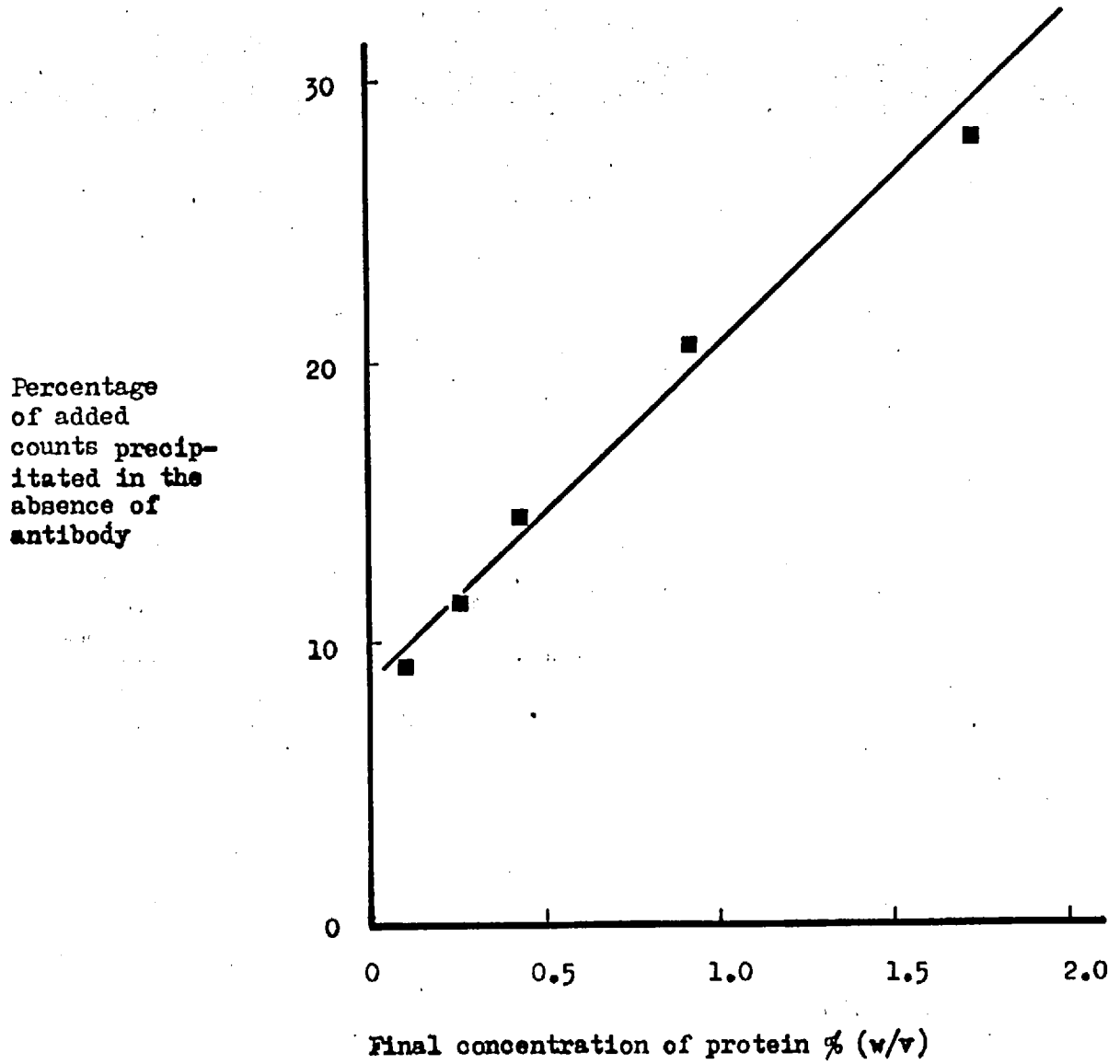


Fig. 11. THE EFFECT OF DIFFERENT CONCENTRATIONS OF ALBUMIN ON THE NON-SPECIFIC BINDING OF A GLUCAGON ASSAY.

Equal volumes (50 µl.) of various veronal assay buffers containing albumin at concentrations from 0.1% to 5% were incubated (in replicates of four) with iodinated glucagon (0.58 ng./ml.) in veronal assay buffer containing the usual concentration of albumin of 0.1% (100 µl.). In this way, the non-specific binding was measured in buffers containing final concentrations of albumin ranging from 0.1% to 1.73%. After two days, the tubes were ethanol precipitated, and counted in an automatic counter.

Richards and Russell Fraser (1967), who studied the effect of normal human serum on the non-specific binding of insulin antibody assays carried out in borate buffer, pH 8.3, using ethanol precipitation as a method of separating bound and free insulin. These authors found that there was an increase in non-specific binding at normal serum concentrations above 1 in 10, which was not inhibited by the presence of unlabelled insulin.

The results of the present study confirm the results of others, and show that ethanol precipitation is not suitable for measuring insulin and glucagon in undiluted serum, because high concentrations of proteins increase the precipitation of free hormone. For assays of serum glucagon it is, therefore, necessary to use one of the many alternative methods for separating bound and free hormone, such as the double antibody technique.

d) The effect of changing the buffer protein from albumin to casein.

An experiment is illustrated in Fig. 12, in which two glucagon assays were carried out simultaneously under identical conditions, except that the radio-immunoassay buffer of one contained casein in place of albumin.

This change did not have a significant effect on the equilibrium of the assay, as determined from B/F versus B plots. The main effect was on the non-specific binding, which was less in the casein-containing medium than in that containing albumin.

The casein used in this experiment was completely denatured during the process of solubilising it and should not, therefore, have contained any peptidase activity. Albumin on the other hand may well have

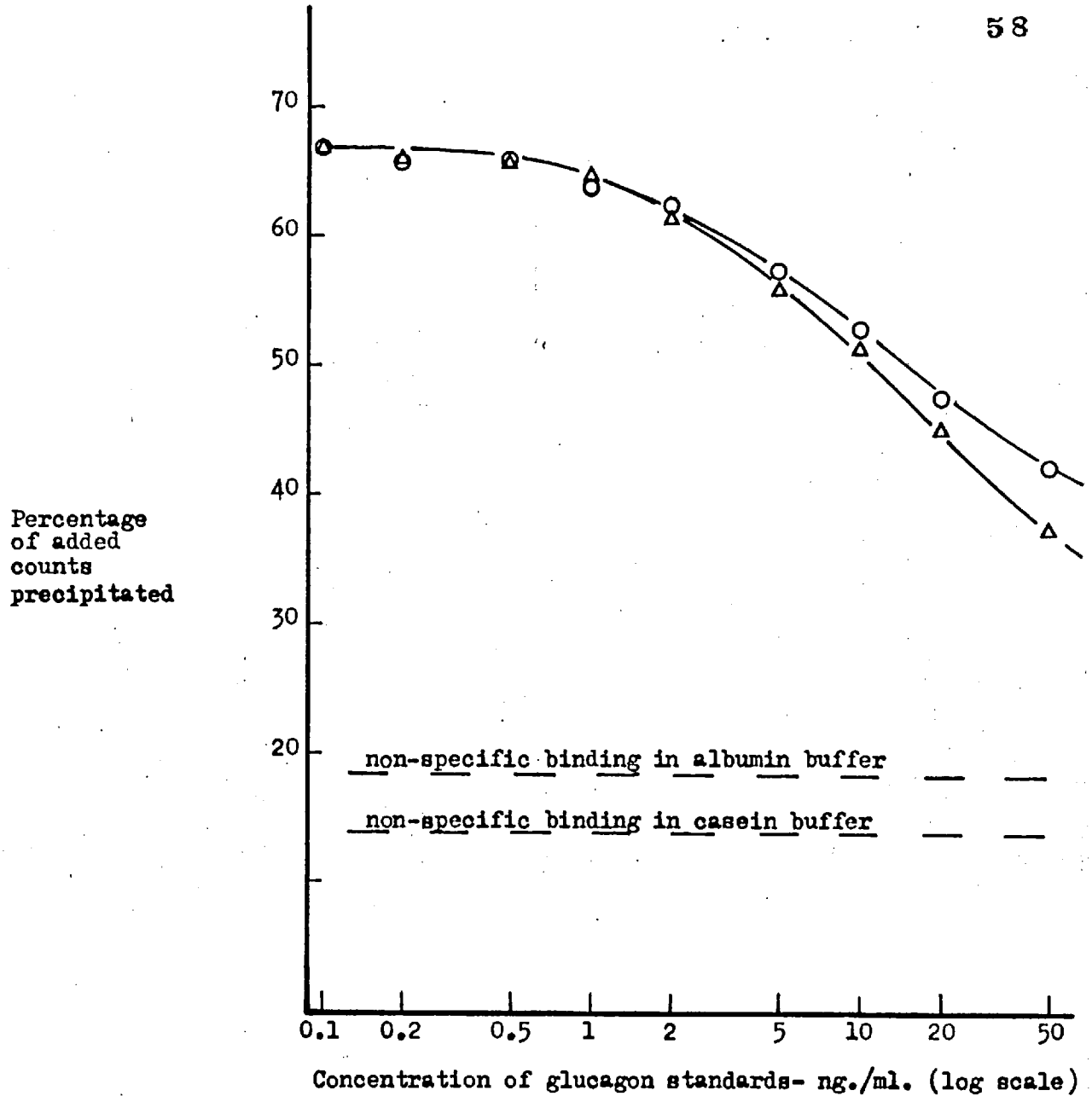


Fig. 12. A COMPARISON OF GLUCAGON IMMUNOASSAYS IN BUFFERS CONTAINING ALBUMIN AND CASEIN.

Two immunoassays were carried out at the same time: in one, all the reagents were diluted in veronal assay buffer, and in the other, the albumin of this buffer was replaced by 0.1% casein, in all the solutions pipetted. The casein was first deliberately denatured by solubilising it in 0.1 M NaOH at 80°, and then adjusting the pH of the cooled solution to 8.6.

The concentration of reagents was the same in both assays. ^{125}I-glucagon was pipetted at a concentration of 1 ng./ml., and the antiserum, which was a pooled serum, was pipetted at a dilution of 1 in 1,000. Replicates of five tubes were used. After three days incubation at 4°, the tubes were ethanol precipitated, and counted in an automatic counter. A maximum of 81% and 82% of the added counts were precipitated in the presence of excess serum in the albumin and casein assays respectively.

(-O-O-) standard curve in albumin-containing buffer
(-Δ-Δ-) standard curve in casein-containing buffer

contained residual peptidase activity, despite the presence of Trasylol in the assay medium. The difference between the non-specific binding in the two buffers may, therefore, have been caused by the hydrolysis of glucagon to ethanol-insoluble fragments by peptidases in the albumin. This peptidase activity was, however, so small that the sensitivity would not be expected to be increased significantly by using higher concentrations of Trasylol, or casein in place of albumin.

2. Immunoassay with different iodinated glucagon fractions.

The method used in this work for separating iodinated glucagon from damaged fragments, i.e. gel filtration through G. 25 Sephadex, was different from that used by the majority of other investigators, who have generally used the cellulose column method of Yalow and Berson (1964), first developed for the purification of iodinated insulin.

It was usually found that at least 80% of the iodinated glucagon in the peak elution fraction from a G. 25 column was precipitated in the presence of an excess of antiserum, provided this was measured immediately after iodination, suggesting that most of the iodinated glucagon purified by these means was fully immunoreactive.

However, there was a possibility that most of the immunoreactive material was damaged during iodination, either by polymerisation or partial breakdown. Such damage, it was considered, might have been so extensive that the peak of eluted radioactivity no longer corresponded to the peak of undamaged glucagon.

This was investigated by incubating similar concentrations of iodinated glucagon from different

fractions from the column with the same non-saturating concentration of antiserum, to investigate whether the material from each fraction was bound to the same extent.

The G. 25 elution profile of the fractions used in this experiment is presented in Fig. 13, and the results of the assay in Table 2. These suggest that the cross-reactivity with antibody was approximately the same for each fraction although there was an increase in non-specific binding in the first fraction (fraction 7) suggesting that this contained more damaged material than subsequent fractions.

3. The temperature of assay.

a) Insulin assay.

Fig. 14 presents B/F versus B plots of a series of insulin assays at different temperatures. The slope of the linear parts of the curves was greater at low temperatures than at high, suggesting that the affinity of the antibody-insulin reaction decreased with increase in temperature. Each curve departed from linearity at high concentrations of bound hormone. If it is assumed that the initial slope of the plots was a valid measure of an 'average equilibrium constant' (K_{ab}), then the data can be used to derive the heat of the reaction (ΔH) by plotting $\log_{10} K_{ab}$ against 1/T. This plot (the van't Hoff plot) should be linear, and of slope $\frac{-\Delta H}{2.303\ R}$. A value of ΔH for the reaction of insulin with antibody of -4.1 kcal./mole was derived in this way. The free energy change at 4^{o}, derived from K_{ab}, was calculated as -15.5 kcal./mole, and the corresponding entropy change as + 41 E.U.

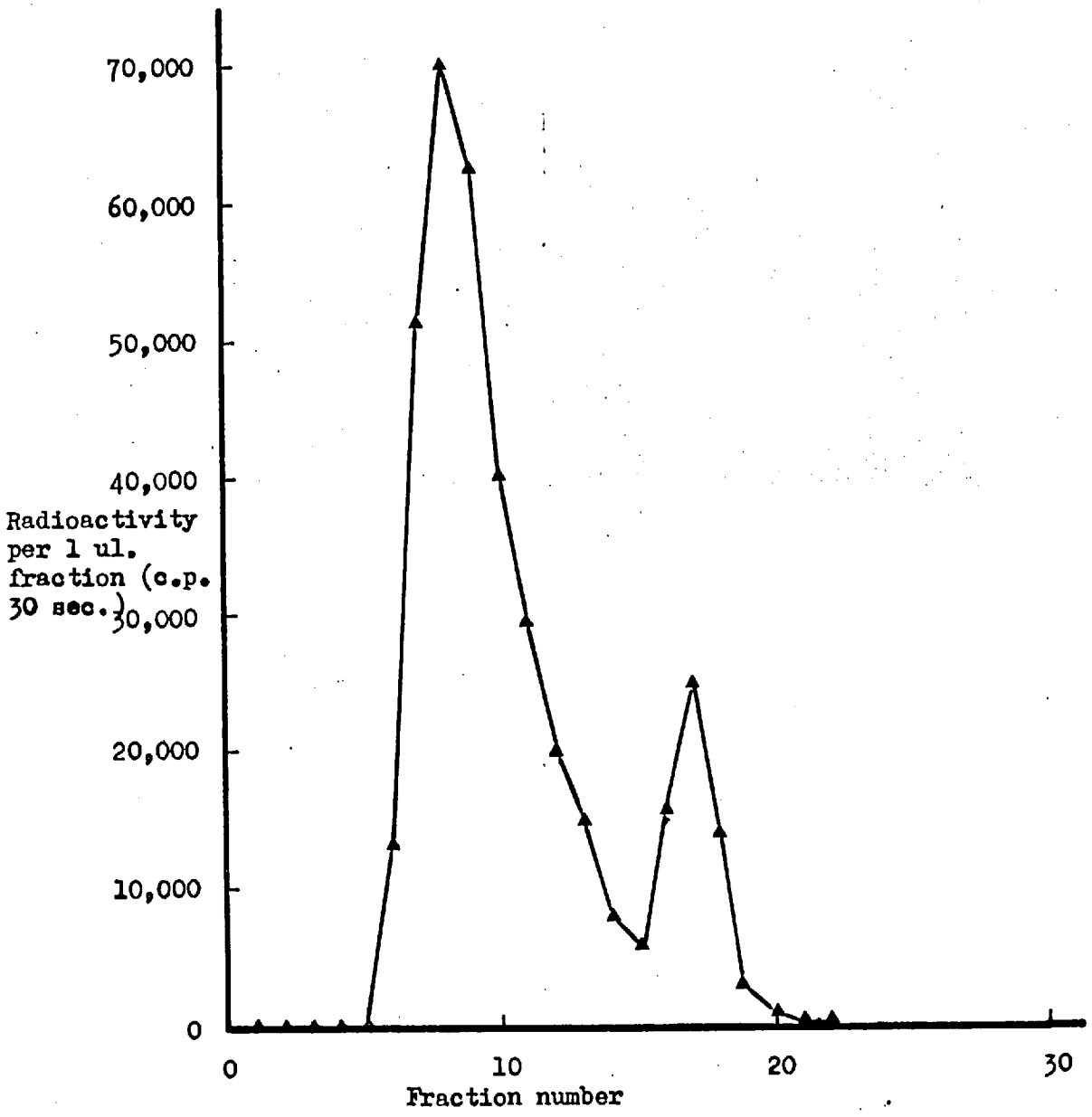


Fig. 13. SEPHADEX FILTRATION OF THE PRODUCTS OF A GLUCAGON IODINATION.

Glucagon (5.0 µg.) was iodinated with ^{125}I (0.989 mC), as described above. The iodination products were transferred to a G. 25 (fine) Sephadex column (21 ml.), pre-equilibrated with 0.01 M veronal-acetate buffer, pH 8.6, containing 1% (w/v) albumin. Fractions (1.2 ml.) were eluted from the column with the same buffer, and equal volumes (1 µl.) taken into Microcaps for counting.

In this experiment, the yield (in terms of the percentage of ^{125}I coupled to glucagon) was 83%, and the product had a specific activity of 163 C/g.

Table 2. THE IMMUNOLOGICAL PROPERTIES OF DIFFERENT ELUTED FRACTIONS OF IODINATED GLUCAGON.

Fraction	Non-specific binding (% of added counts)	% of added counts bound specifically by antibody
7	17.6	42.0
8	12.5	42.9
9	9.0	46.2
10	10.0	43.3
11	9.9	52.1

Glucagon was iodinated and eluted from a G. 25 Sephadex column. (The elution profile from this experiment is presented in Fig. 13) Eluate fractions 7 to 11 (i.e. the main fractions containing eluted glucagon) were diluted to a glucagon concentration of 1.12 to 1.13 ng./ml., and equal volumes (75 µl.) were incubated (in replicates of four) with veronal assay buffer (75 µl.), to measure non-specific binding, and in addition, with serum 4B, diluted 1 in 500 (75 µl.). This dilution was chosen to give a ratio of bound to free hormone of 1.2 in each tube. After incubating for 24 hours at 4°, the tubes were ethanol precipitated, and counted in an automatic counter.

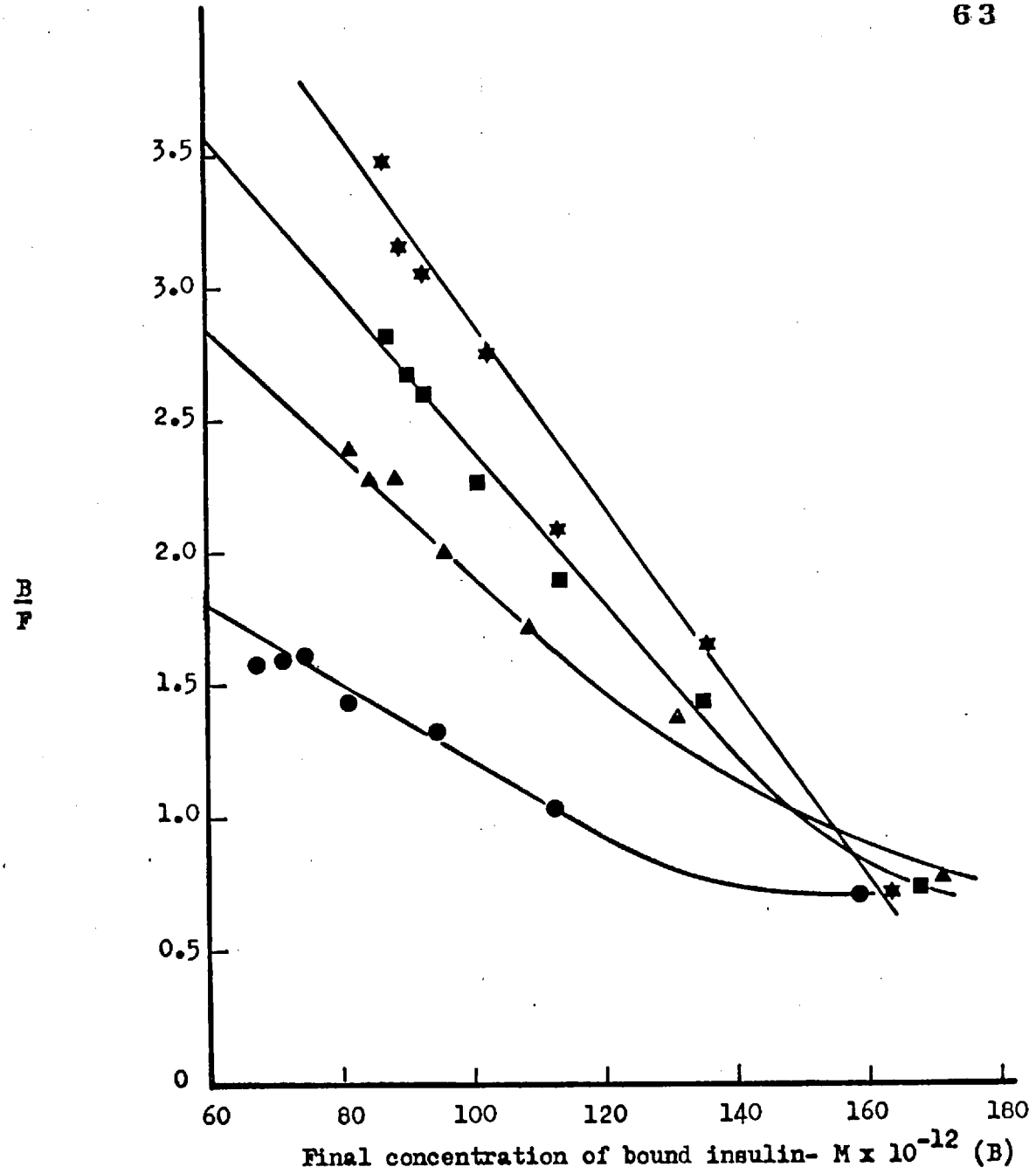


Fig. 14. PLOTS OF THE RATIO OF BOUND TO FREE INSULIN (B/F) AS A FUNCTION OF THE FINAL CONCENTRATION OF BOUND INSULIN (B) FOR INSULIN ASSAYS AT DIFFERENT TEMPERATURES.

Identical assays were incubated at the stated temperatures in stoppered tubes to prevent evaporation. In each assay, serum 8 (50 µl.) was pipetted at a dilution of 1 in 30,000, and iodinated insulin (50 µl.) at an (uncorrected) concentration of 2.5 ng./ml. The standard solutions of unlabelled insulin (50 µl.) were pipetted in replicates of five. After two days incubation, the tubes were rapidly cooled to 4°, and immediately ethanol precipitated, and counted in an automatic counter.

The slopes of the linear parts of the B/F versus B plots were as follows:

Symbol:	Temperature:	Slope(l./M x 10^9)
(-✱-✱-)	4°	34.5
(-■-■-)	15°	29.1
(-▲-▲-)	24°	23.9
(-●-●-)	37°	14.5

b) Glucagon assay.

In an early experiment with serum 4B, a glucagon immunoassay was carried out at three different temperatures. It was found that the initial assay slope was higher (2.33% of the added counts per ng./ml. standard glucagon) when the assay was carried out at 4^{o} than if it was carried out at room temperature (initial slope 1.44%) or at 37^{o} (initial slope 0.74%).

No assessment of maximum specific binding was made in this experiment, so it was repeated, including this measurement at each incubation temperature. The results, in the form of the resulting B/F versus B plots, are found in Fig. 15.

The maximum counts that could bind varied from 62% at 4^{o} to 53% at 37^{o}, showing that glucagon was degraded during incubation to a greater extent at higher temperatures. It was found that the slope of both the assay and B/F versus B plots was greater at 4^{o} than at higher temperatures, as for the insulin assay described above. However, the B/F versus B plots were non-linear and so could not be used to derive a value for the equilibrium constant.

An empirical assessment of the heat of reaction of glucagon with antibody was made by measuring the slopes of the B/F versus B curves at three fixed fixed values of B. The resulting Van't Hoff plots are illustrated in Fig. 16. These plots were approximately parallel straight lines, giving slopes which could be used to calculate values of 'ΔH' of -8.19, -5.78 and -6.25 kcal./mole at B = 200, B = 300 and B = 500 x 10^{-12} M respectively.

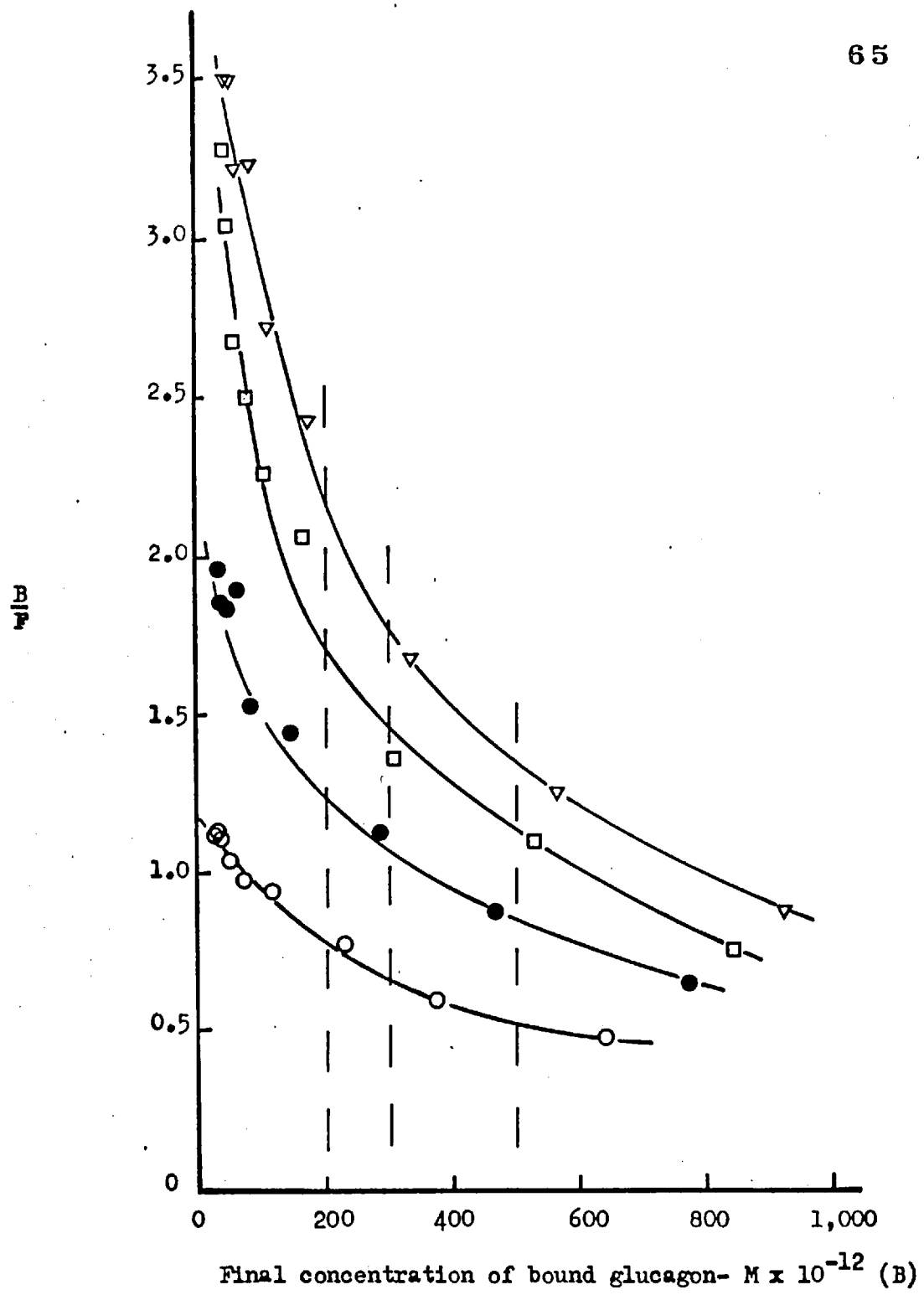


Fig. 15. PLOTS OF THE RATIO OF BOUND TO FREE GLUCAGON (B/F) AS A FUNCTION OF THE FINAL CONCENTRATION OF BOUND GLUCAGON (B) FOR GLUCAGON IMMUNOASSAYS AT DIFFERENT TEMPERATURES.

Identical assays were incubated at the stated temperatures in stoppered tubes to prevent evaporation. In each assay, pooled antiglucagon serum (50 µl.) was pipetted at a dilution of 1 in 1,000, and iodinated glucagon (50 µl.), at an (uncorrected) concentration of 1 ng./ml. The standard solutions of unlabelled glucagon (50 µl.) were pipetted in replicates of five. After three days incubation, the tubes were rapidly cooled to 4°, immediately ethanol precipitated, and counted in an automatic counter.

(▽–▽–▽) 4° (●–●–●) 24°

(□–□–□) 15° (○–○–○) 37°

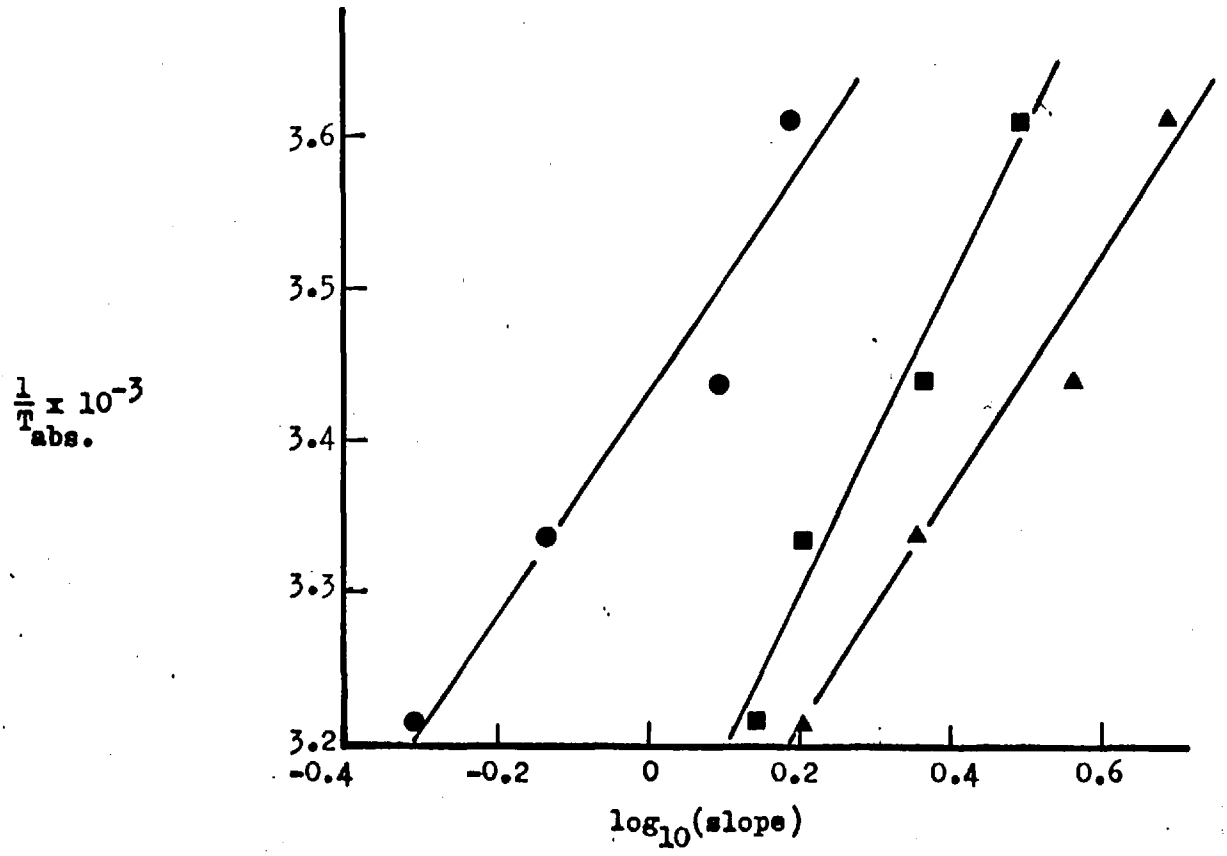


Fig. 16. 'VAN'T HOFF PLOTS' DERIVED FROM DATA FOR GLUCAGON IMMUNOASSAYS AT DIFFERENT TEMPERATURES.

The slopes of B/F versus B plots for glucagon immunoassays at different temperatures were measured at different values of B, and are plotted as a function of the temperature of assay. Experimental details of the assays are presented in Fig. 15 .

(●–●–●) B = 500 M/l. x 10^{-12}

(■–■–■) B = 300 M/l. x 10^{-12}

(▲–▲–▲) B = 200 M/l. x 10^{-12}

c) Immunoassay below the freezing point of water.

The negative heats of reaction indicated that the equilibrium constant and the precision of immunoassays would be increased significantly by incubating at temperatures below 0^{o}, and experiments were attempted to achieve this.

An initial experiment was performed at -12^{o} in a 35% w/v solution of glycerol, which was added to prevent freezing. Reproducibility was low, probably because the high viscosity of glycerol caused the amount of drainage after adding ethanol to vary from tube to tube. There was some evidence that the equilibrium constant of the reaction was somewhat lower than at 4^{o} in buffer, perhaps because glycerol altered the dielectric constant of the incubation mixture and so decreased the intensity of any salt-salt interactions that might have contributed to the stability of the glucagon-antibody reaction. In this respect, other alcohols would be expected to behave similarly.

Similar, but more reproducible results were obtained in an experiment in which 37.5% (v/v) ethanol was added to prevent freezing. Fig. 17 illustrates the resulting immunoassay curves obtained in the presence and absence of added ethanol at 4^{o} and with ethanol at -20^{o}. Ethanol lowered the slope of the assay curve considerably, even more in the sets of tubes incubated at -20^{o} than at 4^{o}.

These experiments showed that there was no gain in attempting to immunoassay at temperatures below 0^{o} using ethanol and glycerol to prevent freezing, because these substances interfere with the reaction of glucagon with antibody.

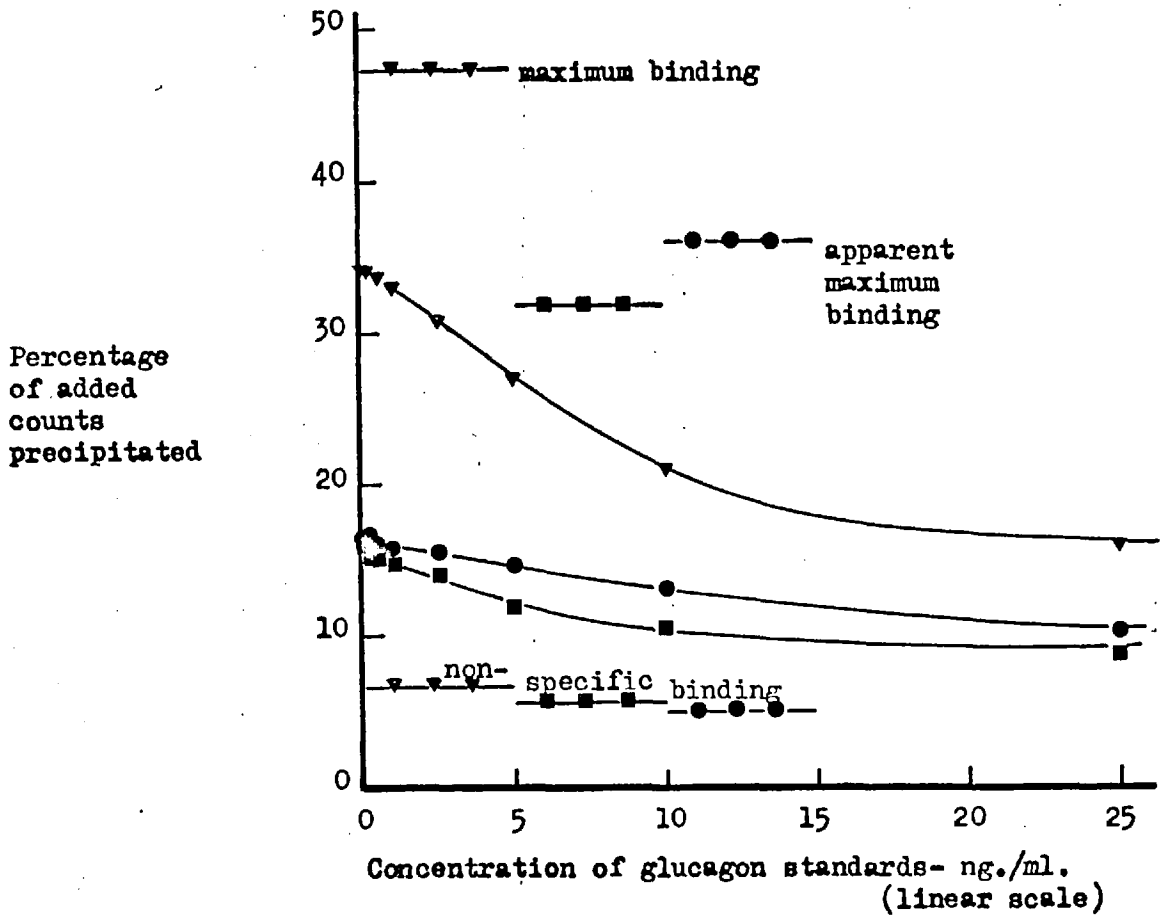


Fig. 17. THE IMMUNOASSAY OF GLUCAGON AT -20°, USING ETHANOL TO PREVENT FREEZING.

Identical assays were incubated in stoppered tubes to prevent evaporation, at 4° (—▼—▼—); at 4°, after adding ethanol (90 µl.) to each completed incubation tube, and mixing the contents carefully (—■—■—); and at -20°, after a similar addition of ethanol (—●—●—). In each assay, serum 4B (50 µl.) was pipetted at a dilution of 1 in 500, and iodinated glucagon at an (uncorrected) concentration of 1 ng./ml. Replicates of five tubes were used. After incubating for three days, the tubes were ethanol precipitated (with 0.65 ml. for the set of tubes without added ethanol, and with 0.56 ml. for the sets already containing ethanol), and counted in an automatic counter.

4. The kinetics of incubation.

a) Assay progress curves.

Figs. 18 and 19 illustrate experiments in which different concentrations of insulin and glucagon antisera were incubated with the respective iodinated hormone for different time periods. It is clear that both antibody-antigen systems showed increase in the rate of reaction with increase in the concentration of antibody.

It would be predicted from this result, although this was not investigated directly, that the rate of reaction should also increase at higher concentrations of total antigen. If an immunoassay is carried out for an insufficient time to reach equilibrium, the assay will be further from equilibrium at low glucagon concentrations than at high, causing it to be of lower initial slope (and therefore sensitivity) than if it had been incubated for a longer period. For this reason it is important that immunoassay samples should be incubated until equilibrium is attained in the tubes with the lowest concentration of total antigen. In the kinetic experiment with insulin antiserum, equilibrium was achieved in all samples by six hours. In contrast, the reaction of glucagon with antiserum was slower, and equilibrium was not achieved at low concentrations of antiserum until twenty four hours. In order to allow sufficient time for equilibrium to be reached, insulin assay samples were usually incubated overnight, and glucagon assay samples for two days or more.

b) Pre-incubation.

Fig. 20 illustrates a glucagon immunoassay in which there was a twenty hour pre-incubation of standards with antiserum before adding iodinated hormone. The

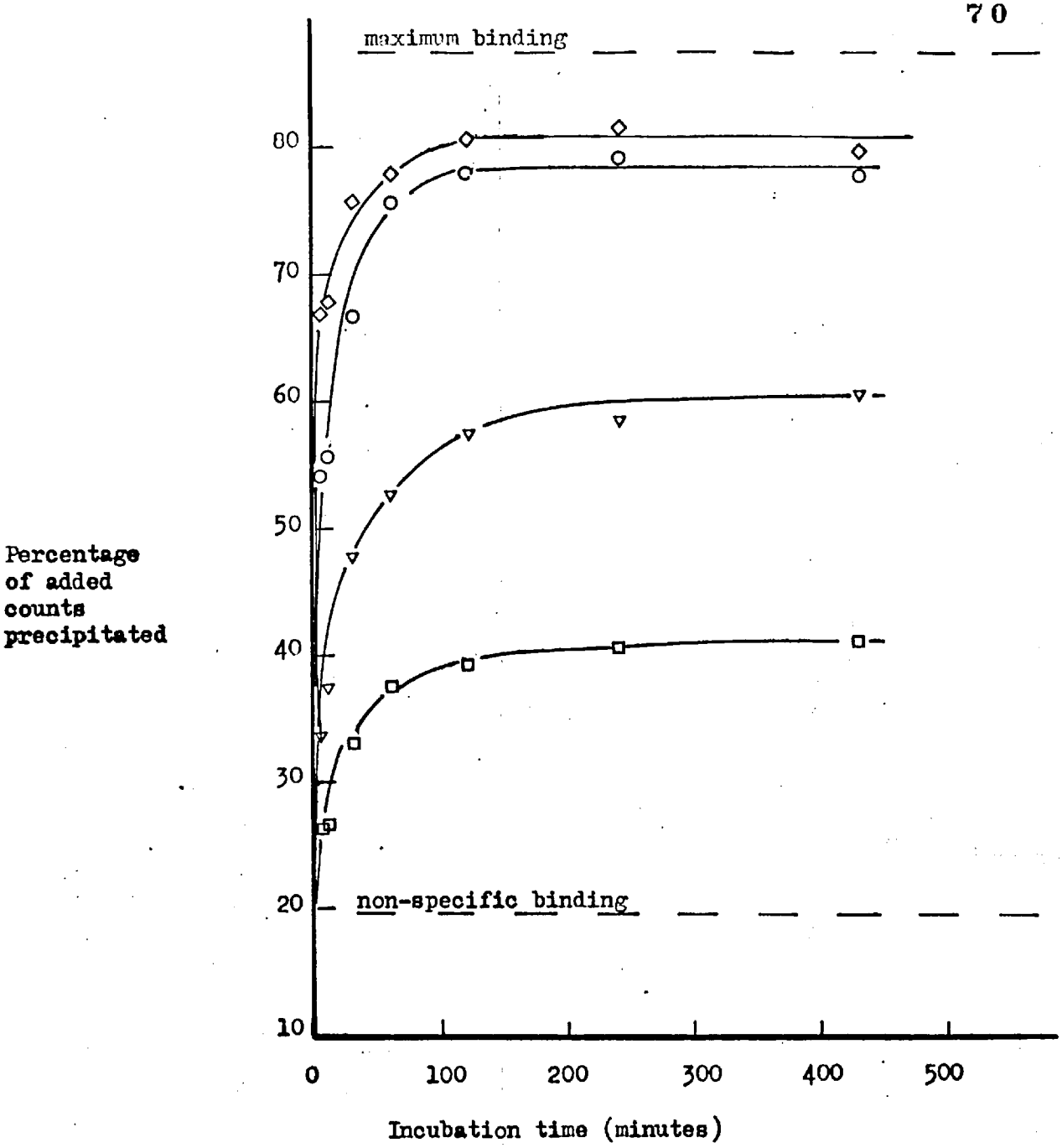


Fig. 18. KINETICS OF REACTION OF IODINATED INSULIN WITH VARIOUS CONCENTRATIONS OF ANTI-INSULIN SERUM.

Equal volumes (75 µl.) of a range of dilutions of anti-insulin serum 8 were incubated at 4°, in replicates of three, with the same volume of iodinated insulin (concentration, uncorrected for damage, 2.5 ng./ml.), for various times from 5 min. to 2 days. At the end of the incubation period, the tubes were ethanol precipitated, and counted in an automatic counter.

(–◇–◇–) antiserum diluted 1 in 10,000
(–○–○–) antiserum diluted 1 in 20,000
(–▽–▽–) antiserum diluted 1 in 50,000
(–□–□–) antiserum diluted 1 in 100,000

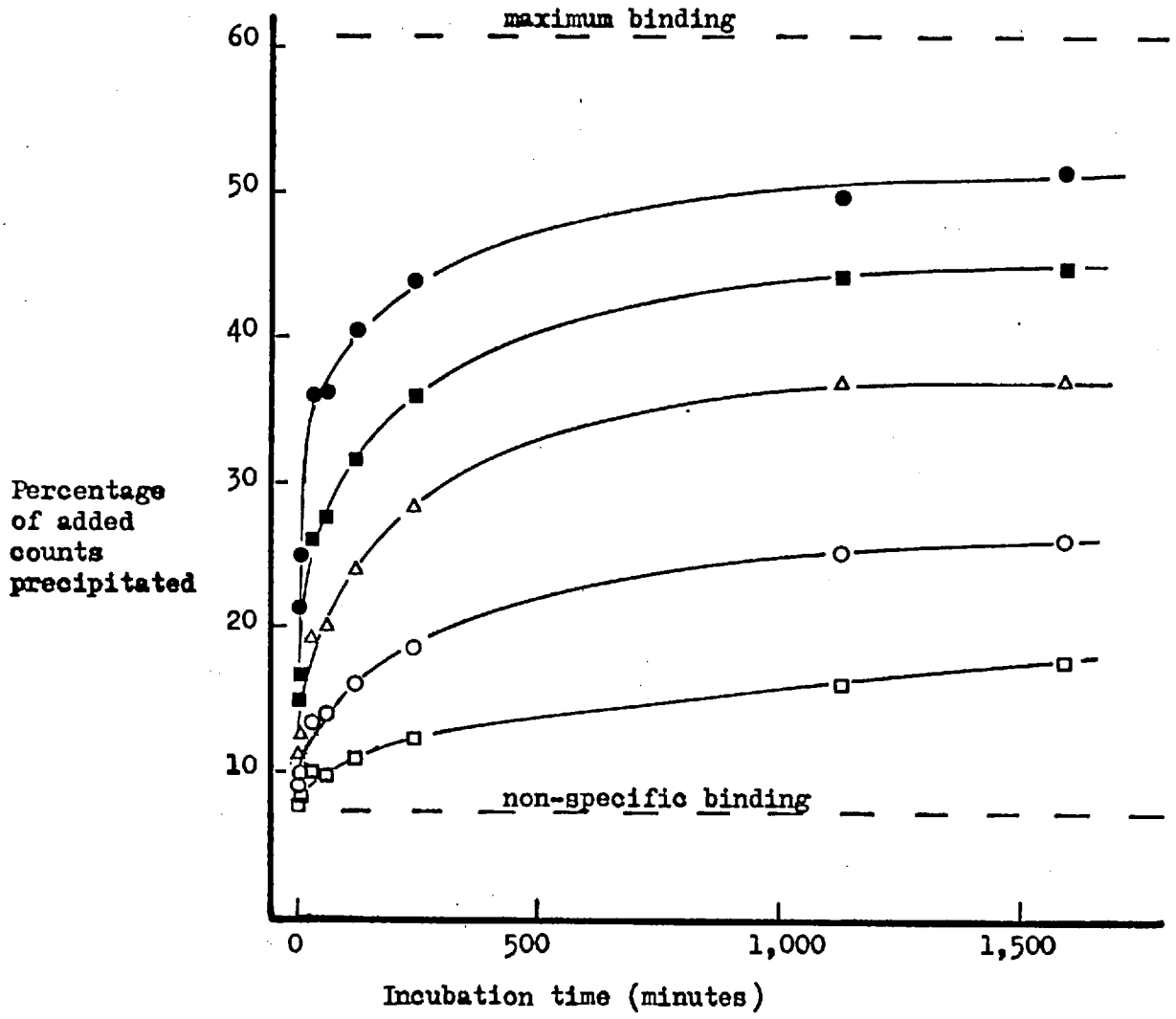


Fig. 19. KINETICS OF REACTION OF IODINATED GLUCAGON WITH VARIOUS CONCENTRATIONS OF ANTIGLUCAGON SERUM.

Equal volumes (75 µl.) of various dilutions of pooled antiserum from 1 in 200 to 1 in 5,000, were incubated, in replicates of three, at 4°, for various times from 5 min. to 4 days, with the same volume of iodinated glucagon (uncorrected concentration, 1.4 ng./ml.). At the end of the incubation period, the tubes were ethanol precipitated and counted in an automatic counter.

(-●-○-) antiserum diluted 1 in 200
(-■-□-) antiserum diluted 1 in 500
(-△-△-) antiserum diluted 1 in 1,000
(-○-○-) antiserum diluted 1 in 2,000
(-□-□-) antiserum diluted 1 in 5,000

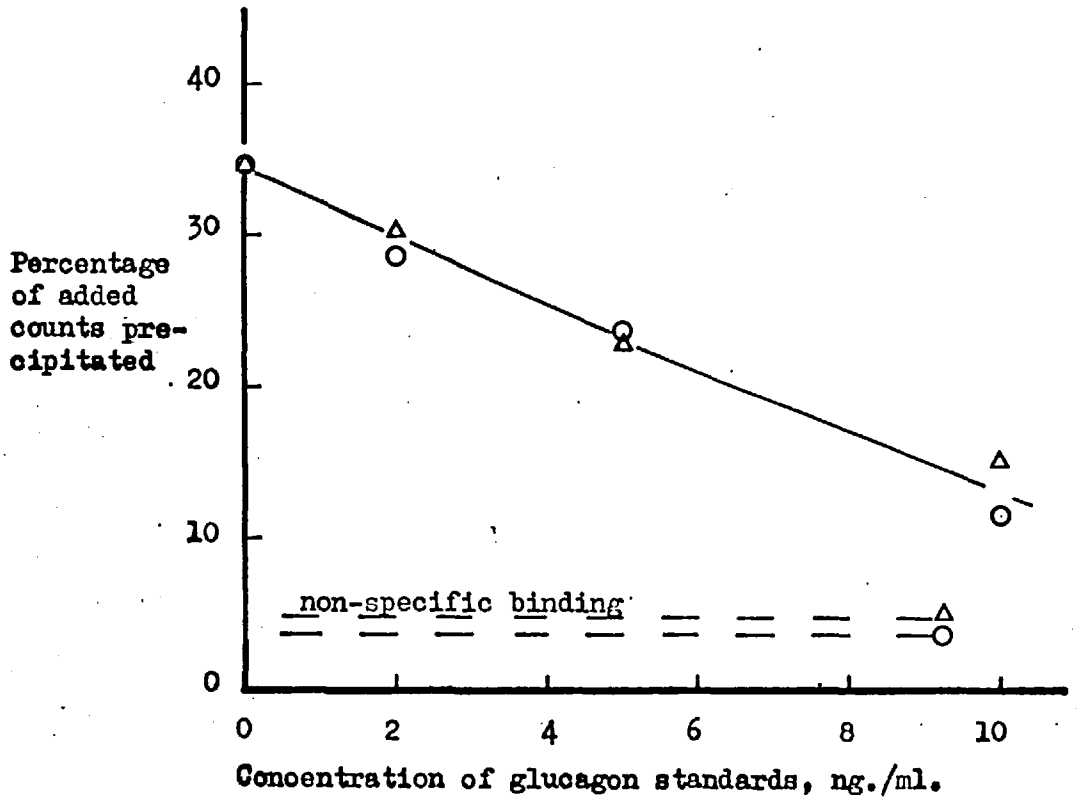


Fig. 20. THE EFFECT OF PRE-INCUBATION OF ANTIBODY WITH UNLABELLED HORMONE ON THE STANDARD CURVE OF A GLUCAGON IMMUNOASSAY.

Two assays were performed. In one, (Δ–Δ–Δ), standard hormone solutions were pre-incubated with antiserum for 20 hours before addition of ^{125}I-glucagon, while in the other, (O-O-O) radioactive and standard glucagon solutions were pipetted before addition of antiserum (as usual). In both assays, rabbit antiserum 4B was diluted 1 in 400, iodinated glucagon was pipetted at a concentration of 1.7 ng./ml., the assay medium was identical to veronal assay buffer, but was of pH 8.2, replicates of three tubes were used, and the total incubation time after adding antiserum was 48 hours. At the end of this period, the tubes were ethanol precipitated, and the precipitates were counted in a manual counter.

experiment was performed with the same reagents and at the same time as an assay without pre-incubation, in the same buffer. There was no apparent difference between the two curves and it seems that there is therefore no improvement to be gained from pre-incubation.

This finding can be explained on the grounds that the low equilibrium constant of the anti-glucagon sera raised in this work was caused by a high rate of dissociation of antibody-antigen complex rather than by low rate of complex formation. This would lead to a rapid exchange of radioactive hormone with cold hormone as soon as this were added to the incubation samples, so that the end results would be the same binding as if radioactive hormone had been added at the beginning of the incubation.

F. THE SEPHADEX FRACTIONATION OF SERA.

The finding of Worobec *et al.* (1967) that glucagon antibodies raised early in an immunisation schedule are mercapto-ethanol sensitive (i.e. are probably 19S), but within a month are replaced by antibodies which are not sensitive to mercepto-ethanol, suggested that antibodies of more than one class may be present in the sera raised during this work. Accordingly, glucagon and insulin antisera were fractionated on Sephadex in an attempt to discover the class or classes of antibodies present, and to investigate whether there was any difference in affinity between antibodies of different classes.

1. Insulin antiserum.

Fig. 21 presents the elution pattern of pooled insulin antiserum, fractionated on G. 200 Sephadex. The highest concentration of antibodies was eluted in a peak centered about fraction 21 (peak B). In addition there was a small peak at fraction 32 (peak D).

The protein of peak B was of higher molecular weight than albumin (mol. wt. 50,000) (peak A) but of lower molecular weight than IgM (mol. wt. 1,000,000) which would be expected to run at the 'front' corresponding in position to peak C. For these reasons peak B probably contained 7S antibodies. It was too broad for there to be a separation of IgG (mol. wt. 140,000) from IgA (mol. wt. 160,000) or IgE (mol. wt. 200,000), both of which would be expected to be present in small proportions only. The absence of a high molecular weight antibody peak contradicts the findings of other workers who report the presence of IgM in insulin antisera. According to Christiansen (1970), IgG

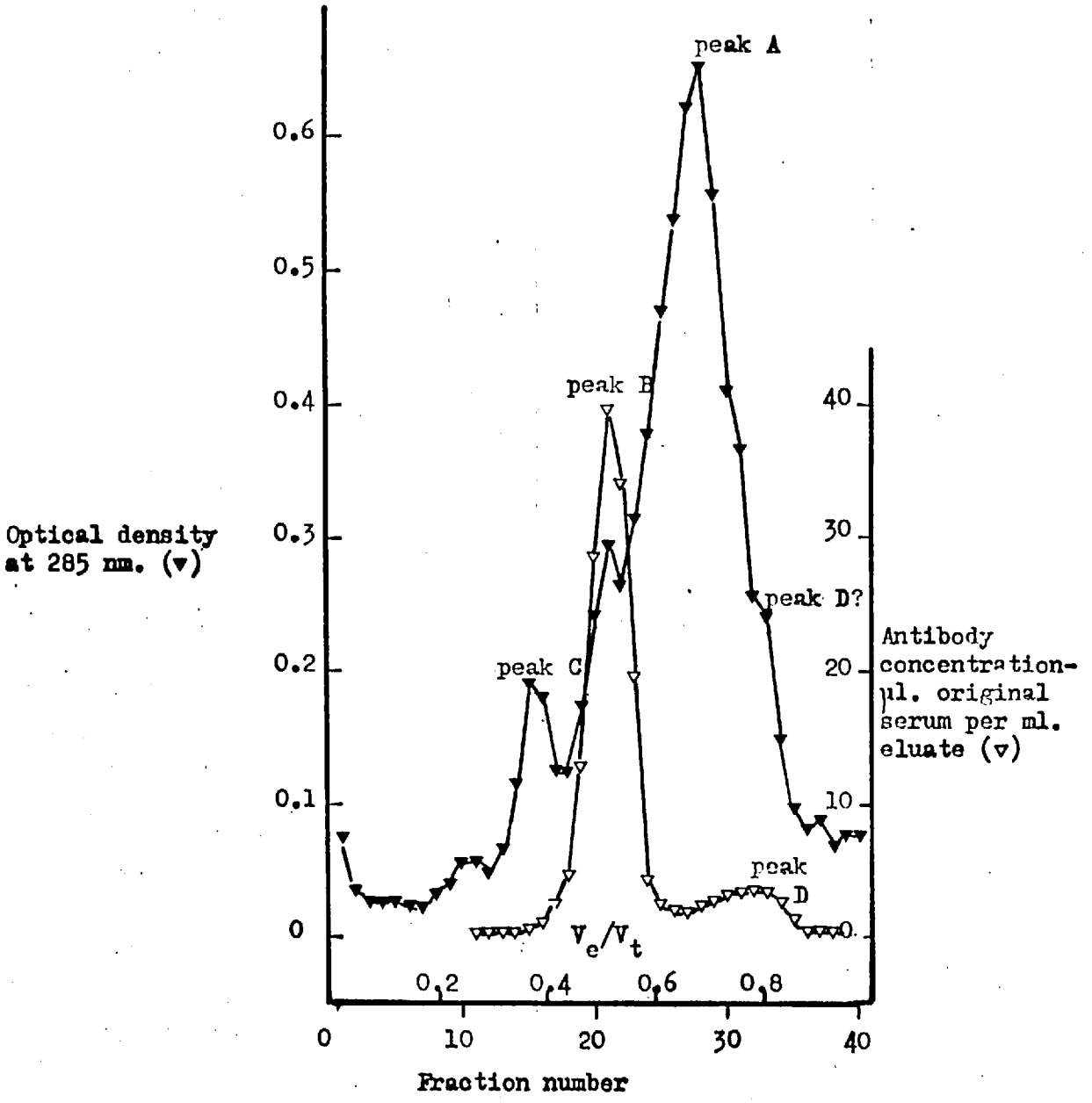


Fig. 21. THE FRACTIONATION OF ANTI-INSULIN SERUM ON G. 200 SEPHADEX.

Pooled anti-insulin serum (0.5 ml.) was fractionated on a column of G. 200 Sephadex (130 ml.), pre-equlibrated with 0.01 M veronal-acetate buffer containing 0.114 M NaCl, and 0.025% (w/v) thiomersalate, by eluting with the same buffer. The optical density of each fraction was measured in 1 cm. cells against the elution buffer, at a wavelength of 285 nm. The fractions were assayed for insulin antibodies at a dilution of 1 in 25.

and IgM insulin antibodies are present in sera in a ratio of about 7 to 1.

Peak D contained immunoglobulins of apparently lower molecular weight than albumin, and must, therefore, have consisted of damaged IgG antibodies similar to F_{ab} fragments.

There was a possibility that the antibodies in peak D were of different affinity from those in peak B. The original serum and fractions 21 and 32 were used in immunoassays. B/F versus B plots derived from these assays were linear at insulin concentrations below 5.0 ng./ml. and had approximately parallel slopes giving equilibrium constants of 16.6, 11.8 and 12.9 $1./M \times 10^9$ respectively.

2. Glucagon antiserum.

The results of a fractionation of glucagon antiserum are presented in Fig. 23, and show that the separation was very similar to that of insulin antiserum, except that there was no minor peak equivalent to peak D, and an additional small shoulder of high molecular weight, indicating the presence of small amounts of IgM. Glucagon antiserum, therefore, like insulin antiserum, probably contained IgG almost exclusively.

Whereas the amount of protein in peak A (which was thought to contain albumin) was the same for both fractionations, that in peak B (corresponding to IgG) was larger for anti-glucagon serum than for anti-insulin serum. The differences may have been due either to the use of different adjuvants in the two species, or to species variation in blood composition. Antiglucagon serum was derived from rabbits treated with complete Freunds adjuvant, while the anti-insulin serum was obtained from guinea pigs treated with silicone

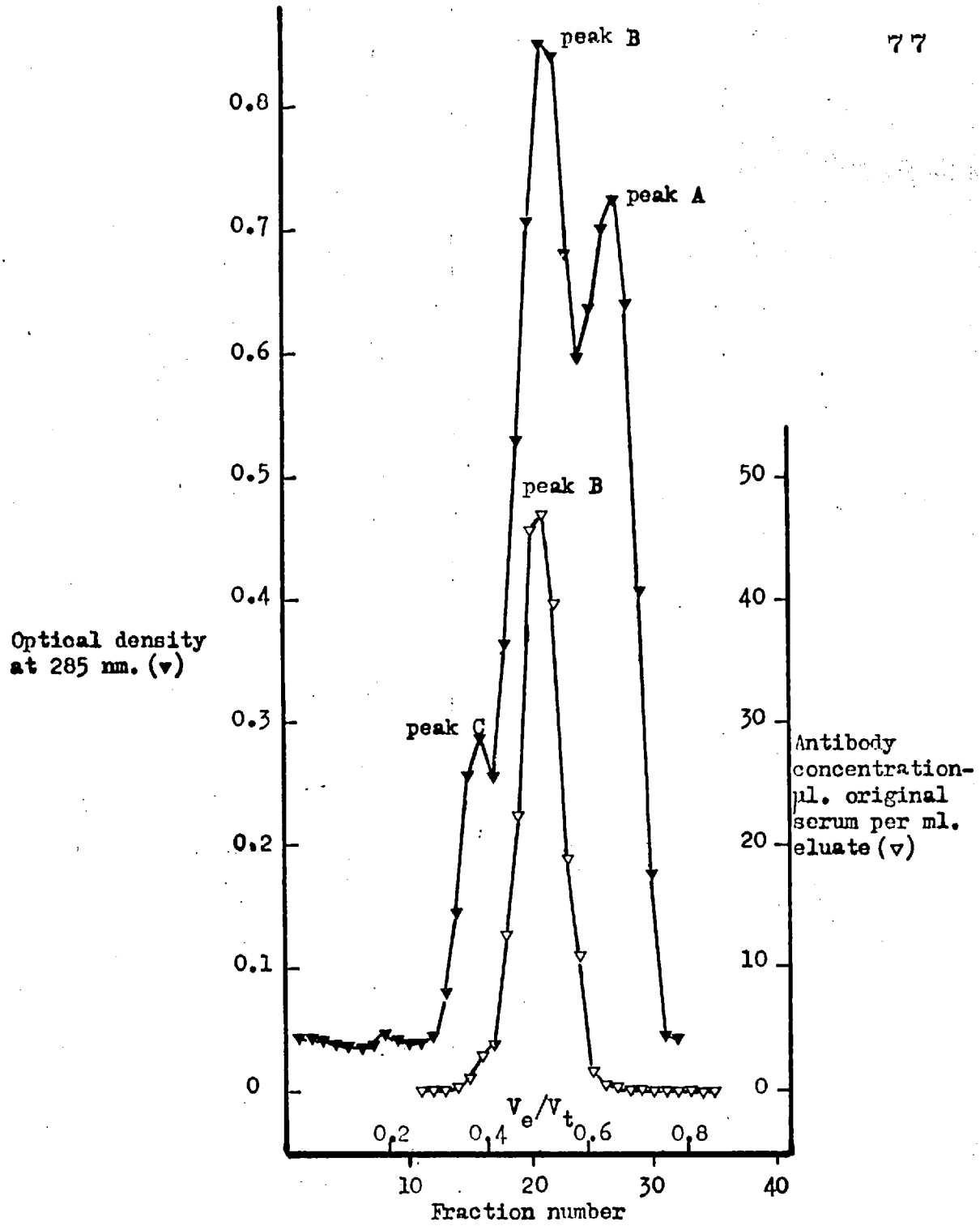


Fig. 22. THE FRACTIONATION OF ANTIGLUCAGON SERUM ON G. 200 SEPHADEX.

Pooled antiglucagon serum (0.5 ml.) was fractionated on a column of G. 200 Sephadex (130 ml.), pre-equilibrated with 0.01 M veronal-acetate buffer containing 0.114 M NaCl, and 0.025% (w/v) thiomersalate, by eluting with the same buffer. The optical density of each fraction was measured in 1 cm. cells against the elution buffer, at a wavelength of 285 nm. The fractions were assayed for glucagon antibodies at a dilution of 1 in 5.

oil/pertussis adjuvant.

As the glucagon antiserum appeared to contain significant amounts of one class of antibody only, immunoassays with various fractions were not attempted.

G. DISCUSSION.

In the present study, the ethanol Precipitation technique of Heding (1966) was extensively modified to make it suitable for the assay of both insulin and glucagon. Ethanol precipitation as a method for separating antibody bound from free hormones has several advantages over many other methods. For example, the precipitation is quantitatively complete, regardless of the concentration of gamma-globulin in the incubation mixture (Heding, 1966. 1971). In this respect, it differs from double antibody precipitation methods in which the relative concentration of the first and second antiserum is critical for a complete precipitation. This means that the method can be used for the quantitative measurement of antibody concentrations as easily as for the measurement of antigen.

Another advantage is that it is extremely rapid, enabling up to 1,000 samples to be prepared for counting in one day. In this respect, it is an improvement on the double antibody assay of Hales and Randle (1963), which involves a large amount of tedious filtering, and the chromatoelectrophoretic methods, in which each assay sample has to be individually spotted onto chromatography paper. However, it is no faster than the double antibody method of Morgan and Lazarow (1963) or any of the methods in which free antigen is adsorbed to a suspended powder.

It was decided to develop the ethanol precipitation method for the measurement of glucagon and insulin not only because of its convenience, but also because it was intended to use it principally for measurement of hormones released from isolated islets of Langerhans, into media containing low amounts of protein.

The assay was not found to be suitable for the measurement of glucagon and insulin in undiluted serum and plasma, because of the high non-specific binding that occurred in media containing high concentrations of proteins (Figs. 10 and 11). This effect of serum on non-specific binding has also been noted (in the case of insulin) by Welborn et al. (1967) and in the case of glucagon by Buchanan and McCarroll (1971).

The glucagon assay developed using ethanol precipitation was found to be very insensitive, in contrast to the insulin assay using the same technique, because the glucagon antisera raised during this work were of much lower binding affinity than insulin antisera.

Szabo and Mahler (1970) have concluded from a study of the influence of various substances on an insulin assay using talc separation, that arginine, leucine and epinephrine all interfere with the binding of insulin to antibody. It was therefore considered that some of the conditions of incubation that were originally used for the assay might not have been optimum for glucagon assay using ethanol precipitation, and that the apparent binding affinity of the sera might be increased by changing these conditions.

A study was undertaken in which the chemical composition of the assay buffer was varied to see if the apparent binding affinity might be thereby increased. Neither the nature of the buffer, nor the concentration and type of protein in the assay had a direct effect upon the binding of glucagon to antibody, although there was a clear effect of each on the non-specific binding. Thus Fig. 8 shows that the hydrogen ion concentration did not influence the sensitivity of glucagon assays over the pH range 6.5 to 9.0. If there had been a significant difference in sensitivity at different pH values this would have been manifest as a divergence

of the experimental curves in this Figure. There was, however, an effect of pH on the non-specific binding, which was lower at more alkaline pH values.

These results are at variance with those of other authors who have investigated the effect of pH on glucagon assays. Thus Grodsky et al. (1961), using a sodium sulphate salt precipitation technique to separate bound and free glucagon, found that the binding of ^{131}I glucagon to antibody increased linearly from pH 5.0 to pH 8.0, was maximum at pH 8.0 to 8.5, and plateaued at higher pH values. Shima and Foa (1968), using a double antibody method, found maximum binding at pH 7.4, with a slight decline to pH 10.0. Schopman et al. (1967), using hydrodynamic flow technique for separation, found a clear maximum binding at pH 9.0 and a sharp decline at higher pH values.

The differences between the results of these various authors and the results of this study are perhaps explained by the difference in the method used to separate bound and free hormone, which may also be influenced by pH in various ways.

Figs. 7 and 9 show assays in tris buffer, veronal acetate buffer, and phosphate buffer. The assays in tris and veronal buffer were identical, whereas in phosphate buffer there was a higher non-specific binding, but the assay was otherwise similar. These results suggest that the specific binding of glucagon to antibody is not influenced by the nature of the buffer in the incubation medium.

A study was carried out in which albumin in the assay buffer was replaced by casein. Albumin inhibits the binding of glucagon to materials such as glass, Sephadex and cellulose, and this fact suggested the possibility that albumin might bind glucagon, causing the equilibrium constant of the glucagon-antibody reaction to be artificially depressed.

Fig. 12 illustrates two assays, one in a casein and the other in an albumin-containing buffer. The assays were carried out at the same time and were otherwise identical. The two curves were very similar, and the slopes of B/F versus B plots of the data were the same. The only slight effect of changing protein was on the non-specific binding, which was found to be significantly lower in the casein assay.

In addition to an investigation of the chemical composition of the immunoassay buffer, the effects of temperature and kinetics of incubation on assay sensitivity were also studied (Figs. 14-20).

The equilibrium constants, and therefore the maximum possible sensitivities, of immunoassays were found to vary with the temperature of incubation, decreasing at higher temperatures. From the data that were obtained in this study it was possible to calculate values for the heat of reaction of glucagon and insulin with antibody (-6 and -4 kcal./mole respectively). These values were similar to values reported by Karush (1956, 1958) for the reaction of p-azo phenyl-β-lactoside and (p-azo benzyl amino)-acetate with specific antibodies (-9.17 and -7.2 kcal./mole respectively). In this latter investigation, antibodies were raised by coupling these substances to bovine gamma globulin, and equilibrium constants were measured at two temperatures by equilibrium dialysis. The reported free energy and entropy changes were however very different from those for insulin obtained in the present work. This was because the equilibrim constants were much lower for the low molecular weight antigens used in that study than for the reaction of either glucagon or insulin with specific antibody.

It can be calculated that a reacton with a heat of reaction of -6 kcal./mole would have an equilibrium constant which was three times as high if carried out

at -25^{o} than at 4^{o}. Attempts were, therefore, made to perform immunoassays at temperatures below 0^{o}, using glycerol and ethanol to prevent freezing. These attempts did not lead to improvement in sensitivity, largely because glycerol and ethanol were found to interfere with antigen-antibody binding (Fig. 17).

One method that has been used to increase immuno-assay sensitivity is to pre-incubate antibody with cold hormone before adding radioactive hormone. By this means the antiserum is partially neutralised when unlabelled hormone is added, so that there are more sites for binding radioactive hormone in samples with a low unlabelled hormone concentration than in those with a high concentration.

The Radiochemical Centre (1967) and Hales and Randle (1963) have both described assays in which unlabelled insulin was pre-incubated with antiserum for six hours before adding radioactive hormone; the latter authors claiming that this leads to a higher initial assay slope, and therefore an increase in sensitivity.

When a twenty hour pre-incubation was carried out for a glucagon assay (the period of twenty hours was chosen deliberately longer than that described for insulin because of the slower kinetics of the glucagon system), the results (Fig. 20) showed that the pre-incubation curve was identical to a comparative curve in which all reagents were added together. There is nothing therefore to be gained from a pre-incubation carried out under the conditions used. One explanation for this may be that there was rapid association and dissociation of glucagon from antibody, so that equilibrium was attained whether pre-incubation was used or not.

The failure of attempts to increase sensitivity significantly by altering the assay conditions suggested attempts to improve the assay by selectively purifying one of the classes of glucagon antibodies using Sephadex filtration. It was considered possible that one of these would be of higher affinity than the others. However, almost all the antibodies from glucagon antisera ran as a single peak, suggesting that they were all of similar molecular weight, and therefore it was not possible to improve the assay by this means either.

To summarise, many different attempts were made during the course of this work to increase the sensitivity and to lower the assay concentration range of the ethanol precipitation assay that was developed for glucagon.

None of these attempts lead to a significant improvement, and it is concluded that the initial slope was not limited by the assay method used, but by the low binding affinity of even the best sera raised during the work.

Subsequent attempts were made to improve the assay sensitivity by raising antisera of higher binding affinity, and purifying high affinity antibodies specifically, using immunoadsorbents. Experiments in which these approaches were tried are described in subsequent Chapters.

CHAPTER 2.

THE PRODUCTION OF GLUCAGON ANTIBODIES.

A. INTRODUCTION.

The sensitivity of an immunoassay depends predominantly on the avidity of antibody binding sites for the antigen assayed, so it is of great importance to produce antiserum with the correct properties. The glucagon immunoassay developed in this study was of insufficient sensitivity to measure low concentrations, and it was, therefore, proposed to vary the method and species of immunisation in an attempt to increase the affinity and concentration of the antibodies obtained.

There are many reports in the literature of different methods of immunisation to produce suitable glucagon antiserum. These are reviewed in Table 3. It is of interest to note that, with few exceptions, there have been no attempts to assess the relative efficiency of the various immunisation procedures either in terms of average titres or equilibrium constants obtained or in terms of the proportion of immunised animals giving a serum with useful titre or affinity. It is, therefore, difficult to evaluate whether any specific method of immunisation is more successful than any other, or whether the production of a suitable antiserum is the result of chance.

Many workers who are active in the field have commented upon the difficulties involved in producing useful antisera to glucagon. Thus Unger and Eisentraut (1969) state that "difficulties in producing a useful antiserum for glucagon immunoassay have plagued many investigators". Yalow and Berson (1969b) state that "porcine, bovine glucagon is so poorly antigenic in both guinea pigs and rabbits that many workers active in the field of glucagon assay have used the one antiserum (produced by Assan *et al.*,

Table 3. METHODS OF PRODUCING GLUCAGON-ANTIBODIES

Abbreviations:

R	rabbit	SC	subcutaneous
GP	guinea pig	FP	footpad
CFA	complete Freund's adjuvant	IM	intramuscular
IFA	incomplete Freund's adjuvant	IV	intravenous
PVP	polyvinyl pyrrolidone	s	sensitivity
D	daily	r	range
W	weekly (3D every three days)		
M	monthly (6M every six months, etc.)		
I	irregularly		

Reference	Quantity of total glucagon administered per injection per animal, and the nature of the immunogen used	Species immunised	Injection sites	Frequency of injection	Range or sensitivity of immunoassay (pg./ml.)
Unger, Eisentraut, McCall & Madison (1961)	1 mg. glucagon in CFA	R	SC & FP	M and less frequently	s = 100
Grodsky, Hayashida, Peng & Geschwind (1961)	3-10 mg. glucagon coupled to ovalbumin with bis-diazobenzidine, in CFA	R	SC	W, then M then I	s = 500
Assan, Rosselin, Drouet, Dolais & Tchobroutsky (1965)	1-2 mg. glucagon either coupled to rabbit serum albumin or guinea pig serum albumin with 'Morpho C.D.I.', or coupled to PVP. Emulsified in CFA	R & GP	SC & FP	W	s = 100
Probst & Colwell (1966)	0.3-0.6 mg. alum-precipitated glucagon in CFA	R & GP	multi-site SC & FP	2W & M	s = 200,000
Braun (1966)	glucagon. Quantity unstated	GP	multiple portal	unstated	used for injection
Lawrence (1966)	glucagon in CFA quantity unstated	R & GP	unstated	W?	s = 300
Heding (1967)	2 mg. alum-precipitated glucagon in CFA, then alum-precipitated glucagon in IFA	R	SC	W, then M	r = 1,000 - 10,000

Table 3. cont.

Reference	Quantity of total glucagon administered per injection per animal, and the nature of the immunogen used	Species immunised	Injection sites	Frequency of injection	Range or sensitivity of immunoassay (pg./ml.)
Schopman Hackeng & Steendijk (1967)	2.5-5 mg. glucagon adsorbed to $Al(OH)_3$, in CFA, and subsequently, free glucagon	R	SC for $Al(OH)_3$ ppd. glucagon then IV	M	s = 200
Worobec, Locke, Hall, Ertl, Ernst & Deininger (1967)	1 mg. glucagon emulsified in a mixture of CFA and beeswax	R	IM	2D	s = 100
Hazzard, Crockford, Buchanan, Vance, Chen & Williams (1968)	1 mg. glucagon in CFA	GP	SC	3D	s = 400
Lomsky, Langr & Vortel (1968)	2.5 mg. glucagon coupled to PVP & emulsified in CFA	R	SC	M	used for immunofluorescent studies
Assan, Rosselin, Tchobroutsky & Freychet (1969)	1-2 mg? glucagon coupled to human serum albumin with 'Morpho C.D.I.', or coupled to PVP, and emulsified in CFA	R	unstated	unstated	s = 15
Heding (1969)	1 mg. glucagon self-coupled with carbodi-imide, in CFA	R	SC	W	s = 100
Nonaka & Foa (1969)	Methods of Assan et al. (1965)	R	SC, FP & IM	W & more often?	r = 1,000 - 50,000
Grey, McGuigan & Kipnis (1970)	1.5-3 mg. glucagon coupled to hemocyanin with diethyl malonimidate, and emulsified in CFA	R & GP	FP	3M	s = 200 used for injection
Goldfine & Ryan (1970)	1 mg. glucagon coupled to rabbit serum albumin or guinea pig serum albumin, emulsified in CFA	R & GP	IM	W	s = 10 pg. or less (vol. not stated)

Table 3. cont.

Reference	Quantity of total glucagon administered per injection per animal, and the nature of the immunogen used	Species immunised	Injection sites	Frequency of injection	Range or sensitivity of immunoassay (pg./ml.)
Katsilambros, Rahman, Hinz, Fubganger, Schroder, Staub & Pfeiffer (1970)	method of Heding (1969)	unstated	unstated	unstated	s = less than 140
Leclercq-Meyer, Mailhe & Malaisse (1970)	3 mg. glucagon coupled to ovalbumin and emulsified in CFA	R	IM	3W	s = 30
Buchanan (1971)	1 mg. glucagon coupled or bound to various materials, including carbon & albumin, and then emusified in CFA	R	normal methods, also spleen & lymph	2W (6W then M for some animals)	s not stated but glucagon free & coupled to albumin gave highest titres
Cuatrecasas, Illiano & Green (1971)	40 µg. glucagon coupled to poly-L-lysine, emulsified in CFA	poly-L-lysine responder guinea pigs	FP, then intradermal	2W-M	dissociation constant $= 1\text{-}5 \times 10^{9}$ l./M

1965) with sufficient sensitivity to permit measurement of plasma hormone". Assan, Rosselin and Tchobroutsky (1969) state that "it has become obvious that methodological problems involving the sensitivity and specificity of the assay remain unsolved", and "many of the immune sera obtained do not exhibit a binding energy compatible with an immunoassay".

In most of the successful immunisation methods reviewed in Table 3 glucagon was covalently coupled to a carrier protein as immunogen, and it was proposed to immunise animals to glucagon linked to a carrier protein in this study also. A synthetic copolymer containing glutamate, tyrosine and alanine was provided for this purpose by Professor M. Sela of the Weizmann Institute. It was considered that the use of a carrier protein containing large amounts of tyrosine might have advantages over the use of natural carriers such as albumin, globulin, ovalbumin or haemocyanin, as it has been shown that attachment of peptides containing L-tyrosine to gelatine (which is a very poor antigen) enhances its antigenicity without changing the specificity of the antibodies formed (Arnon and Sela, 1960).

Synthetic copolymers have been used as carriers to enhance the antigenicity of many different haptens. (for a review see Sela, 1966). Of particular relevance are the results obtained by Benacerraf, Ojeda and Maurer (1963), who used a copolymer of similar composition to that used in the present study* to raise antibodies to arsanilic acid. All fifty nine guinea pigs immunised with a conjugate of arsanilic acid coupled to a copolymer containing L-glutamate, L-alanine and L-tyrosine produced antibodies to arsanilic acid, but those immunised with free arsanilic acid, or arsanilic acid coupled

*See Appendix for chemical composition.

to a copolymer containing D-amino acids in the same proportion gave no antibodies. Recently Cuatrecasas, Illiano and Green (1971) have achieved highly effective immunisation in guinea pigs using glucagon coupled to poly-L-lysine, confirming the value of synthetic co-polymers in raising antibodies to this hormone. Such studies suggested that glucagon linked to a synthetic copolymer might be a more effective immunogen than glucagon alone. It was, therefore, proposed to study the immune response to free glucagon, and glucagon linked to a synthetic copolymer.

It was also proposed to measure the immune response to glucagon immunogens when administered in different adjuvants. Mansford (1967) has shown that guinea pigs immunised to insulin in a silicone oil emulsion give a more consistent immune response and higher titres than when Freund's adjuvant emulsion was used. Wright, Makulu and Posey (1968) have shown that adjuvants containing killed H. pertussis organisms are very useful for raising high potency anti-insulin serum. It was, therefore, decided to carry out a limited study of the effectiveness of pertussis-silicone oil emulsion when used to raise antibodies to glucagon.

In contrast, it was not considered profitable to study the response to change in the route of administration. The biological variation in response to any immunisation technique for a poor antigen is very large. Thus Hurn and Landon (1971) have attempted to compare the response to intramuscular, intranodal and intraperitoneal routes of immunisation to ACTH. When assessed in terms of the number of rabbits responding to give significant titre after two injections, the intra-muscular technique was as effective or more effective than intranodal or intraperitoneal administration, although the three antisera with the highest effective affinity were produced by animals immunised intranodally. However,

despite the immunisation of nearly one hundred animals, the difference between the groups could not be regarded as statistically significant.

Hence a comparative study of different immunisation methods is not likely to yield definitive results unless very large numbers of animals are used, in excess of the numbers normally available.

B. MATERIALS.

1. Preparation of glucagon-copolymer conjugates.

The method used was that of Goodfriend, Levine and Fasman (1964). Synthetic copolymer (240 mg.)*, dissolved in 25% v/v dimethyl formamide (4 ml.), was added to a solution of glucagon (80 mg.) in 1 ml. of the same solvent. 16% w/v 'Ethyl C.D.I.' (1 ml.), also dissolved in 25% dimethyl formamide (1 ml.), was then added to the mixture.

After standing for two hours at 37°, the solution, which underwent no visible change, was dialysed against three changes of distilled water for twenty four hours and lyophyllised.

Recovery of protein was usually 90 - 105% by weight. The product was almost completely water-soluble, unlike glucagon itself, which precipitates if dialysed under the same conditions, indicating that the added glucagon had become completely conjugated. Amino acid analysis of a typical preparation indicated that 25.6% of the dry weight of the resulting glucagon-copolymer consisted of glucagon.

2. Preparation of emulsions.

a) Glucagon-copolymer emulsion.

The emulsion used for the immunisation of rabbits and goats contained glucagon-copolymer conjugate (100 mg.) dissolved in water (4 ml.) and emulsified with Freund's complete adjuvant (16 ml.).

For the immunisation of chickens and guinea pigs, glucagon-copolymer (80 mg.) was dissolved in water (4 ml.) and emulsified with Freund's adjuvant (16 ml.).

*See Appendix for chemical composition and physical properties of copolymer.

b) Emulsions containing unconjugated glucagon.

Glucagon (10 mg.) was dissolved in 0.25 M glycine-NaOH buffer, pH 8.8 (2.0 ml.), by warming to 37^{o}, and the solution was emulsified with Freund's complete adjuvant (8.0 ml.).

c) Insulin emulsion.

The method used was that of Mansford (1967). Emulsion constituents were added in proportion to the amounts quoted below:

Insulin (90 mg.) was dissolved in a solution of 0.8% phenol (w/v) in 0.03 M HCl (10 ml.). A suspension of H. pertussis containing 8 x 10^{10} killed organisms per ml. (5 ml.) was added, followed by an oil mixture (30 ml.) containing:

20 parts by volume silicone oil
1 part by volume Arlacel A (mannide mono-oleate)
20 parts by volume Isocreme.

The resulting suspension was emulsified. For subsequent injections, the suspension of H. pertussis was replaced by an equal volume of water.

d) Emulsification.

Emulsification was achieved by pumping oil-antigen mixtures backwards and forwards through an 18 gauge needle connected to a syringe at each end, two hundred times, using an air pump at maximum pressure of 30 lbs. per sq. in.

C. METHODS.

1. Immunisation of rabbits and goats with glucagon.

The immunisation schedule was the same whether an animal received unconjugated glucagon, or glucagon coupled to copolymer. Each animal received 1 ml. of emulsion in about twenty small doses, equivalent to a total of 1.25 mg. glucagon for the glucagon-copolymer injections, and 1.0 mg. glucagon for the injections of unconjugated glucagon.

The injections were made intradermally or subcutaneously down the flank and in the back of the neck, and were repeated at irregular intervals of about one month. A fortnight after the third injection the animals were bled. Rabbits were bled from the marginal ear vein or by heart puncture, while goats were bled from the jugular vein.

2. The immunisation of guinea pigs and chickens with glucagon.

Guinea pigs were injected in one site in the back of the neck with glucagon-copolymer emulsion (0.5 ml.). The immunisation schedule was identical to that for rabbits, and after the third injection the animals were bled by heart puncture.

The technique adopted for the immunisation of chickens was similar, in that they were treated with 0.5 ml. of emulsion at each injection, and according to the same immunisation schedule as guinea pigs. However, the injections were administered in small doses subcutaneously. The animals were bled from the wing vein.

3. The immunisation of guinea pigs with insulin.

On the first injection, each guinea pig received a subcutaneous injection in one site in the back of the neck of complete silicone oil-insulin emulsion (0.5 ml.), and thereafter incomplete emulsion (0.5 ml.). After each injection an interperitoneal injection of 25% glucose (5 ml.) was administered to counteract possible hypoglycemia, and the drinking water was replaced by 5% (w/v) glucose.

Each animal was bled after the second or third injection by heart puncture.

4. The treatment of immune blood samples.

Sera were allowed to clot at room temperature and then at 4° for about six hours, and were then centrifuged in a bench centrifuge. The aspirated serum was then either frozen, and stored at -25°, or stored at 4° after adding thiomersalate to a concentration of 0.025% w/v.

In the case of rabbits bled by heart puncture, the technique involved the intravenous injection of heparin, to give plasma as the product. This was stored at 4° for several months (after adding thiomersalate) to allow complete clotting to occur, and was then clarified by centrifugation, and frozen.

D. RESULTS.

1. A comparison of different immunisation methods in rabbits.

a) Immunisation with glucagon and glucagon copolymer.

In an initial study, two rabbits were immunised with glucagon coupled to bovine serum albumin, and the response compared with that of two rabbits immunised with glucagon coupled to synthetic copolymer. As can be seen from the results obtained after two immunisations (Table 4) antibodies were produced by both of the animals immunised with copolymer and one animal immunised with glucagon-albumin, suggesting that glucagon covalently linked to protein was potentially a useful immunogen for raising glucagon antibodies. Further immunisations were, therefore, performed in which animals were injected with glucagon copolymer.

Table 5 presents a detailed study of the response of six rabbits injected on three occasions with glucagon in comparison with seven rabbits injected with glucagon-copolymer at the same time. It will be seen from the results that there was relatively little difference between equilibrium constants of sera in the two groups, but it is of interest to note that the two sera with highest affinity were amongst those from animals immunised with glucagon copolymer. There is slight evidence that animals immunised with glucagon gave a higher titre of antibody, as two animals injected with glucagon-copolymer failed to produce any detectable antibody while all animals injected with glucagon gave a response. There appears to be no correlation between titre and equilibruum constant in either group: the two sera of highest equilibrium

Table 4. THE IMMUNISATION OF RABBITS WITH GLUCAGON LINKED TO ALBUMIN, COMPARED WITH THE RESPONSE TO GLUCAGON LINKED TO SYNTHETIC COPOLYMER.

Animal	Immunogen	Amount of conjugated glucagon administered	Total radioactivity precipitated specifically (% of maximum)		
			Serum diln. 1 in 20	1 in 200	1 in 2,000
1A	glucagon-copolymer	2 mg.	19.8	zero	zero
1B	glucagon-copolymer	1 mg.	87.2	14.7	zero
2A	glucagon-albumin	2 mg.	53.8	17.5	3.1
2B	glucagon-albumin	1 mg.	zero	zero	zero
NA	-	-	zero	zero	zero
NB	-	-	zero	zero	zero

Two rabbits were immunised by multi-site intradermal injection of glucagon covalently conjugated to bovine serum albumin (Fraction V) by means of Ethyl C.D.I.* The immunogen was administered as a suspension in Freund's adjuvant, and the animals were injected on days 1 and 48, and were bled on day 77. Similarly, two other rabbits were immunised by the same method with glucagon coupled to synthetic copolymer with Ethyl C.D.I.**, and emulsified in Freund's adjuvant. The animals were injected on days 1 and 48, and were bled on day 72.

Antibody titres were assessed by incubating dilutions of each serum (75 µl.) with an equal volume of iodinated glucagon (1.8 ng./ml.) in triplicate, for 12 hours at 4°. The tubes were then ethanol precipitated and counted.

Sera from uninjected animals were chosen for controls.

*Bovine serum albumin (100 mg.) + glucagon (10 mg.) + Ethyl C.D.I. (100 mg.)

**Synthetic copolymer (36 mg.) + glucagon (12 mg.) + Ethyl C.D.I. (100 mg.)

Table 5. A COMPARISON OF GLUCAGON AND GLUCAGON-COPOLYMER AS IMMUNOGENS.

Serum	Immunogen	Equilibrium constant* ($1./M \times 10^9$)	Titre* (µg. glucagon bound per ml. undiluted serum)	Number of assays used in calculating results
pooled	-	2.45 ± 0.33	1.89 ± 0.27	6
1	glucagon	1.54 ± 0.14	3.56 ± 1.19	3
2	glucagon	0.46 ± 0.04	10.54 ± 8.91	3
3	glucagon	0.78 ± 0.02	14.64 ± 5.14	3
4	glucagon	3.16 ± 1.89	0.84 ± 0.18	3
5	glucagon	2.89 ± 1.60	6.19 ± 3.14	3
6	glucagon	1.64 ± 0.89	3.22 ± 1.82	4
8	glucagon-copolymer	-	zero	-
9	glucagon-copolymer	2.05 ± 0.42	12.51 ± 2.29	3
10	glucagon-copolymer	-	zero	-
11	glucagon-copolymer	1.32 ± 0.31	1.08 ± 0.78	4
12	glucagon-copolymer	3.67 ± 1.29	3.84 ± 1.39	3
13	glucagon-copolymer	0.88 ± 0.72	0.55 ± 0.26	3
14	glucagon-copolymer	6.06 ± 1.92	1.92 ± 0.36	3

*value ± standard deviation

For legend, see next page.

Table 5 cont.

Seven rabbits (nos. 1-7) were immunised with glucagon emulsified in Freund's complete adjuvant, and the immune response compared with that given by a group of seven rabbits immunised similarly with glucagon-copolymer. Each animal received multisite subcutaneous injections of glucagon (1 mg.) or glucagon-copolymer (5 mg.- equivalent to 1.3 mg. glucagon) on days 1, 55 and 86, and was bled on day 107. One animal died after the first injection from non-specific causes (no. 7).

The antibody content of each serum was assessed by incubating equal volumes (50 µl.) of a range of dilutions (1 in 50, 1 in 100, 1 in 500, 1 in 2,000, and 1 in 10,000) with the same volume of iodinated glucagon (1.3 ng./ml.), and each of three standard solutions of unlabelled glucagon (0, 2.5 and 10 ng./ml.), for three days at 4°. The tubes were then ethanol precipitated and counted. Replicates of four tubes were used.

Five immunoassay curves were obtained in this way for each serum, and values for the equilibrium constant and titre were calculated from the corresponding B/F versus B plots. The values obtained were somewhat inaccurate, as each B/F versus B plot was of three points only. Some of the assay curves were of too high or too low counts to give useful data. For these reasons, average values, derived from assay curves falling within the range 30-80% of the added bindable counts precipitated, are presented in the accompanying table. For comparative purposes, a pooled serum was assayed over a wide range of different dilutions, and the corresponding values for titre and affinity are also presented.

constant were produced in average amounts, while, on the contrary, the two sera with lowest affinity were produced in large quantity.

The results of this assay were typical. During the course of this work ninety two rabbits were injected at least three times with glucagon copolymer, and the properties of the sera from each assessed on several occasions. None of these animals produced a serum better than that from rabbit 4B, which could be used to measure approximately 1 ng./ml. glucagon.

b) Immunisation for different periods.

Table 6 presents the results of an assay in which the properties of sera from animals injected for different times were assessed by incubating a range of different dilutions with iodinated glucagon in the presence and absence of unlabelled hormone.

There was no clear correlation between the affinity of the antibodies raised, as judged by the drop in counts in the presence of 2 ng./ml. unlabelled glucagon, and the length of the immunisation procedure. Two out of five animals in group A, which received six injections, showed a drop in counts greater than 10% at one of the concentrations of antibody assayed, while there was a comparable drop in seven out of eighteen sera from animals in group B, which received two injections.

However, group A animals were of higher titre than group B. The percentage of counts bound specifically at 1 in 1,000 dilution in the absence of unlabelled hormone was approximately twice as high in the animals receiving six injections (31.1 $\pm$ 6.4 (SD) for group A) than in animals receiving three injections (15.9 $\pm$ 10.4 (SD) for group B). The difference

Table 6. THE ASSAY OF SERA FROM RABBITS IMMUNISED FOR DIFFERENT PERIODS.

Serum diln.		1 in 10		1 in 100		1 in 1,000	
Unlabelled glucagon conc. ng./ml.		0	2	0	2	0	2
Serum no.	No. of immun-isations	Counts precipitated specifically (% of added counts)					
1	6	54.0	52.5	47.2	46.5	38.9	37.0
2	"	60.3	62.0	49.3	49.9	26.4	25.7
3	"	59.8	58.4	49.5	47.1	24.1	21.2
4	"	62.8	62.0	52.1	51.6	29.8	27.7
5	"	57.7	59.1	50.7	49.5	36.6	32.3
6	2	63.0	62.0	51.0	51.0	24.5	19.5
7	"	44.2	46.2	24.6	27.2	6.9	6.9
8	"	41.1	42.8	15.5	13.7	2.8	1.3
9	"	67.5	66.7	57.6	56.2	21.5	19.7
10	"	53.3	53.6	31.4	34.7	12.7	13.6
11	"	48.1	48.7	26.5	27.1	12.8	12.0
12	"	51.7	49.6	22.4	24.0	4.0	4.2
13	"	53.0	53.2	37.5	35.9	17.2	15.0
14	"	58.7	59.0	38.4	39.4	14.5	11.3
15	"	53.2	52.6	33.8	31.4	13.0	9.1
16	"	35.4	33.7	16.2	13.6	3.2	3.4
17	"	53.0	53.3	32.4	33.6	13.7	13.0
18	"	60.4	61.2	53.6	50.7	37.2	35.2
19	"	54.6	55.8	47.3	50.5	40.8	40.7
20	"	62.1	64.9	41.9	39.7	12.6	11.0
21	"	51.5	51.7	34.2	34.1	16.8	16.7
22	"	58.9	59.8	38.9	39.2	11.4	11.9
23	"	55.2	53.3	45.4	40.9	21.5	18.5

Two groups of rabbits were immunised according to the following schedules:

The animals receiving six immunisations were immunised with glucagon-copolymer (4 mg.: equivalent to 1 mg. glucagon) emulsified in Freund's complete adjuvant, on days 1, 22, 58, 100, 142, and 184 by multisite subcutaneous injection. They were bled on day 198, and the serum assayed shortly afterwards. The group consisited originally of six animals, but one died of non-specific causes before bleeding.

The animals receiving two immunisations were injected as above on day 1 and day 42, and were bled on day 56. There were 18 animals in the group.

Sera were assayed for antibody content as follows:
Identical volumes (50 µl.) of a range of dilutions (1 in 10, 1 in 100, and 1 in 1,000) of each serum were incubated in replicates of three with iodinated glucagon (1.1 ng./ml.) (50 µl.), and with the same volume of standard solutions of unlabelled glucagon (0 and 2 ng./ml.). After two days incubation, the tubes were ethanol precipitated and counted.

was significant at the 1% level by the student's t test.

c) A comparison of the response using emulsions of complete Freund's adjuvant, and pertussis-silicone oil adjuvant.

The interpretation of the results of this experiment, which are presented in Table 7, is complicated by the fact that there was a high mortality in the group of animals receiving Freund's adjuvant within two days of one of the injections. In this experiment a higher ratio of water to oil was used with this adjuvant than was usual. This may have resulted in a more rapid release of immunogen, followed by a fatal allergic reaction in animals which had responded with production of antibodies.

However, the sera from animals injected with pertussis/silicone oil emulsion were not of higher titre or apparently of higher affinity than those from animals injected with Freund's adjuvant, so it may be deduced that there is no clear advantage in using pertussis/silicone emulsion.

2. Immunisation of other species.

a) Guinea pigs.

Table 8 presents the results of an experiment in which seventeen guinea pigs were immunised three times with glucagon-copolymer conjugate in Freund's adjuvant. All guinea pigs responded with the production of small amounts of antibody, which from most animals was apparently of lower affinity than antiserum from rabbits, suggesting that guinea pigs give an inferior immune response. However, no attempt was made, on

Table 7. THE IMMUNE RESPONSE TO GLUCAGON-COPOLYMER EMULSIFIED IN PERTUSSIS-SILICONE OIL ADJUVANT, IN COMPARISON WITH THE RESPONSE WHEN EMULSIFIED IN COMPLETE FREUND'S ADJUVANT.

Serum	Adjuvant	Average titre (relative to pooled serum, %)	Equilibrium constant (relative to pooled serum)
61B	Freund's	1.3	lower
61G	Freund's	6.6	the same
62B	Freund's	2.6	the same
64B	Freund's	15.0	lower
66R	Freund's	12.3	lower
66K	Freund's	5.2	lower
68B	pertussis-silicone	2.0	lower
69B	pertussis-silicone	12.0	lower
69Y	pertussis-silicone	7.4	lower
70B	pertussis-silicone	4.2	lower
70Y	pertussis-silicone	5.5	lower
71Y	pertussis-silicone	45.4	lower

Six rabbits were immunised with glucagon-copolymer (4 mg.) in pertussis-silicone oil adjuvant (1 ml.) on day 1, and with a similar emulsion, without H. pertussis organisms, on days 20 and 51. A similar group of 14 rabbits were immunised with the same amount of glucagon-copolymer, emulsified in Freund's complete adjuvant (1 to 1 v/v with water) on days 1, 28, and 59. Of this latter group, 8 died within a week of the second injection. The deaths may, therefore, be related to the immunisation proceedure.

The animals were bled respectively on days 102 and 110. For antibody assay, each serum was diluted 1 in 100, 1 in 500, 1 in 2,000 and 1 in 10,000, and equal volumes (75 µl.) incubated for two days with the same volume of iodinated glucagon diluted to 1 ng./ml. The samples were ethanol precipitated, and the counts compared with those given by a range of two-fold dilutions of a pooled serum, from 1 in 50 to 1 in 10,000. The comparative serum was of higher antibody content than that normally used.

In the accompanying table, an indication is given of the equilibrium constant of each serum, relative to the pooled serum chosen for comparison. This was estimated from the comparative shape of each antibody dilution curve*, relative to that of the pooled serum. Sera giving shallower curves than the pooled serum were assumed to be of lower equilibrium constant, while sera giving steeper slopes were assumed to be of higher equilibrium constant.

*plotted on a log. scale

Table 8. THE IMMUNISATION OF GUINEA PIGS WITH GLUCAGON-COPOLYMER.

Serum	Antibody content relative to pooled comparison serum,%	Affinity, relative to pooled comparison serum
R1	6.8	lower
G1	5.1	the same
V1	5.4	the same
U2	13.4	lower
G2	10.8	lower
R2	2.0	lower
U3	4.3	lower
G3	11.5	lower
V3	5.7	lower
R3	2.0	lower
G4	3.1	lower
R4	1.9	lower
U4	1.1	lower
V5	7.0	lower
G5	18.6	lower
R5	2.0	lower
U5	3.2	the same

Guinea pigs were immunised with glucagon-copolymer (2 mg.: equivalent to 0.5 mg. glucagon) emulsified in complete Freund's adjuvant, on days 1, 32 and 56, and were bled on day 69. Of the original group of 20 animals, 3 died from non-specific causes.

For assay, each serum was diluted 1 in 200, 1 in 500, 1 in 1,000, 1 in 2,000 and 1 in 5,000, and equal volumes of each dilution (75 µl.) incubated with the same volume of iodinated glucagon (1.0 ng./ml.). After 1 day, the tubes were ethanol precipitated, and the counts compared with those given by a range of 2-fold dilutions of a pooled serum of low affinity.

An indication of the equilibrium constant of each serum was obtained from the shape of the antibody log dilution curves, as described for the results of Table 7.

practical grounds, to immunise by multisite subcutaneous injection, and it may be that the lower titres and lower affinities were a consequence of a different immunisation route.

(Guinea pigs with relatively high equilibrium constant or titre (V_1, V_2, G_2, G_3, V_5 and G_5 were chosen) were re-injected and subsequently bled again but with no further improvement in titre or equilibrium constant.)

b) Goats.

Two goats were immunised with glucagon-copolymer in Freund's adjuvant by Professor M. Sela and Staff of the Weizmann Institute. The results of the assay of the sera that were produced are presented in Table 9. One animal gave serum of a low titre, while the other gave a good response, yielding a serum with about 10% of the antibody content of a typical serum from a 'responder' rabbit. The apparent affinity of sera from both animals was lower than either pooled serum or serum 4B.

These results suggest that goats may be a useful animal in which to raise glucagon antibodies. Considerations of animal husbandary ruled out a more extensive study of the immune response in this species however.

c) Chickens.

Two chickens were immunised at monthly intervals, and bled two weeks after the second and third immunisations. No antibodies were apparently produced by either animal after either bleeding. After the completion of these experiments, it was reported by

Table 9. THE IMMUNISATION OF GOATS WITH GLUCAGON-COPOLYMER.

Day	Assay (1) Antibody content relative to pooled rabbit serum (%)		Assay (2) Antibody content relative to serum 4B (%)
	goat 43	goat 50	goat 50
27	1.7	11.8	19.0
35	1.7	7.0	22.6
42	1.5	10.6	27.9
51	-	-	26.1
62	-	-	17.5

Two goats (nos. 43 and 50) were immunised with glucagon-copolymer (4 mg.: equivalent to 1 mg. glucagon) on days 1, 11 and 20, and were bled on the days indicated above.

Two assays were carried out on different shipments of serum:
Assay 1. Each serum was diluted 1 in 100, 1 in 500, 1 in 2,000 and 1 in 10,000, and equal volumes (75 µl.) incubated for two days with the same volume of iodinated glucagon (1 ng./ml.). The samples were ethanol precipitated, and the counts compared with those given by a range of two-fold dilutions of pooled serum from 1 in 50 to 1 in 10,000.
Assay 2. (Goat 50 only). This assay was identical, except that the serum was diluted 1 in 100, 1 in 200, 1 in 500, and 1 in 1,000, a different batch of iodinated glucagon (1.2 ng./ml.) was used, and the comparative serum was 4B (diluted 1 in 50 to 1 in 10,000).

Hurn and Landon (1971) that chicken immunoglobulins have different physiochemical properties from mammalian immunoglobulins, and it is possible that chicken gamma globulins are not precipitated by ethanol. This could account for the negative results obtained in the antibody assay. It is not clear, therefore, whether there was in fact no immune response, or whether the apparent lack of response was caused by unsatisfactory assay conditions for antibodies from this species.

E. DISCUSSION.

During this work, different immunological procedures were used in an attempt to raise high affinity glucagon antibodies. The immune response of rabbits to glucagon covalently linked to both a synthetic and a natural carrier, and the response to unconjugated glucagon were investigated. In addition, guinea pigs, goats and chickens were immunised with glucagon linked to a synthetic copolymer.

As reported by other workers, the immune response was found to be very variable. Thus in the case of the experiment reported in Table 5, which was the most detailed experiment in this work, the coefficients of variation of the values obtained for titre and equilibrium content with glucagon and glucagon-copolymer were as follows:

Immunogen	Coefficient of variation (%)	
	titre	equilibrium constant
glucagon copolymer	157	75
glucagon	80	62

This very large variability precludes definitive conclusions as to whether one immunogen gave more useful sera than any other that was administered, or whether there was any difference in the immune response in different species. In the case of chickens and goats, where only two animals were immunised in each case, even tentative conclusions cannot be reached.

In most of the routine immunisations that were performed, glucagon copolymer emulsified in Freund's adjuvant was administered to rabbits by multisite subcutaneous injection. There is no evidence to suggest that the use of any other species, procedure

or immunogen would have been of any advantage whatsoever. Despite the use of over one hundred animals none of the sera that were raised was of sufficient equilibrium constant for the sensitivity required. This suggests that the production of high affinity antibodies is a matter of complete chance, with a very small percentage of animals responding usefully.

The main conclusions that can be drawn with some assurance from this study are:

1. Rabbits gave more useful sera than guinea pigs,
2. Continued immunisations increased antibody titre,
3. There are considerable difficulties in producing antisera of sufficiently high affinity to give the the sensitivity required in immunoassay techniques.

CHAPTER 3.

THE SEPARATION OF ANTIBODIES TO INSULIN AND GLUCAGON USING IMMUNOADSORBENTS.

A. INTRODUCTION

1. Theoretical considerations.

The initial purpose of preparing glucagon immunoadsorbents was to purify antibodies of high affinity from sera containing predominantly low affinity antibodies. It was proposed to achieve this by incubating a large excess of serum with immunoadsorbent, so that high affinity antibodies would be preferentially bound, and could be recovered by elution with acid. It is relevant, therefore, to consider the evidence for the existence of high affinity glucagon antibodies in the sera produced during this work.

Serum from an animal immunised against a foreign protein would be expected to contain some antibodies of very high equilibrium constant. However, if the antigenic determinant sequences of the potential antigen are identical or very similar to those of a protein produced by the species immunised, there will be no production of high affinity antibodies, as the animal will be tolerant to the antigen. Antibodies, according to the clonal selection theory of acquired immunity (Burnet, 1959), are manufactured by the descendants of 'clone precursor cells', each of these being capable of the synthesis of a very limited number of types, or possibly only one type, of antibody molecule. Tolerance is the consequence of the specific elimination of clone precursor cells that produce antibodies against endogenous proteins during the early stages of development.

Whether tolerance will be induced towards glucagon depends, therefore, amongst other considerations, on the degree of similarity between the structure of glucagon from the species from which it was extracted (in this work, beef or pig) and the species in which it is intended to raise antibodies.

The primary sequence of glucagon has been established for pig (Behrens and Bromer, 1958), but not for other mammalian species. However, glucagon from those mammalian species which have been studied cross-reacts immunologically with porcine or bovine glucagon, suggesting that the structure is very similar or identical in each. Further evidence suggesting that the structure of glucagon from different mammalian species may be the same comes from recent work by Sundby and Markussen (1971), who have shown that crystalline rat glucagon has the same amino acid composition as porcine glucagon. The fact that glucagon is of low molecular weight also suggests that there may be less variability in sequence than with higher molecular-weight polypeptide antigens, such as insulin or growth hormone.

Experimental evidence that anti-glucagon serum contains antibodies with different equilibrium constants comes from the shape of immunoassay curves. According to Berson and Yalow (1959) antigen-antibody reactions at equilibrium that give a non-linear curve for the B/F versus B plot contain more than one order of antibody combining sites. Many of the B/F versus B plots that were obtained during the course of this work were linear over a certain range of glucagon concentration, but all departed from linearity at higher concentrations, suggesting the presence of very low affinity antibodies.

Neither theoretical nor experimental considerations, however, provide definitive evidence for the existence of high affinity antibodies in the sera raised during the course of this work.

It was nevertheless proposed to investigate the use of immunoadsorbents to purify high affinity antibodies because of the potential value of such antibodies in improving immunoassay sensitivity.

2. Immunoadsorbents.

The development of immunoadsorbents has been reviewed by Silman and Katchalski (1966) and will therefore only be discussed briefly. Campbell, Luescher and Lerman (1951) prepared an immunoadsorbent by coupling bovine serum albumin to the m-diazobenzyl oxymethyl ether of cellulose, and, although several adsorbents were described in subsequent years, little progress was made until 1961. Gurevich, Kuzouleva and Tumanova (1961) found that the properties of the immunoadsorbent of Campbell et al. (1951) could be changed by reprecipitation from ammoniacal copper solution, so that the immunoadsorbent would bind more protein, thus giving a product with a greater capacity for adsorbing antibodies to the coupled antigen.

Recently there have been significant advances in the development of new immunoadsorbents which include the use of ethyl chloroformate to couple and cross-link protein antigens (Avrameas and Ternynck, 1967), and a method for the activation of cross-linked dextrans such as Sephadex and Agarose so as to use these materials as protein carriers (Axen, Porath and Ernback, 1967; Porath, Axen and Ernback, 1967). For example, using these methods Cuatrecasas (1969a, b) has prepared an insulin immunoadsorbent that retains full biological action, in that when it is incubated with fat cells, the metabolism of ^{14}C glucose increases to the same extent as if an equivalent amount of free insulin had been used.

Immunoadsorbents containing insulin have been prepared by Miles and Hales (1968a, b) using the procedure of Gurevich et al. (1961), and have been used by these authors as the basis for an 'immunoassay'. The principle employed was the competition of labelled insulin antibody (prepared by use of the immunoadsorbent) for immunoadsorbent-bound and free insulin. It is this

immunoadsorbent which was largely used in the procedures discussed below.

A suitable immunoadsorbent to carry out the fractionation of antiserum should preferably have the following characteristics:

1. The antigen must be covalently coupled to the immunoadsorbent matrix by a linkage sufficiently stable for it to remain bound under conditions used to elute adsorbed antibodies.
2. The covalent coupling of antigen to the matrix must not cause a loss of antigenicity, either through denaturation of the antigen, or by obscuring the antigenic determinant sites of the molecule.
3. It must be possible to wash any linked antigen from the immunoadsorbent so that there is no neutralisation of antibodies by free antigen during adsorption.
4. The immunoadsorbent should be precipitated easily by centrifugation, yet sufficiently finely divided for most of the antigen molecules to be presented to the external aqueous medium.
5. The physical properties of the immunoadsorbent must not cause denaturation of antibodies during elution.

The two immunoadsorbents prepared during this work did not fulfil these criteria in several respects. For example, only a small proportion of the antigen used was still immunologically active when linked to the immunoadsorbent. In addition, there was a significant loss of adsorbed antibodies which could not be recovered on elution.

Despite these disadvantages, it was possible to prepare purified glucagon antibodies for the first time by the use of these immunoadsorbents.

B. THE PREPARATION OF IMMUNOADSORBENTS

1. Bromoacetyl cellulose hormone conjugates.

a) Synthesis of bromoacetyl cellulose.

Bromoacetyl cellulose was synthesised and conjugated to glucagon and insulin by techniques reported by Robbins, Haimovich and Sela (1967). Part of this paper is reproduced below:

"Powdered cellulose (Whatman) was washed with acetone and dioxan and then dried in vacuo over phosphorus pentoxide to a constant weight. A solution of 100 g. of bromoacetic acid in 30 ml. of absolute dioxan was added to 10 g. of cellulose powder and the suspension stirred at room temperature for 20 hours in a tightly stoppered 1 l. round bottom flask. Bromoacetyl bromide (75 ml.) was added and the flask connected to a sodium hydroxide trap which prevented the entrance of water vapor and neutralized the hydrobromic acid which was evolved. This solution was stirred vigorously for 10 to 12 hours and then slowly poured with vigorous stirring into 7 l. of deionized water which had previously been cooled to 4°. The stirring was continued uninterrupted for 15 minutes. The precipitated cellulose was washed exhaustively with deionized water and then in turn with 0.1 M sodium bicarbonate and deionized water over a sintered glass funnel. It was found that if the bromoacetyl cellulose had been dried extensively, its physical properties were changed, and the extent of conjugation with the antigens was reduced. The bromoacetyl cellulose was, therefore, stored at 4° in a moist state. To determine the extent of substitution, a sample of the cellulose was dried to a constant weight over phosphorus pentoxide in vacuo and the content of bromine analysed....."

Bromoacetyl cellulose synthesised in this study had a bromine content of 11 to 14% of the dry weight, and, after centrifuging, was kept in a damp condition containing 12 to 16% (w/w) of dry material.

b) Conjugation of glucagon to bromoacetyl cellulose.

Glucagon (10 mg.) was dissolved in phosphate-citrate buffer, pH 2.4 (1 ml.) and conjugated to wet bromoacetyl cellulose (162.5 mg. equivalent to 25 mg. dry material).

The mixture was stirred vigorously for 30 hours at room temperature, after adding Antifoam A, and toluene to prevent bacterial growth. It was then centrifuged at 10,000 x g for 10 minutes, resuspended in 0.1 M sodium bicarbonate buffer, pH 8.9 (10ml.)and allowed to stand at 4^{o} for 24 hours with occasional stirring. At this pH the chemical bonds between the bromoacetyl cellulose and the antigen were formed. The suspension was centrifuged at 10,000 x g for 10 minutes, the supernatant discarded, and cellulose resuspended in 0.05 M 2-aminoethanol/0.1 M sodium bicarbonate buffer, pH 8.9, to block any unreacted bromine, and allowed to stand at 4^{o} for another 24 hours.

c) Conjugation of insulin to bromoacetyl cellulose.

Insulin (50 mg.) in phosphate-citrate buffer, pH 2.6 (5 ml.) was reacted with bromoacetyl cellulose (2 g. wet weight); otherwise the conjugation was as described for glucagon.

2. Diazocellulose hormone conjugates.

a) Synthesis of diazocellulose.*

These methods are described by Kabat and Meyer (1961), from the work of Gurevich et al. (1961). N-(m-nitrobenzoxy) methyl pyridinium chloride was reacted with specially prepared cellulose to give the product 'nitro cellulose'. This was reduced to 'amino-cellulose' and was then solubilised and precipitated from cuprammonium hydroxide, in order to increase the surface area. The finely divided 'amino-cellulose' was then diazotised and reacted with insulin or glucagon. The resulting immunoadsorbent was washed exhaustively.

i. Synthesis of N-(m-nitrobenzoxy) methyl pyridinium chloride.

M-nitrobenzyl alcohol (6 g.) and dry benzene (35 ml.) were added to paraformaldehyde (4.8 g.) in a round bottom flask. Dry HCl gas was passed into the stirred mixture for two hours. After standing stoppered overnight, the upper organic layer, which had by this time separated from the mixture, was dried over anhydrous Na_2SO_4, and the benzene that it contained was removed on a rotary evaporator. The residue was vacuum distilled twice at ca. 1 mm. pressure (b.p. 150-160°; then 150-153°). The light-yellow oily product (m-nitrobenzoxy methyl chloride) (5 g.) was mixed with pyridine (15 g.). Over the next 24 hours, a yellow amorphous precipitate slowly formed. This was filtered, washed with petrol ether, and dried.

* This term refers throughout the work to the m-diazobenzoxy methyl ether of cellulose.

ii. Preparation of cellulose.

Cotton wool of the purest Boots absorbent grade (115 g.) was refluxed for 1 hour with absolute ethanol (4 l.) to which had been cautiously added acetyl chloride (28 ml.). The residue was washed with ethanol, and then dispersed in water. After resuspending and sedimenting the product from water several times, it was washed again in ethanol, and dried thoroughly in vacuo.

iii. Synthesis of m-nitrobenzoxy cellulose ('nitro cellulose').

Cellulose powder (50 g.) was mixed with N-(m-nitrobenzoxy) methyl pyridinium chloride (7 g.) dissolved in 2.5% sodium acetate in water (100 ml.). The resulting paste was dried at 60°- 80° in a vacuum oven. The powdered product was then heated to 125° for 40 minutes, washed thoroughly with water, dried at 80°, washed with benzene twice, and finally dried in a vacuum oven.

iv. Reduction of 'nitrocellulose' to 'aminocellulose'.

The product of the previous stage was stirred with 15% sodium dithionite (300 ml.) at 50°-60° for 30 minutes. It was then washed on a sintered funnel with water (1 l.), followed by 30% acetic acid (3 l.), and finally with water (1 l.). It was then dried in a vacuum oven at 40°.

v. Reprecipitation of aminocellulose.

The dried 'aminocellulose' was dissolved in cuprammonium hydroxide (100 ml.) prepared by mixing reagents in the order stated below:

61 ml. NH_4OH of density 0.880
11.52 g. $CuSO_4.5H_2O$
1 g. sucrose
3.69 g. NaOH
water to 100 ml.

The solution, after centrifuging a small amount of insoluble material, was diluted to 20 l. with water. A black sediment, consisting of a mixture of $Cu(OH)_2$ and precipitated 'aminocellulose' formed overnight. The precipitate was decanted and washed thoroughly with water. $Cu(OH)_2$ was removed by washing with 30% acetic acid (10 l.). The product was finally washed with water (10 l.), and lyophilised.

vi. Determination of the amino group content of 'aminocellulose' and the preparation of 'diazocellulose'.

In order to use the correct ratio of immunoadsorbent to hormone for coupling, it was necessary to titrate the 'aminocellulose' to determine its amino group content.

40 mg. quantities were suspended in a stirred solution of 5% HCl containing 5% KBr, cooled to 4°, and 0.05 M $NaNO_2$ was introduced in small aliquots from a 1 ml. burette. After allowing 10 minutes for the reaction to go to completion, the solution was tested for excess HNO_2 using starch-iodide paper as external indicator.

For the diazotisation, 200 mg. quantities of 'aminocellulose' were suspended in stirred 5% HCl (2.5 ml.), and a tenfold excess of $NaNO_2$ was added. The mixture was left to stir at 4° in a closed container to avoid evaporation of HNO_2, for 30 minutes. Solid urea was added in small quantities until all the HNO_2 had been destroyed, as judged by the external starch-iodide test.

b) The coupling of protein to 'diazocellulose'.

The techniques described in this section were identical to, or adapted from, the methods of Miles and Hales (1968a).

The diazotised product of the previous stage was washed three times with water at 4° and then three times with cold borate buffer (0.1 M). An amount of glucagon or insulin equivalent to half the free amino content of the original aminocellulose was then added, dissolved or suspended in borate buffer (2.5 ml.).

The mixture was allowed to stir for two days at 4° and glycine (0.5 g.) was added as a solid to the mixture to remove excess diazo groups by coupling. Finally, after standing for two days at 4°, the immunoadsorbent was washed at 4° five times in distilled water, five times in phenol/acetic acid/water (1:1:1; w/v/v), a further three times in water, and finally three times in phosphate buffer containing non-immune guinea pig and rabbit serum, of the composition:

Phosphate: 40 mM, pH 7.5
NaCl: 0.9% (w/v)
Thiomersalate: 0.025% (w/v)
Bovine plasma albumin (fraction V): 0.1% (w/v)
Non-immune guinea pig serum: 0.15% (v/v)
Non-immune rabbit serum: 0.15% (v/v)

(This buffer is described as N.I.S. phosphate buffer in the remainder of the thesis. Reference is made elsewhere to N.I.S. veronal and N.I.S. borate buffers, which are identical in composition, except that 40 mM phosphate buffer was replaced by 40 mM pH 8.6 veronal or borate buffer respectively).

C. THE DEVELOPMENT OF PURIFICATION TECHNIQUES USING INSULIN ANTIBODIES AND IMMUNOADSORBENTS.

1. Outline of the work described.

Insulin was used as a model protein to develop methods to prepare glucagon immunoadsorbents because it was available in large quantities and had similar physical properties to glucagon and because an assay was already developed for both insulin and insulin antibodies.

In an initial attempt to prepare an immunoadsorbent, insulin was coupled to bromoacetyl cellulose using the methods of Robbins et al. (1967). The immunoadsorbent prepared by these methods could be used to adsorb insulin antibodies, but it was not possible to elute these off again with dilute acid.

An immunoadsorbent of insulin coupled to the m-diazobenzoxy methyl ether of cellulose was then prepared, following the publication by Miles and Hales (1968a) of a method of washing the product free of uncoupled insulin. This immunoadsorbent was successfully used to recover insulin antibodies by elution with dilute acid.

It was subsequently found that bromoacetyl cellulose-insulin conjugates could also be used to recover adsorbed antibodies by acid elution provided that they were washed in the same way as the immunoadsorbent of Miles and Hales. This suggested that as originally prepared the bromoacetyl cellulose immunoadsorbent still contained free insulin.

These techniques were used, with slight modifications, to prepare glucagon immunoadsorbents. The preparation of glucagon immunoadsorbents and bromoacetyl cellulose-insulin immunoadsorbent have not previously been reported. The experiments in which their properties were investigated are described in chronological sequence.

2. The properties of the first bromoacetyl cellulose-insulin immunoadsorbents to be prepared.

a) Details of preparation.

In the first experiment, a bromoacetyl cellulose preparation (2 g. wet weight, equivalent to 335 mg. dry weight) containing 11.88% bromine was coupled to various quantities of insulin (from approximately 0.5 mg. to 50 mg.). The physical adsorption stage was carried out at pH 2.6, which is the most alkaline pH on the acid side of neutrality at which insulin is soluble.

After carrying out the chemical coupling step, each of the products was washed exactly as described by Robbins et al. (1967). The washing procedure involved four washes in 0.15 M NaCl, then six washes in 8 M urea, followed by six washes in 0.1 M acetic acid, and finally two washes in 0.15 M phosphate buffer, pH 7.4. The immunoadsorbents were then made to a final suspension volume of 20.0 ml. with phosphate buffer.

b) Adsorption and elution of antibodies from the immunoadsorbents.

Antibodies were adsorbed from different anti-insulin sera using three of the immunoadsorbents prepared as described above. Attempts were made to elute the adsorbed antibodies using 0.1 M acetic acid.

The results of this experiment are recorded in Table 10 and indicate that insulin antibodies were adsorbed almost completely by the immunoadsorbent with highest insulin content, and to a lesser extent by the immunoadsorbents with lower insulin content, but that there was negligible recovery of antibodies in any of the acetic acid eluates.

Table 10. AN INITIAL ATTEMPT TO RECOVER INSULIN ANTIBODIES USING BROMOACETYL CELLULOSE INSULIN CONJUGATES.

Immunoadsorbent preparation*	Fraction assayed	Serum number	Antibodies recovered (as a percentage of the total added)
1	supernatant after adsorption	4	2.4
		5	1.7
		7	3.1
	acetic acid eluate	4	0.1
		5	0.8
		7	2.1
2	supernatant after adsorption	4	3.2
		5	53.3
		7	0.8
	acetic acid eluate	4	0
		5	0.1
		7	0.8
3	supernatant after adsorption	4	75.8
		5	107.0
		7	45.4
	acetic acid eluate	4	0
		5	0.8
		7	0.4

*Suspension 1:
50 mg. insulin coupled to 2 g. (wet weight) bromoacetyl cellulose
Suspension 2:
5 mg. insulin + 2 g. bromoacetyl cellulose
Suspension 3:
0.5 mg. insulin + 2 g. bromoacetyl cellulose

Each immunoadsorbent suspension (1 ml. of a total of 20 ml. total suspension volume) was incubated with each of the anti-insulin sera at 4° for 2 hours with stirring. After centrifuging, each precipitated immunoadsorbent was washed with 0.15 M NaCl twice, and incubated with 0.1 M acetic acid at 37° for 1 hour, to elute bound antibodies. After centrifuging, the eluates were dialysed overnight against 0.1 M veronal-acetate buffer, pH 8.6. The supernatant sera and neutralised eluates were incubated at several different dilutions with iodinated insulin (2.5 ng./ml.), for two days at 4°. The tubes were then ethanol precipitated, and counted. The antibody content of each set of replicates was calculated from a comparison with the radioactivity precipitated by various dilutions of the original sera. The results presented above are average values for assays at four dilutions.

Two explanations were advanced for the failure to elute antibodies from bromoacetyl cellulose insulin immunoadsorbents. The first was that the elution conditions were too severe, causing the eluted antibodies to be denatured. Alternatively, the conditions were too mild, so that antibodies were not being eluted at all.

These possibilities were investigated by changing the conditions of elution, which was attempted under more severe conditions, using glycine-HCl (pH 2.2, 0.2 M) and 5.5 M KI in tris buffer (pH 8.2, 0.05 M) (these reagents were first used to elute antibodies by Avrameas and Ternynck, 1967), and 0.01 M, 0.1 M and 1 M HCl as eluants. Elution was also attempted under very mild conditions using 0.01 and 0.001 M acetic acid. However there was no increase in the recovery of eluted antibodies.

Successful attempts were then made to prepare a new immunoadsorbent of insulin coupled to m-diazobenzoxy cellulose.

3. The preparation and properties of diazocellulose insulin immunoadsorbent.

a) Details of preparation.

The immunoadsorbent was prepared as described above. The preliminary diazotisation of 'aminocellulose' showed it to have an amino group content of 50 μ equiv./g., about a third of the value obtained by Miles and Hales (1968a)for a similar preparation.

The appropriate amount of insulin (100 mg.) suspended in borate buffer, was then coupled to diazocellulose (400 mg.), which was then washed exhaustively. The total immunoadsorbent was finally suspended in N.I.S. borate buffer (20 ml.).

b) The immunoreactive insulin content of the immunoadsorbent.

Before attempting to recover antibodies from the immunoadsorbent, it was necessary to assess its immunoreactive insulin content. Various dilutions were incubated with anti-insulin serum, and the supernatants were then assayed for residual antibodies. This assay is presented in Table 11.

It can be calculated from the results that were obtained that the total immunoadsorbent contained approximately 230 µg. of immunoreactive insulin. As it was prepared by reacting 100 mg. insulin, the yield of immunoreactive material coupled to the immunoadsorbent was very low (0.23%). This suggests either that most of the added insulin was not bound by the coupling procedure, or alternatively that it was bound in such a way that relatively few molecules retained antigenicity.

c) The elution of bound antibodies from the immunoadsorbent.

It was estimated, from the results given in Table 11 that one volume of immunoadsorbent suspension would bind antibodies present in approximately three volumes of the antiserum used. In the first experiment to recover bound insulin antibodies, immunoadsorbent was accordingly incubated with serum in this ratio, and adsorbed antibodies were recovered by elution with HCl (0.01 M) through a millipore filter. The results of the antibody assay of various fractions from this experiment are presented in Table 12, and indicate that about 60% of the adsorbed antibodies were recovered in the first eluate fraction. These results contrast with those of the initial experiments to recover adsorbed antibodies from bromoacetyl cellulose insulin immunoadsorbent

Table 11. THE ADSORPTION OF INSULIN ANTIBODIES DIAZOCELLULOSE INSULIN IMMUNOADSORBENT.

Dilution of added immunoadsorbent	Percentage of added antibodies present in each supernatant after adsorption	
undiluted	1.2	3.4
1 in 3	7.5	8.7
1 in 10	53.4	69.0
1 in 30	83.4	104.0

Equal volumes of a sample of pooled guinea pig anti-insulin serum, and immunoadsorbent suspensions diluted in N.I.S. borate buffer, were incubated at 4° for 12 hours. After centrifuging, the supernatants were aspirated, and assayed for unadsorbed antibodies as previously described. Each supernatant was assayed at two dilutions, and both results are recorded above.

Table 12. THE ELUTION OF ANTIBODIES ADSORBED TO DIAZOCELLULOSE INSULIN.

Sample	Antibodies recovered in the sample as a percentage of the total added	
supernatant after adsorption	3.3	3.1
first HCl eluate fraction	62.0	58.5
second HCl eluate fraction	0.8	0.8

Equal volumes of immunoadsorbent (washed in N.I.S. phosphate buffer and then diluted 1 in 3 relative to the original suspension) and pooled serum (the same serum as used in the previous experiment) were incubated at 4° for 2 hours, with shaking. The mixture was then centrifuged, the supernatant aspirated, and the immunoadsorbent precipitate transferred to a Millipore filter. The precipitate was washed copiously with N.I.S. veronal buffer, and then with water. 0.01 N HCl (5 ml.) was added, and allowed to drip through the filter into N.I.S. veronal buffer (35 ml.). The elution was repeated several times with fresh HCl and buffer. The supernatant and eluates were assayed for antibody at two dilutions, and both results are recorded above.

in which there was negligible recovery.

It was thought that the poor recovery in these latter experiments could be caused by the presence of uncoupled insulin on the immunoadsorbent. This explanation was tested by washing bromoacetyl cellulose immunoadsorbent with phenol/acetic acid/water, and then carrying out an antibody adsorption and elution.

This experiment is described in the following section.

4. The separation of insulin antibodies using phenol/acetic acid/water-washed bromoacetyl cellulose-insulin immunoadsorbent.

The immunoadsorbent prepared with the largest ratio of insulin to cellulose was washed six times in phenol/acetic acid/water (1:1:1; w/v/v), twice in distilled water, and finally six times in N.I.S. phosphate buffer, pH 7.6. Most of the immunoadsorbent was lost during washing with phenol/acetic acid/water, as it was partially soluble in this solvent. What remained of the immunoadsorbent was suspended in N.I.S. phosphate buffer (5 ml.). Antibodies were adsorbed and eluted from a small amount of the suspension, in an experiment similar to that with diazocellulose insulin discussed above. Results of the assay of the antibody content of various eluted fractions are presented in Table 13, and show that about 50% of adsorbed antibody was recovered on elution. This result is similar to that achieved using diazocellulose immunoadsorbent.

The possible explanation for the failure to recover all adsorbed antibodies in this and other experiments are discussed subsequently.

Table 13. THE ELUTION OF ANTIBODIES ADSORBED TO PHENOL/ ACETIC ACID/WATER - WASHED BROMOACETYL CELLULOSE INSULIN IMMUNOADSORBENT.

Sample	Antibodies recovered in the sample as a percentage of the total added		
supernatant after adsorption	2.0	2.2	2.2
first HCl eluate fraction	47.8	54.6	54.5
second HCl eluate fraction	0.3	0.2	-

Pooled anti-insulin serum (5 ml.) was added to washed immunoadsorbent (0.4 ml.), suspended in 5 ml. N.I.S. phosphate buffer. After stirring for 2 hours at 4°, the mixture was centrifuged, and the supernatant aspirated. The precipitate was washed and eluted as previously described for insulin diazocellulose immunoadsorbent, and the fractions were assayed for antibody at three dilutions.

D. THE PROPERTIES OF GLUCAGON IMMUNOADSORBENTS.

1. The techniques used to adsorb and elute glucagon antibodies.

These were minor modifications of the methods developed using insulin antibodies as presented above. The experimental conditions were very similar for each adsorption and elution and are described in the Tables in which the results are presented.

2. The properties of diazocellulose glucagon immunoadsorbents.

Glucagon immunoadsorbent was prepared by coupling glucagon (30 mg.) to aminocellulose (200 mg.). The product was rigorously washed with phenol/acetic acid/water, water, and N.I.S. phosphate buffer, and was finally suspended in N.I.S. phosphate buffer (20 ml.).

A titration of glucagon antiserum against different amounts of the immunoadsorbent (Table 14) showed that only 0.15% of the added glucagon was bound to the immunoadsorbent in an immunoreactive form. This is a slightly lower yield than for diazocellulose insulin.

Antibodies were then adsorbed and eluted from diazocellulose glucagon immunoadsorbent in an experiment in which two equal amounts of immunoadsorbent were incubated with a five-fold and a fifty-fold excess of serum respectively. The results of this experiment are presented in Table 15 , which shows there was some loss of antibody as not all of the adsorbed antibody could subsequently be eluted.

The immunoreactive behaviour of the eluted antibodies in this experiment was investigated, and is described later.

Table 14. THE ADSORPTION OF GLUCAGON ANTIBODIES TO DIAZOCELLULOSE GLUCAGON IMMUNOADSORBENT.

Dilution of added immunoadsorbent	Percentage of added antibodies remaining in the supernatant after adsorption		
undiluted	0.5	0.5	-
1 in 3	1.9	2.1	2.8
1 in 10	20.0	22.5	27.8
1 in 30	57.6	67.0	-
1 in 100	78.3	84.2	-
1 in 300	79.2	105.6	-
1 in 1,000	78.0	103.0	-

Various dilutions of diazocellulose glucagon immunoadsorbent (suspended in a total volume of 20 ml.) were incubated with equal volumes of serum 4B, at 4° for 12 hours, with gentle shaking. The mixtures were centrifuged, and the residual antibodies were measured in the supernatants at two or three different dilutions.

Table 15. THE ELUTION OF ANTIBODIES ADSORBED TO DIAZOCELLULOSE GLUCAGON IMMUNOADSORBENT.

Sample	Vol. serum used	Antibodies recovered in the sample as a percentage of the total added
supernatant after adsorption	1 ml.	74.6 68.5
first HCl eluate fraction	1 ml.	10.0 10.3 10.3 10.4 10.0 10.5
supernatant after adsorption	10 ml.	67.9 83.8
first HCl eluate fraction	10 ml.	1.3 1.2 1.2 1.4

Diazocellulose glucagon immunoadsorbent was washed and diluted with N.I.S. phosphate buffer to one fifth the concentration of the original suspension. Equal amounts (0.5 ml.) were incubated respectively with 1 ml. and 10 ml. pooled antiglucagon serum. After shaking for two days at 4°, the mixtures were centrifuged, the supernatants aspirated, and the immunoadsorbents washed with N.I.S. phosphate buffer, and then distilled water. Bound antibodies were eluted from the immunoadsorbents on Millipore filters with 0.01 M HCl (3 ml.), into immunoassay buffer (7 ml.). Assays were then carried out on the supernatants and neutralised eluates, at several dilutions each, to measure the recovery.

3. The properties of bromoacetyl cellulose glucagon immunoadsorbents.

Bromoacetyl cellulose glucagon immunoadsorbent was prepared by coupling glucagon (10 mg.) to bromoacetyl cellulose (164 mg. wet weight, equivalent to 25 mg. dry weight: bromine content 14% dry weight).

A preliminary experiment was performed to determine the immunoreactive glucagon content of the immunoadsorbent, and the yield (i.e. the ratio of the immunoreactive glucagon content of the immunoadsorbent to the amount of glucagon added for coupling) was found to be slightly lower than that of the synthesis of diazo-cellulose glucagon. The results of this experiment are presented in Table 16.

An adsorption and elution of glucagon antibodies was carried out with the concentration of the reactants adjusted so that about 15% of the added antibodies would be bound by the immunoadsorbent.

The results of this experiment are presented in Table 17 and show that approximately the same percentage of adsorbed antibodies was recovered on elution using bromoacetyl cellulose glucagon as was recovered using diazocellulose glucagon.

The immunoreactive properties of the eluted antibodies were then investigated, and are described in a subsequent section of this thesis.

Table 16. THE ADSORPTION OF GLUCAGON ANTIBODIES TO BROMOACETYL CELLULOSE GLUCAGON IMMUNOADSORBENT.

Dilution of added immunoadsorbent	Percentage of added antibodies remaining in the supernatant after adsorption	
undiluted	4.6	5.6
1 in 3	31.8	37.6
1 in 10	73.0	76.4
1 in 30	84.8	92.0
1 in 100	91.0	100.0
1 in 300	100.0	100.0

Equal volumes of a sample of pooled antiglucagon serum, diluted 1 in 2 in N.I.S. phosphate buffer, and immunoadsorbent suspensions, diluted in the same buffer, were incubated with stirring at 4° for 12 hours. After centrifuging, the supernatants were aspirated and assayed for unadsorbed antibodies as previously described. Each supernatant was assayed at two dilutions, and both results are recorded above.

Table 17. THE ELUTION OF GLUCAGON ANTIBODIES FROM BROMOACETYL CELLULOSE GLUCAGON IMMUNOADSORBENT.

Sample	Antibodies recovered in the sample as a percentage of the total added	Average
supernatant after adsorption	69 73 80 87 94	81
NaCl wash (1)	0.7 0.9 0.9 1.0	0.9
NaCl wash (2)	0.3 0.3 0.2 0.2	0.2
neutralised HCl eluate (1)	8.9 7.2 7.0 7.0 9.2	7.9
neutralised HCl eluate (2)	0.5 0.5 0.2 0 0	0.2
neutralised HCl eluate (3)	0 0 0 0	0
	Total	90.2

Bromoacetyl cellulose glucagon immunoadsorbent suspension (1 ml.) was washed five times with albumin (5% w/v in phosphate buffer, pH 7.5), to remove any free glucagon. Pooled serum (20 ml.) (a different batch from that used in the experiment of Table 16) was added to the precipitate, and the mixture was stirred at 4° for four hours. The precipitate was recovered by centrifuging, and was washed twice with 0.15 M NaCl. 0.01 M HCl (4 ml.) was then added, and the mixture left to stand at room temperature for 5 minutes before centrifugation. The elution was repeated several times. Each acid eluate was immediately neutralised by adding a mixture containing 0.6 M NaCl, 0.04 M NaOH, and 0.1 M glycyl-glycine buffer, pH 7.4. This mixture, when diluted 1 in 5 with 0.01 M HCl, (as in this experiment), gives a neutral, approximately isotonic, solution. (The eluates in all subsequent experiments involving elution by centrifugation were neutralised in the same way.)

The various fractions were assayed for antibody content at four or five dilutions. The results using the highest concentration of antibody are recorded first, then in sequence, with the results at the lowest antibody concentration presented last.

E. THE PROPERTIES OF ELUTED ANTIBODIES.

1. Antibodies eluted from glucagon immunoadsorbents.

The objective of the experiments described in this section was to use glucagon immunoadsorbents to isolate glucagon antibodies of high affinity, by specifically adsorbing these from an excess of serum of low affinity. Such purified antibodies should give more sensitive immunoassays than the serum from which they were derived.

In the experiment presented in Table 12, already discussed in the previous section, attempts were made to purify high affinity antibodies by these means. Equal amounts of diazocellulose glucagon immunoadsorbent were incubated with a five-fold and a fifty-fold excess of serum respectively. The adsorbed antibodies were eluted, the recoveries measured, and the equilibrium constants of each of the eluates and the original serum measured by means of an immunoassay at two different dilutions. As can be clearly seen from comparing the initial slopes and shapes of the immunoassay curves that were obtained (presented in Fig. 23) there was no significant difference in the equilibrium constants of the eluted antibodies (1.83 and 1.79 x 10^9 1./M, with a fifty-fold excess of serum, and 1.85 and 1.57 x 10^9 1./M, with a five-fold excess of serum) and the serum from which they were derived (1.70 and 2.18 x 10^9 1./M).

The experiment was repeated using bromoacetyl cellulose glucagon immunoadsorbent (Table 17). Each fraction, i.e. the original serum used, the eluted antibodies and the supernatant that remained after adsorption was used in an immunoassay after being diluted to a concentration giving approximately the same binding in the absence of unlabelled hormone. The immunoassay curves are presented in Fig. 24.

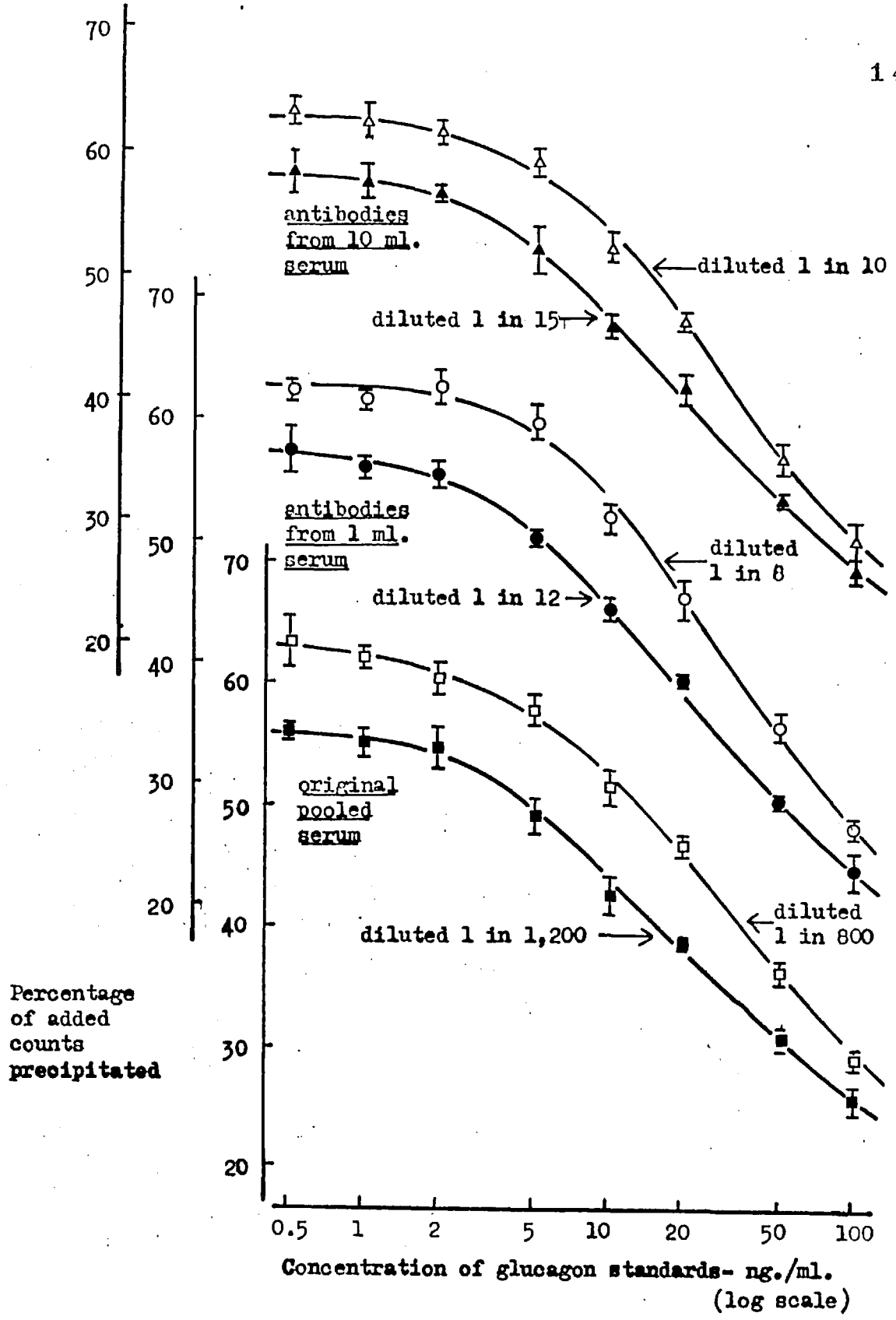


Fig. 23. IMMUNOASSAY WITH ANTIBODIES ELUTED FROM DIAZOCELLULOSE GLUCAGON IMMUNOADSORBENT

Antibodies were adsorbed from two different volumes of pooled antiglucagon serum (1 ml. and 10 ml. respectively), with identical quantities of diazocellulose glucagon immunoadsorbent. Bound antibodies were eluted with dilute HCl. Details of the adsorption and elution are presented in Table 15 .

The eluted antibodies and the original serum were diluted to equivalent concentrations, and used for immunoassay at two dilutions, one 1.5 times the other in each case.

Length of incubation: 3 days No. of replicates: 5
Conc. of ^{125}I-glucagon pipetted (uncorrected for damage): 1.13 ng./ml.
Non-specific binding: 12.1 % added counts
Maximum binding: 85.9 % added counts

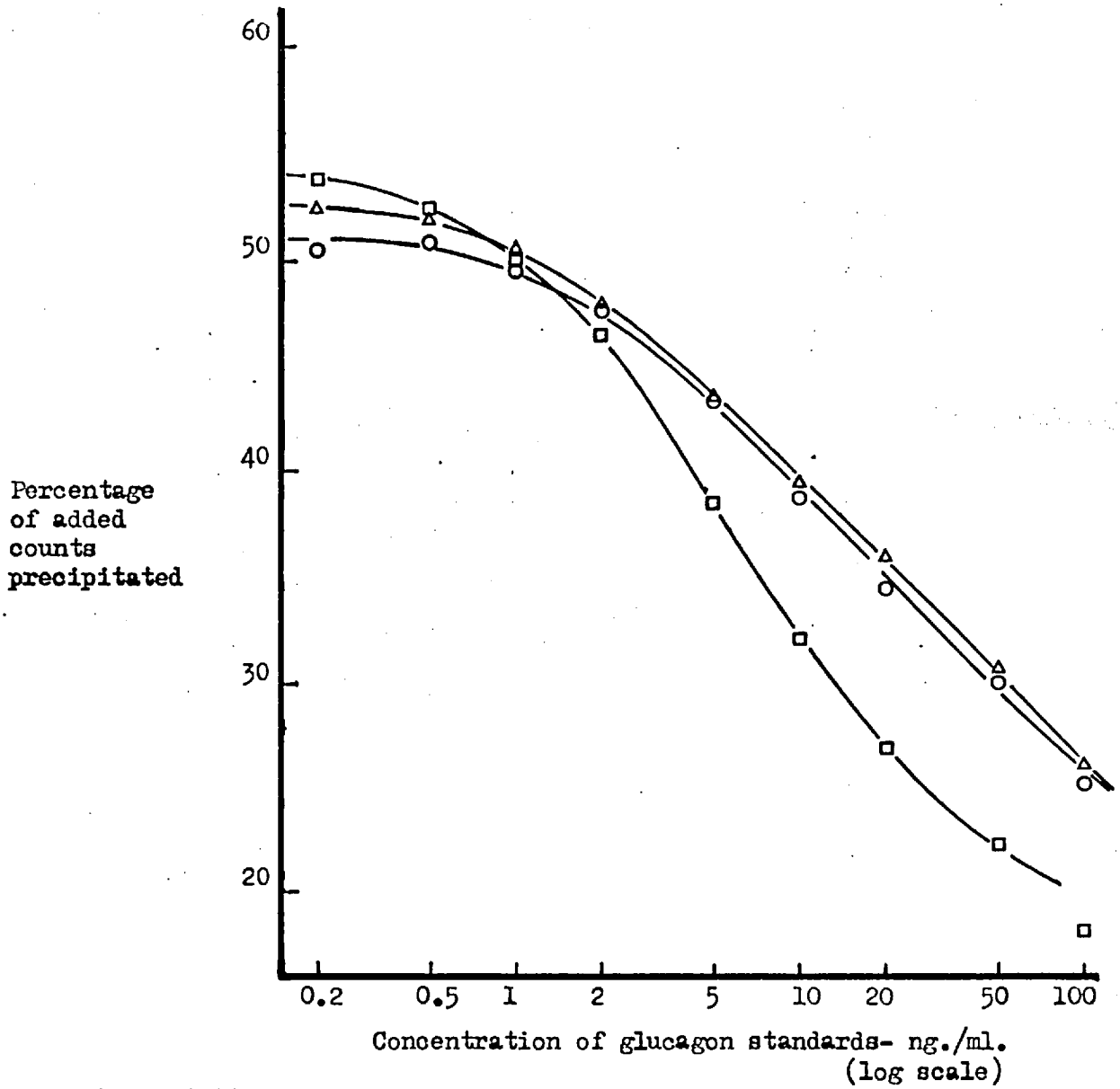


Fig. 24. IMMUNOASSAY WITH ANTIBODIES ELUTED FROM BROMOACETYL CELLULOSE GLUCAGON IMMUNOADSORBENT.

Antibodies were adsorbed from an excess of pooled anti-glucagon serum with bromoacetyl cellulose immunoadsorbent, and were eluted with dilute HCl. Details of the adsorption and elution are presented in Table 17 .

The eluted antibodies, supernatant serum, and the original serum were diluted to equivalent concentrations, and used for immunoassay.

Length of incubation: 3 days
conc. of ^{125}I-glucagon pipetted (uncorrected for damage): 1.0 ng./ml.
non-specific binding: 13.9% added counts
maximum binding: 69.4% added counts

(–O–O–) serum, diluted 1 in 300
(–□–□–) antibodies from first elution, diluted 1 in 110
(–Δ–Δ–) supernatant serum, diluted 1 in 285

As can be seen, there is very little difference in shape between the curve from the pooled serum used for adsorption, and the supernatant after adsorption. This similarity is reflected in the equilibrium constants that were obtained by plotting the data as B/F versus B: 5.0×10^9 1/M for the original serum and 6.5×10^9 1/M for the supernatant after elution.

In contrast the eluted antibodies had an equilibrium constant of 11.1×10^9 1/M, which represents an increase to about double the value for the serum and supernatant. This is perhaps only a small increase when it is considered that the eluted antibodies represent 8% of the total in the original serum (see Table 17). Although the eluted antibodies gave an immunoassay curve with a steeper initial slope than the serum from which they were derived, the sensitivity (0.58 ng./ml. for the original serum and 0.23 ng./ml. for the eluate) was not sufficient to allow plasma levels of glucagon to be measured accurately.

It is possible that diazocellulose glucagon immunoadsorbent and bromoacetyl cellulose glucagon immunoadsorbent have different selective properties because there was no purification with the former, but a significant (if small) purification with the latter. However a different serum was used in each case, so no firm conclusions could be drawn from these experiments.

The possibility that the avidity of diazocellulose glucagon was the same for both high and low affinity antibodies was tested by repeating the adsorption and elution with this immunoadsorbent, using an excess of an equal mixture of a high affinity and low affinity serum.

The results of this experiment and B/F versus B plots derived from immunoassays of the mixed serum and eluate fraction, are presented in Fig. 25. The equilibrium constant of the eluted antibodies was almost twice as high as that of the original mixed serum,

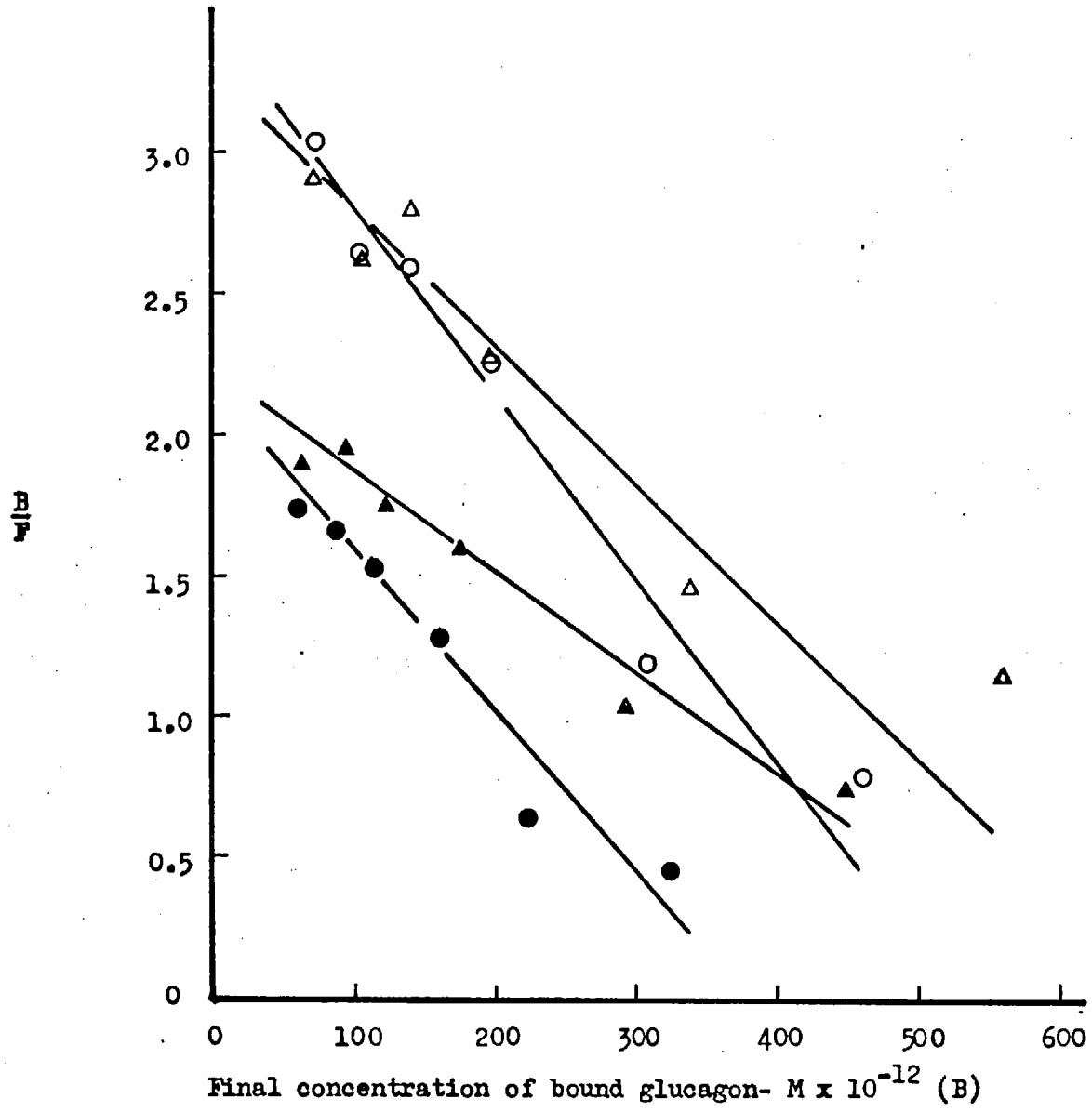


Fig. 25. THE PURIFICATION OF GLUCAGON ANTIBODIES FROM A MIXTURE OF A HIGH AND LOW AFFINITY SERUM, USING DIAZOCELLULOSE GLUCAGON IMMUNOADSORBENT.

A small amount of immunoadsorbent (0.2% of the total batch) was incubated with a mixture of 'high affinity' serum 4B (1 ml.), and 'low affinity' pooled serum (0.18 ml.). The pooled serum contained 5.6 x the concentration of antibodies compared with 4B, so that an equal amount of antibodies were present from both sera. After overnight incubation at 4°, the supernatant was separated by centrifugation, the immunoadsorbent washed with saline, and bound antibodies eluted into immunoassay buffer, using a Millipore filter. The various fractions were assayed for antibody content. The eluate contained 10.3% of the added antibodies, and the supernatant 61.1%, showing that there was a considerable excess of antiserum.

The eluate and pooled serum were both used at equivalent concentrations in immunoassays. The following equilibrium constants were derived from the B/F versus B plots of these assays, presented in the figure above:.

Sample	Assay dilution	K (1/M x 10^9)
mixed serum	1 in 333 (▲–▲–▲)	3.46
	1 in 200 (△–△–△)	5.46
eluted antibodies	1 in 4.13 (●–●–●)	6.11
	1 in 2.47 (○–○–○)	8.28

suggesting that this immunoadsorbent had some degree of specificity for high affinity antibodies.

These experiments demonstrate that it is possible to selectively concentrate antibodies of high affinity using glucagon immunoadsorbents, but that the extent of the purification is slight, and is not sufficient to enable the assay to be used to measure plasma glucagon levels.

2. Antibodies eluted from insulin immunoadsorbents.

Tables 12 and 13 demonstrate the results of experiments in which immunoadsorbents were used to purify insulin antibodies. In this case, the principle objective was to concentrate the antibodies rather than to prepare high affinity antibodies, and therefore a much higher ratio of immunoadsorbent to antibody was used compared with similar glucagon experiments.

Conditions were chosen in which most of the antibodies present were bound, leaving only 2-3% in the supernatant after adsorption, and giving a recovery of 50-60% of the added antibodies on elution. The eluted antibodies would therefore be expected to have the same equilibrium constant as the original serum, and if the immunoadsorbents were selective for high affinity antibodies, the non-adsorbed antibodies should have a lower equilibrium constant.

Figs. 26 and 27 present immunoassay curves using the fractions assayed for antibody content in these experiments. The maximum binding of iodinated insulin was not measured for any of the assays, so that values for the equilibrium constants of the fractions tested cannot be derived. However a comparison of the assay curves that were obtained suggests that the equilibrium constants of the first eluates were almost identical to those of the original sera, as would be expected, while the supernatants were clearly of lower

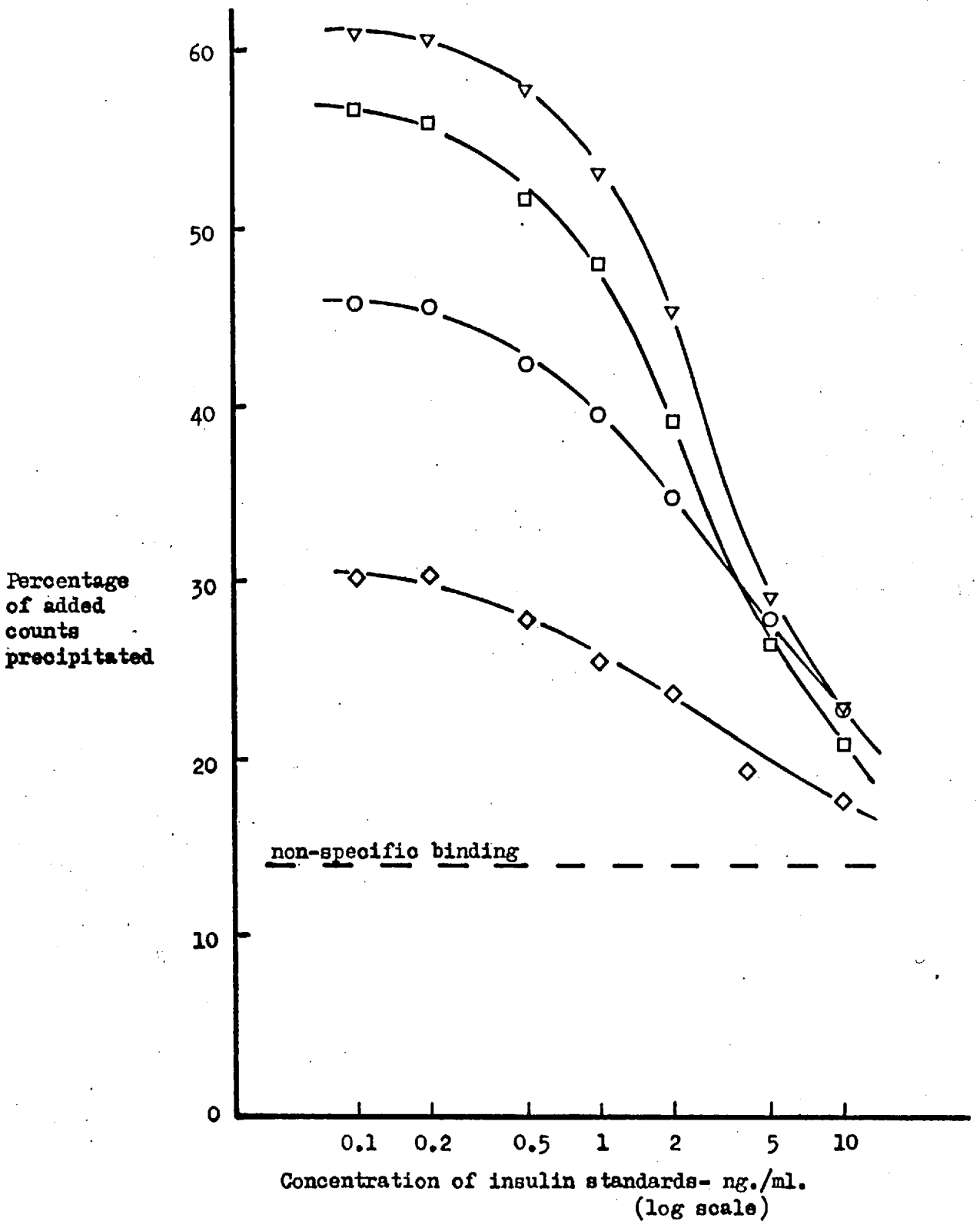


Fig. 26. INSULIN IMMUNOASSAY USING FRACTIONS FROM AN ELUTION EXPERIMENT WITH DIAZOCELLULOSE INSULIN IMMUNOADSORBENT.

Antibodies were adsorbed from pooled anti-insulin serum, and eluted with two successive batches of dilute HCl. Details of the adsorption and elution are presented in Table 12.

The original serum, supernatant serum, and both eluates were diluted to equivalent concentrations, and used in the immunoassays presented above.

(-▽-▽-▽-) original serum, diluted 1 in 5,000
(-O-O-O-) supernatant serum, diluted 1 in 80
(-□-□-□-) first eluate, diluted 1 in 30
(-◇-◇-◇-) second eluate, undiluted

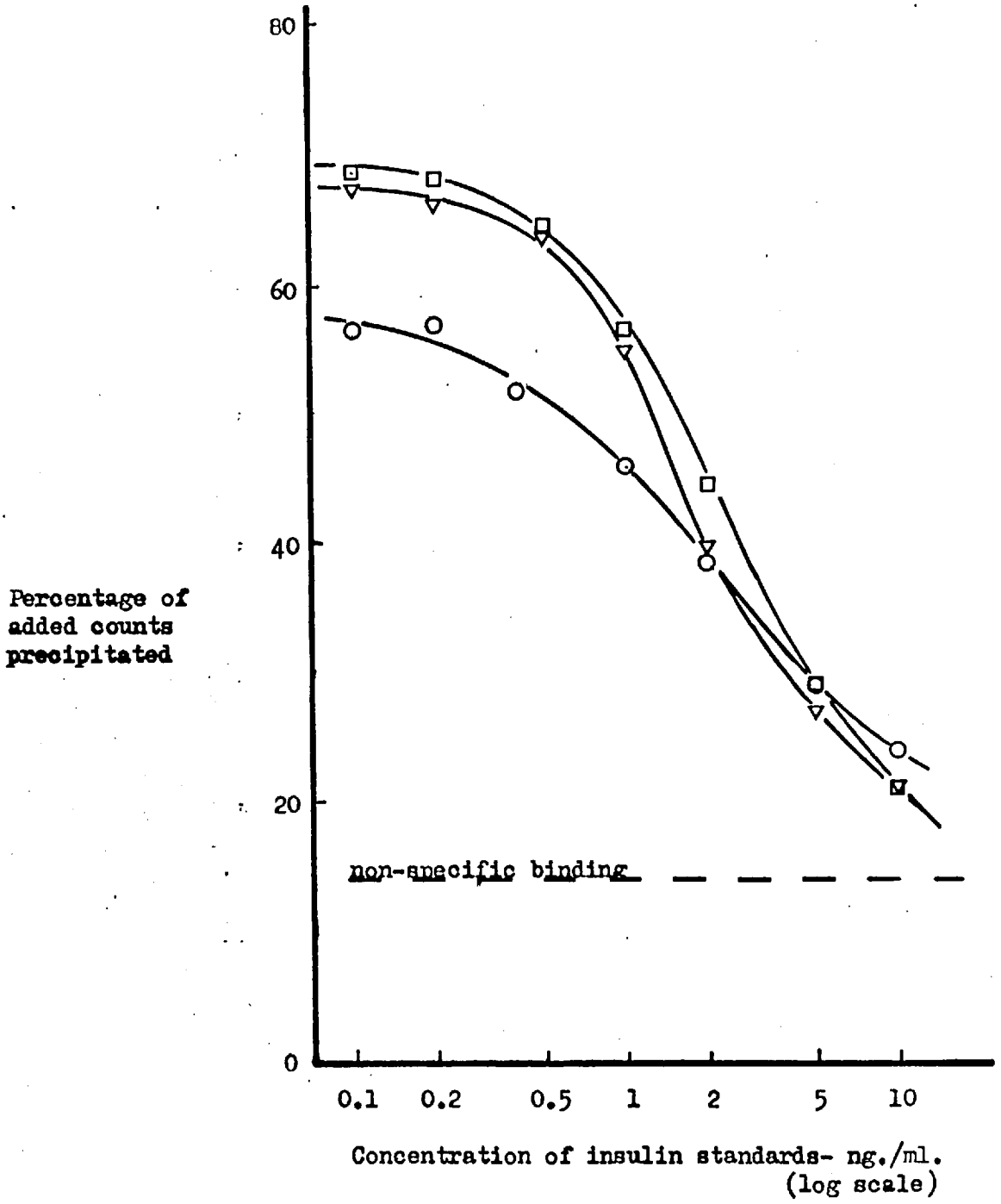


Fig. 27. INSULIN IMMUNOASSAY USING FRACTIONS FROM AN ELUTION EXPERIMENT WITH BROMOACETYL CELLULOSE INSULIN IMMUNOADSORBENT.

Antibodies were adsorbed from pooled anti-insulin serum, and eluted with two successive batches of dilute HCl. Details of the adsorption and elution are presented in Table 13 .

The original serum, supernatant serum, and first eluate were diluted to equivalent concentrations, and used in the immunoassays presented above.

(▽-▽-▽) original serum, diluted 1 in 5,000
(○-○-○) supernatant serum, diluted 1 in 54
(□-□-□) first eluate, diluted 1 in 26

equilibrium constant. However, the difference was small in both cases.

The results suggest that insulin immunoadsorbents as well as glucagon immunoadsorbents were only partially selective for high affinity antibodies.

F. ADSORPTION AND ELUTION.

1. The specificity of adsorption.

The specificity of adsorption was tested by attempting to adsorb and then elute glucagon antibodies from an immunoadsorbent of insulin coupled to diazo-cellulose. If adsorption were totally non-specific, a good recovery of antibodies would be expected, whichever hormone were linked to the immunoadsorbent.

The results of this experiment are presented in Table 18 and show that only a very small amount of the added antibody (0.12%) was recovered in the eluate, although there was an apparent disappearance of 25% of the total antibody during adsorption. It is possible that the eluted antibody was bound by glucagon contaminating the insulin used to prepare the immunoadsorbent, rather than by non-specific adsorption.

2. The recovery of antibody from immunoadsorbents.

The total recovery of added antibody was never 100%, as can be seen from the results of any of the elution experiments presented above. In fact, in circumstances in which a large amount of immunoadsorbent was used, the recovery was sometimes as low as 30%.

Possible explanations for this are as follows:

1. Antibodies were denatured during elution.
2. The elution procedure did not remove all bound antibodies.
3. There was release of free glucagon during elution which neutralised some antibodies, lowering the recovery.

The second and third explanations would account for the lack of success in purifying high affinity anti-

Table 18. THE CROSS REACTIVITY OF GLUCAGON ANTIBODIES WITH INSULIN IMMUNOADSORBENT.

Sample	Vol. serum used	Antibodies recovered in the sample as a percentage of the total added
supernatant after adsorption	0.5 ml.	49 68 56 88
HCl eluate	0.5 ml.	0.0025
supernatant after adsorption	2.0 ml.	68 82 80 83
HCl eluate	2.0 ml.	0.118 0.113 0.115

Two different volumes of a pooled antiglucagon serum (0.5 ml. and 2 ml. respectively) were incubated with diazocellulose insulin immunoadsorbent (0.2 ml. volumes of a 1 in 3 dilution of the original preparation in N.I.S. phosphate buffer) for three days, at 4°, with shaking. The mixtures were then centrifuged, the supernatants aspirated, and the immunoadsorbents washed with buffer and water. Bound antibodies were eluted into veronal immunoassay buffer (7 ml.) with 0.01 M HCl (3 ml.), using a Millipore filter. The various fractions were diluted and assayed for antibody as previously described.

bodies from low affinity sera, and experiments to investigate these possible explanations for incomplete recovery were therefore carried out.

Table 19 records the results of an experiment to measure the rate at which glucagon antiserum was denatured at pH 2.0. Serum was adjusted to the required pH, incubated for various times at 4^{o}, and then neutralised and assayed for residual antibodies. About one third was found to have disappeared after 52 hours incubation, suggesting that denaturation due to low pH accounted for the disappearance of at least a small proportion of the antibodies that were not recovered on elution.

It is possible that immunoadsorbents may have caused denaturation directly, as they consist of finely divided surface-active material. Neutralised acid eluates were usually found to precipitate material on standing. As neither insulin nor glucagon generally form precipitates with specific antibodies it was possible that the precipitated material consisted of aggregated globulin, denatured at the immunoadsorbent surface.

This was investigated by attempting to redissolve the precipitate and assess the antibody content. The precipitates in pooled eluates from an experiment to purify glucagon antibodies were washed once in NaCl (2 ml.: 0.15 M), and partially dissolved in HCl (1.6 ml.: 0.01 M). Undissolved material, perhaps 50% of the precipitate, was removed by centrifuging, and the supernatant was neutralised in the same way as for eluate fractions. On neutralisation there was reprecipitation, so the supernatant was re-centrifuged. It was then assayed, and found to contain 4% of the antibodies of the serum from which it was originally derived.

Table 19. THE DENATURATION OF GLUCAGON ANTIBODIES BY ACID.

Incubation time (hours)	Recovery of antibodies as a percentage of the amount present in the original diluted serum.
1	85.6
2	81.4
8	74.7
23	66.9
52	67.1

Antiglucagon serum (50 µl.) was made to 1 ml. with 0.15 M NaCl, and was then adjusted to pH 2.0 on a pH meter, with 2M HCl. After incubating for 15 minutes at room temperature, the acidified serum was incubated for the remainder of the times indicated at 4°. Samples (200 µl. each) were diluted 1 in 10 with veronal assay buffer, and assayed for antibodies at three different dilutions.

In order to calculate recoveries, it was assumed that the volume of acidified serum was exactly 1 ml., after adjusting the pH. As the volume was slightly larger than this value, the recoveries are not completely accurate in absolute terms.

The same experiment was repeated at pH 2.5 and more alkaline pH values, but there was no denaturation under these conditions.

The material recovered in this way contained antibodies with a reduced solubility, as normal gamma globulins do not precipitate from dilute salt solutions such as neutralised eluates. It is not possible to estimate the proportion of denatured antibodies that were resolubilised by the procedure described above. The amount of antibody present in the precipitate may have been much higher than the 4.1% resolubilised, and could account for a large proportion of the added antibodies (70% of the total) that disappeared during adsorption and elution in this particular experiment.

Table 20 presents the results of two experiments to investigate whether high affinity antibodies were still bound to immunoadsorbent after elution. In both experiments antibodies were bound and then eluted from the same sample of glucagon immunoadsorbent several times. It was found that a higher proportion of the added serum was bound to the immunoadsorbent on the second and subsequent adsorption than on the first, establishing that elution was essentially complete. If antibodies were still bound to the immunoadsorbent, less would be removed from successive batches of serum, until the immunoadsorbent was saturated, when it would cease to bind antibodies at all. These two experiments established, therefore, that a quantitatively significant proportion of bound antibodies did not remain bound after elution.

Experiments were performed to measure the amounts of free glucagon and insulin released during elution by incubating the respective immunoadsorbents with HCl (0.01 M) after washing them with 10% albumin to remove any free hormone. It was found that quantities of hormone equivalent to 2.3% and 0.4% respectively of the immunoreactive hormone content of the immunoadsorbent were released by this procedure (Table 21). These results suggest that the release of free hormone

Table 20. THE ADSORPTION AND ELUTION OF ANTIBODIES SEVERAL TIMES FROM THE SAME IMMUNOADSORBENT SUSPENSION.

Experiment 1.

Sample	Antibodies recovered in the sample as a percentage of the total added in each cycle.	
	cycle 1	cycle 2
supernatant	12.3	6.5
NaCl wash 1	0.3	0.3
NaCl wash 2	0.1	0.1
NaCl wash 3	0.1	0.2
HCl elution 1	23.0	49.5
HCl elution 2	9.6	16.9
HCl elution 3	2.5	4.1
HCl elution 4	0.8	2.3
N.I.S. phos. buffer wash	0.1	0.1
total recovery	36.6	73.5

Experiment 2.

Sample	Antibodies recovered in the sample as a percentage of the total added in each cycle.					
	cycle 1	cycle 2	cycle 3	cycle 4	cycle 5	cycle 6
supernatant	7.0	4.6	3.2	2.2	1.9	2.1
HCl elution 1	19.1	19.8	17.7	15.3	12.2	12.6
HCl elution 2	6.2	9.3	14.0	13.8	16.0	19 2
total recovery	32.4	33.8	34.9	31.2	30.1	33.9

Washed, freshly prepared diazocellulose glucagon immunoadsorbent (10 ml.) was washed four times with 4% albumin in N.I.S. phosphate buffer, to remove any free glucagon. Low activity, pooled anti-glucagon serum was added, and the mixture was stirred at 4° for 12 hours. It was then centrifuged at 6,000 g, and the precipitate was washed twice with 0.15 M NaCl (2 ml.). 0.01 M HCl (1.6 ml.) was then added, and the pellet was carefully resuspended. After standing at room temperature for 5 minutes, the suspension was centrifuged, and the supernatant aspirated, and immediately neutralised as previously described, using 0.4 ml. of neutralisation buffer. The elution was repeated several times.

The immunoadsorbent was then washed with N.I.S. phosphate buffer, and was used to adsorb and elute antibodies from fresh serum once again (experiment 1), and 5 more times (experiment 2). In experiment 1, all fractions were assayed for antibody content, while in experiment 2, only the fractions expected to contain large amounts of antibody were assayed.

Table 21. THE ELUTION OF COVALENTLY BOUND INSULIN AND GLUCAGON FROM DIAZOCELLULOSE IMMUNOADSORBENTS.

Immunoadsorbent	insulin	glucagon
Total hormone released (µg.)(average value of assays at four dilutions).	0.71	1.61
Immunoreactive insulin or glucagon content of the suspension (µg.)	193.5	71.2
Percentage of total immunoreactive hormone released.	0.4	2.3

Insulin and glucagon immunoadsorbent suspensions (10% and 8% of each preparation respectively) were washed five times with 4 ml. quantities of bovine serum albumin (10% w/v) dissolved in N.I.S. phosphate buffer, to remove any free insulin or glucagon. The suspensions were then washed twice with 0.15 M NaCl (4 ml.). 0.01 M HCl (4 ml.) was added, and the suspended immunoadsorbents were left to stand at room temperature for five minutes (to simulate the conditions of elution), before being centrifuged at 4°. The supernatants were neutralised, and the concentration of glucagon and insulin released was measured by immunoassay at four different dilutions.

The batches of immunoadsorbent used in these experiments were of much larger immunoreactive content than those prepared at the beginning of the work.

was not a major contributing factor in determining the apparent equilibrium constant of antibodies eluted from immunoadsorbents in experiments in which an excess of antiserum was used.

3. Elution of neutralised antibodies.

One possible explanation for the low equilibrium constants and titres of the glucagon sera raised in this work was that most of the antibodies were neutralised by endogenous glucagon released from the pancreases of immunised animals. This would result in a decrease in the slope of immunoassay curves, as the true amounts of unlabelled hormone in each sample would be larger than the amounts added.

An experiment was performed in which attempts were made to recover neutralised antibodies by acidifying serum (to dissociate the antibodies from glucagon) and readjusting the pH to 7.3 in the presence of an excess of immunoadsorbent. If a large proportion of the total glucagon antibody in the original serum were combined with glucagon, then the eluted antibodies would be of higher equilibrium constant than the serum from which they were derived, as they would represent an average sample of the total antibody population (bound and free) present in the serum.

It is clear, however, that the slopes of the B/F versus B plots obtained by use of eluted antibodies (Fig. 28) were lower than those in which the original serum was used, suggesting that the eluate was of lower equilibrium constant than the serum from which it was derived.

It may be that these anomalous results were caused by the release of large amounts of free glucagon from the immunoadsorbent during elution, thereby lowering

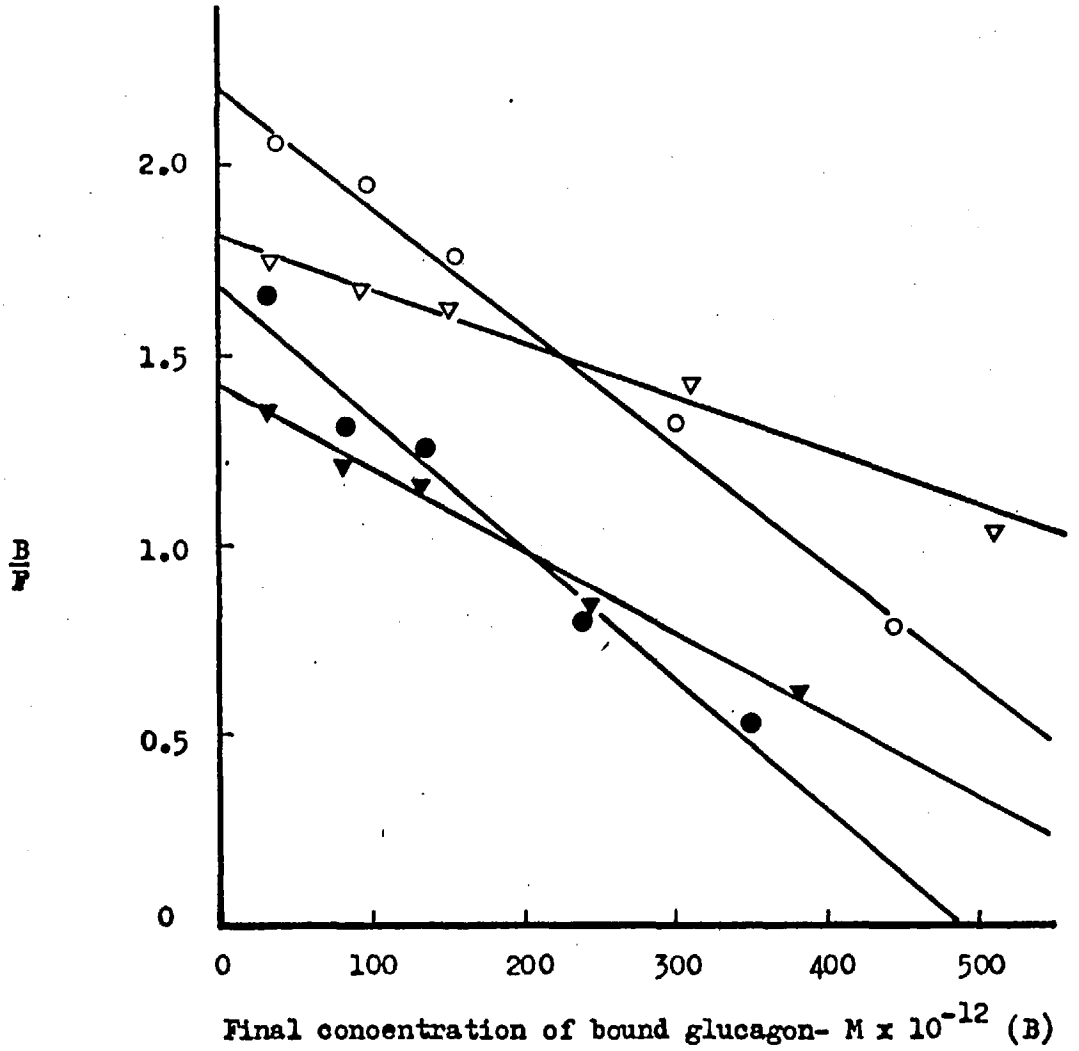


Fig. 28. AN EXPERIMENT TO TEST FOR THE PRESENCE OF ENDOGENOUSLY BOUND GLUCAGON ANTIBODIES IN SERUM.

Pooled antiglucagon serum (0.25 ml.) was diluted to 1.5 ml. with 0.15 M NaCl, and acidified to pH 2.0 using a pH meter. After standing at room temperature for 20 minutes, a large excess of albumin-washed glucagon diazocellulose immunoadsorbent (2.5% of the total batch, in 1 ml. N.I.S. phosphate buffer) was added, and the mixture immediately neutralised to pH 7.3 by adding 5 M NaOH (10 µl.). After stirring for 16 hours at 4°, the antibodies were eluted from the immunoadsorbent through a Millipore filter, with 0.01 M HCl (4 ml.). The neutralised eluate and supernatant were assayed for antibody content. The eluate contained 27.5% of the added antibodies, and the supernatant 0.45%, showing that there was an excess of immunoadsorbent over antiserum.

The eluate and pooled serum were both used at two equivalent concentrations in immunoassays. The following equilibrium constants were derived from the B/F versus B plots presented in the accompanying figure:

Sample	Assay dilution	K (1/M x 10^9)
pooled serum	1 in 800 (O-O-O)	3.20
	1 in 1,200 (●-●-●)	3.53
eluted antibodies	1 in 2.2 (▽-▽-▽)	1.72
	1 in 3.3 (▼-▼-▼)	2.13

the apparent equilibrium constant of the eluted antibodies. In this experiment, there was a much larger ratio of immunoadsorbent to serum than in all other experiments as the adsorption took place with considerable immunoadsorbent excess. The experiment was inconclusive as it did not establish whether neutralised antibodies were present in serum or not. What was shown was that neutralised antibodies could not be recovered using diazocellulose glucagon immunoadsorbent.

It would be of considerable interest to repeat this experiment using an immunoadsorbent in which there was no release of glucagon by hydrolysis during adsorption and elution, as the possibility that antibodies to endogenous hormones are present in sera in a partially neutralised form has not been extensively investigated.

G. DISCUSSION

One objective of the present work was to selectively separate high affinity glucagon antibodies present in sera, and to use these in a glucagon immunoassay so as to increase the sensitivity sufficiently to measure plasma levels of glucagon.

Techniques for separating insulin antibodies were tried first, to be used as a model applicable to the problem of purifying high affinity glucagon antibodies, as the separation of insulin antibodies by the use of immunoadsorbents (Miles and Hales, 1968a) was already described in the literature and insulin antiserum was available in large amounts.

The results presented above show that it was not possible to separate antibodies of sufficient affinity to significantly increase the sensitivity of the glucagon immunoassay. There was evidence, however, that glucagon immunoadsorbents were at least partially selective for high affinity antibodies. This could be concluded from the results presented in Fig. 25, in which an excess of a mixture of two sera containing antibodies of greatly differing affinity was treated with diazocellulose glucagon immunoadsorbent. The eluted antibodies were of higher equilibrium constant than the serum from which they were derived. The same results were obtained in an experiment (Fig. 24) in which a pooled serum was treated with bromoacetyl cellulose glucagon immunoadsorbent.

Having established that glucagon immmnoadsorbents were partially selective for high affinity antibodies, there remained alternative explanations for the failure to obtain useful separation of high affinity antibodies from sera. In the first place, it was possible that the elution conditions failed to remove

high affinity antibodies from the immunoadsorbent. The experimental findings, however, showed that elution was complete. In the case of glucagon, two experiments were performed in which adsorption and elution of antibodies was carried out several times from the same immunoadsorbent. The results (Table 20) showed that the capacity of the immunoadsorbent to adsorb antibodies was not diminished with re-use, i.e. immunoreactive sites on the immunoadsorbent were not becoming blocked by antibody that had failed to be eluted. In the case of insulin, evidence for the complete elution of high affinity antibodies was provided by two experiments in which immunoassay curves using antibodies eluted with two successive batches of dilute HCl were compared (Figs. 26 and 27). The second eluates were not apparently of higher affinity than the first, suggesting that high affinity antibodies were not remaining bound to the immunoadsorbent, to be eluted on further incubation with dilute HCl.

Another explanation was that free glucagon or insulin was released from the immunoadsorbent during elution, neutralising high affinity antibodies, but not low affinity antibodies. This would account for the low total recovery of antibody in elution experiments and could explain why recoveries were higher with Millipore filtration than with elution by centrifugation. With the former technique, the immunoadsorbent was in contact with dilute HCl for a much shorter time.

Experimental support for this explanation comes from the results presented in Fig. 28. This experiment was designed to test whether endogenously neutralised antibodies were present in serum and for this purpose the immmunoadsorbent was used in large excess. If free glucagon were released from immunoadsorbent during

elution the use of large amounts of immunoadsorbent would give partially neutralised eluates of apparently lower equilibrium constant than the serum from which they were derived. The equilibrium constant of the eluted antibodies was in fact lower than that of the original serum in this experiment, whereas in other experiments in which a lower ratio of immunoadsorbent to serum was used, the eluted antibodies had the same affinity, or higher affinity, than the original serum.

Experiments were performed to measure the amount of insulin and glucagon released from diazocellulose immunoadsorbents under the conditions used for elution (Table 21). After incubating with dilute HCl, there was release of only a small proportion of the immunoreactive hormone content of the immunoadsorbents, suggesting that the release of free hormone during elution would only account for the low affinity of eluted antibodies in experiments in which very high ratios of immunoadsorbent to antiserum were used.

An alternative explanation to account for difficulties encountered in purifying high affinity antibodies was that the affinity of antibodies for free hormone was not related to the affinity for immunoadsorbent-bound hormone. A small molecule such as glucagon may only have a few amino acid sequences which act as antigenic determinants, and only antibodies to one of these sequences may be of high affinity. If the structure were changed either chemically or conformationally by coupling the hormone to immunoadsorbent, antibodies for glucagon itself would only react with very low affinity to the modified determinant. Such structural changes are in fact likely to occur during chemical coupling. Both insulin and glucagon bind to cellulose with great avidity. This binding may cause conformational changes that are maintained

when the hormones are coupled to the immunoadsorbent. Chemical modification will occur if an antigenic site is directly involved in coupling. Diazocellulose couples through tyrosine hydroxyl groups and aminocellulose through ε-lysyl and terminal amino groups, both of which may be part of antigenic determinant sequences.

The arguments presented above presuppose that high affinity antibodies to glucagon (and low affinity antibodies to insulin) are present in the sera used for these experiments in significant amounts. If the proportion of total antibody with a high binding affinity were very small, then there would be insufficient enrichment on elution to make a difference to immunoassay sensitivity.

There are no obvious means of measuring the proportion of antibodies of different affinity in sera, so it is not possible to predict the degree of enrichment of high affinity antibodies that would be expected using immunoadsorbents with the same selectivity as glucagon itself. It is not possible therefore to specify whether attempts to obtain a useful enrichment of high affinity antibodies in eluates in these experiments failed because of an absence of high affinity antibodies in the sera, or because of a lack of selectivity of the immunoadsorbents used. It may be that the complete explanation is a combination of both factors.

To summarise, results suggest that the immunoadsorbents made in this study were specific for antibodies to the antigens that they contained, and that the elution conditions used were suitable to remove all bound antibodies. Difficulties in obtaining high affinity antibodies cannot therefore be ascribed to faulty elution technique or to lack of specificity of antibodies for antigens coupled to immunoadsorbents.

CHAPTER 4.

THE INTRAVENOUS ADMINISTRATION OF ANTIBODIES.

A. INTRODUCTION.

During this work glucagon antibodies were successfully purified from low titre sera using immunoadsorbents, and it was proposed to investigate whether any metabolic changes could be observed in animals made glucagon deficient by injection of these antibodies.

At the time when these experiments were performed the use of anti-glucagon serum to produce acute glucagon deficiency had been reported by one worker only, and this report was in a very preliminary and incomplete form. This author (Braun, 1966) claimed that administration of anti-glucagon serum to rats caused a transient hypoglycemia. In contrast, acute insulin deficiency produced by the intravenous injection of guinea pig antiserum to pork insulin (anti-insulin serum, AIS) has been extensively investigated since AIS was first produced (Moloney and Coval, 1955).

AIS administered intravenously in doses approximately equal to antibody equivalents of 1.5 to 2 units of insulin/Kg. has been found to cause a prompt rise in blood glucose to 200 mg./100 ml. or above in a wide range of mammals, including mouse (Moloney and Coval, 1955), rabbit, rat and cat (Armin, Grant and Wright, 1960), and dog, pig and sheep (Armin, Cunningham, Grant, Lloyd and Wright, 1961). The response is transient in most mammals, with blood glucose at a maximum at approximately one hour, although in some species, e.g. sheep, the response has been found to take somewhat longer (four hours).

Armin *et al.* (1960) have shown that in rats the degree and duration of hyperglycemia is a function of the dose of anti-insulin serum injected, although the

initial rate of increase in blood glucose was found to be approximately the same, whatever the dose. These results have been extended by Wright, Rivera-Calimlim and Malaisse (1966) who have investigated the kinetics of the hyperglycemic response to AIS, and the disappearance of insulin antibodies from the plasma of injected rats. These authors have shown that most of the injected antibody is no longer present in a free form in the plasma by the time blood glucose values have reached a maximum. There was little decline in the plasma concentration of antibody in alloxan-diabetic rats similarly injected with AIS, showing that the disappearance was caused by neutralisation of the antibodies by endogenously released insulin, and not primarily by removal from the circulation. These results have been confirmed by Moody, Jeffcoate and Volund (1970), who found that the concentration of free insulin in the blood of starved rats injected with AIS intraperitoneally fell to a very low level by one hour, and thereafter increased to supranormal values, indicating that there was an excessive release of insulin even when all antiserum had been neutralised, presumably in response to hyperglycemia.

Gregor, Martin, Williamson, Lacy and Kipnis (1963) have shown that when a dose of AIS was injected into rats which theoretically was sufficient to completely deplete the pancreas of insulin, it did not in fact lower the pancreatic insulin content to below 40% of the initial value. This suggests that the pancreas may respond to the injection of insulin antibodies both by releasing stored insulin, and by a rapid re-synthesis of the hormone. These authors have also shown that it is necessary to administer sufficient antiserum to neutralise the total quantity of stored insulin, in order to obtain a maximum increase in the level of blood glucose.

It was decided, by analogy to these experiments with insulin antibody, to inject sufficient glucagon antibodies to neutralise all the glucagon-like immunoreactive material present in the animals used. It was not possible, however, to measure the absolute amounts of total glucagon-like immunoreactive material with any certainty, because the gut contains immunoreactive material as well as the pancreas (Samols, Tyler, Megyesi and Marks, 1966; Unger, Ketterer and Eisentraut, 1966; Valverde, Rigopoulou, Marco, Faloona and Unger, 1970).

Gut 'glucagon' is not identical to pancreatic glucagon either physico-chemically or immunologically, but usually cross-reacts extensively with antibodies to glucagon, although to different extents with different sera (Unger, Aguilar-Parada, Muller and Eisentraut, 1970).

In the present work it was not possible to estimate the correct amount of glucagon antibodies to neutralise the total 'glucagon' content of the rat because the cross-reactivity of antibodies purified for these proposed experiments was not known. It was therefore judged appropriate to inject approximately twice the quantity of antibodies necessary to neutralise the total pancreatic glucagon content of the experimental animals. In earlier studies, it was found that the pancreas of a 300 g. rat contained approximately 4 µg. glucagon (see Results section of Chapter 5).

B. METHODS.

1. Injection and bleeding techniques.

Experiments were normally performed on male Sprague-Dawley rats weighing 300 g. These were injected in a tail vein, and bled by cutting off 2 mm. of the end of the tail, or, in the case of animals given ether anaesthetic, by inserting a needle into another of the four veins of the tail in the posterior end, and allowing blood to drip out. A final blood sample was always taken from the heart immediately after the animals had been bled from the tail, allowing comparative measurements on blood obtained from these two sources.

In most experiments, the animals were kept under continuous anaesthesia by an initial intra-peritoneal injection of a 6% solution of pentabarbitone sodium - "Nembutal" (0.30 ml.), followed by small additional quantities (0.05 ml.) at the slightest signs of consciousness. In some of the insulin antibody injection experiments, the animals were lightly anaesthetised with ether while being injected or bled, and were allowed to recover consciousness between operations.

2. Measurement of blood glucose.

Each blood sample (50 µl.) was first de-proteinised by mixing with 5% $ZnSO_4 7H_2O$ (1 ml.), and then adding an equal quantity of 0.15 M $Ba(OH)_2$. After remixing, the samples were centrifuged and the clear supernatants assayed for glucose. (The zinc sulphate and barium hydroxide solutions were standardised to give a neutral supernatant on mixing equal volumes).

The de-proteinised extract was then assayed for glucose by the 'glucose oxidase' method of Marks and Lloyd (1963), using O-toluidine hydrochloride as the electron donor. Standard blood glucose samples were 'de-proteinised' in the same way as experimental samples before assay.

C. RESULTS.

1. An assessment of the quantity of glucagon antibodies administered.

The glucagon antibodies used in these experiments were extracted from 120 ml. of a pooled serum, in 20 ml. volumes using diazocellulose glucagon immunoadsorbent. This was re-incubated with a fresh batch of serum following each acid elution to a total of six times. The antibody recovered in the acid eluate fractions (1) and (2) from each cycle and that left in each of the supernatants was measured, and shown to represent a recovery of approximately 33% in each case. The first two neutralised acid eluates from each of the six cycles were pooled and concentrated by vacuum dialysis to 4.62 ml. The antibody content of the concentrated material was measured by the comparative method at five different dilutions and it was shown to contain 6.6 times the concentration of antibodies of the original serum from which it was derived. As the eluted antibodies can be presumed to have the same equilibrium constant as the original serum, this figure may be taken as an accurate indication of the concentration achieved.

The original serum was assayed for antibody content using B/F versus B plots on four occasions and with two different batches of iodinated glucagon. The B/F versus B plots that were obtained were non-linear, but the initial slopes could be extrapolated to give approximate estimates of the minimum antibody content. The values that were obtained were:

iodination (1) - 2.28 µg./ml. and 1.23 µg./ml.

iodination (2) - 1.70 µg./ml. and 1.83 µg./ml.

It can be calculated from the average of these values

that 1 ml. of the concentrated antibodies contained sufficient binding sites to neutralise a minimum of 11.7 µg. of glucagon.

2. Metabolic response to the injection of glucagon antibodies.

Table 22 presents the results of blood glucose measurements over a period of two hours in rats previously deprived of food for four hours from 10 a.m. and injected with 0.5 ml. or 1.0 ml. of purified glucagon antibodies or the same volume of saline (control animals).

Two experimental animals (nos. 4 and 9) and one control animal (no. 5) showed a decrease in blood glucose concentration greater than 15%, and it was concluded that there was no measureable effect of injected glucagon antibodies on blood glucose levels under the conditions of the experiment.

One possible explanation for the absence of an effect of glucagon antibodies on blood glucose levels was that the intravenous tail vein injection technique was faulty. To investigate this possibility, sera from the animals which had received antibodies were assayed for apparent antibody content. The results of these assays are presented in Table 23, and suggest that there was a possible failure of injection technique in animals nos. 3 and 4, which had a low (and increasing) concentration of glucagon antibodies in the serum. However, animals nos. 9, 10 and 11 had high serum antibody levels consistent with the distribution of injected antibody in a volume of 14.9 15.7 and 30.2 ml. of serum half an hour after injection respectively. The first two of these values are equal to the plasma volume of a 300 g. rat, suggesting that

Table 22. BLOOD GLUCOSE VALUES OF RATS INJECTED WITH PURIFIED GLUCAGON ANTIBODIES.

Time (hrs.) after injection			before	0.5	1.0	1.5	2.0	2 hr. heart (h) or femoral vein (f) blood gluc.
Rat no.	Injected material	Injection volume (ml.)	Blood glucose values (mg./100 ml.) obtained from tail-vein blood (average of duplicate measurements)					
1	saline	0.5	72.4	81.8	83.2	87.3	85.8	121.4 (f)
2	saline	0.5	108.7	100.4	105.1	102.0	103.5	134.7 (f)
3	antibody	0.5	104.7	97.1	99.1	92.6	91.5	111.0 (h)
4	antibody	0.55	103.5	90.5	85.7	died	-	-
5	saline	1.0	117.9	98.1	100.0	105.8	93.9	89.7 (h)
6	saline	1.0	95.1	107.8	103.8	99.8	92.5	107.3 (h)
7	saline	1.0	81.5	96.8	86.8	95.6	102.5	112.2 (h)
8	saline	1.0	95.5	91.9	86.5	89.7	99.0	124.7 (h)
9	antibody	1.0	116.3	99.5	83.3	80.5	82.1	84.0 (h)
10	antibody	1.0	99.8	98.4	95.6	111.7	98.8	106.5 (h)
11	antibody	1.0	96.3	80.9	85.1	89.7	90.3	111.1 (h)

Male Sprague-Dawley rats (280-340 g.) were starved 4-5 hours from 10 a.m., and then anaesthetised with Nembutal (0.3 ml.) injected interperitoneally. Successive small doses were administered subsequently to keep the animals anaesthetised. The rats were bled by cutting the tail, and immediately afterwards, saline (control animals) or antibody (experimental animals) was injected into the tail vein. The posterior end of the tail was clamped manually during the injection to ensure that antibodies did not leak through the cut end. Further bleedings were then made at intervals of 30 min., and the animals were in addition bled from the heart or femoral vein immediately following a final tail bleeding at 2 hours.

The blood was analysed for glucose after deproteinisation. Duplicate determinations were made. These were found to differ by an average of 4%.

Table 23. ANTIBODY CONTENT OF THE SERUM OF RATS INJECTED WITH GLUCAGON ANTIBODIES.

Rat number	3	4	9	10	11
Antibody injection volume (ml.)	0.5	0.55	1.0	1.0	1.0
Time (hours) after injection	Apparent content of glucagon antibodies, relative to the original solution of purified antibodies (%)				
0.5	0.10	0.10	6.72	6.37	3.31
1.0	0.16	0.13	5.77	5.24	3.11
1.5	0.19	-	5.61	5.21	2.96
2.0	0.20	-	5.09	5.14	2.64
heart puncture	0.21	-	4.93	4.19	2.67

Blood from the cut end of the tails of rats injected with purified glucagon antibodies (further details of this experiment are described in the previous Table) was allowed to clot at room temperature for 1 hour, and was then centrifuged. The supernatant sera were diluted 1 in 20, 1 in 50, 1 in 100, 1 in 200, 1 in 500 and 1 in 1,000, and equal volumes (75 µl.) of each dilution were incubated with the same volume of iodinated glucagon (1.0 ng./ml.). After 36 hrs., the tubes were ethanol precipitated and counted. The antibody content of each dilution was measured by comparison with a dilution curve of the purified antibodies. This curve is presented in Fig 2 . Values of the antibody content of each serum, calculated for each dilution assayed, did not differ by more than 20% from the average results presented above.

the injected antibodies were not appreciably re-distributed from the blood over the period of the experiment.

There was at most a small fall in the apparent antibody content of the serum between half an hour and two hours in animals nos. 9, 10 and 11, suggesting that the administered antibodies were not extensively neutralised during this period by endogenous glucagon. However, this conclusion is tentative, as it is possible that the method of antibody assay may not discriminate completely between free and neutralised antibodies if these are of low affinity. Neutralised antibodies would be expected to re-equilibrate with added radioactive hormone during the assay.

3. Metabolic response to the injection of insulin antibodies.

An alternative explanation for the failure of injected glucagon antibodies to cause hypoglycemia was that the properties of the antibodies had been changed by the purification procedure. It was considered possible, for example, that there was denaturation during elution, which changed the glucagon binding properties of the eluted antibodies.
To test this, insulin antibodies were purified by an identical technique, injected into rats, and the blood glucose response measured.

The results of these experiments are presented in Table 24 , which shows that purified antibodies were capable of neutralising endogenous insulin, as each animal showed hyperglycemia of 200 mg./100 ml. glucose or above, which was maximal one hour or one and a half hours following injection.

Further experiments were performed using insulin

Table 24. BLOOD GLUCOSE VALUES OF RATS INJECTED WITH PURIFED INSULIN ANTIBODIES.

Rat number	1	2	3
Time (hours) after injection	Blood glucose values (mg./100 ml.) (average of duplicate determinations)		
0	165	138	124
0.5	239	191	152
1.0	348	217	242
1.5	202	260	182
2.0	136	160	74

Male Sprague-Dawley rats (150-180 g.) were anaesthetised with ether and bled from the tail-vein. Insulin antibodies, purified using immunoadsorbent and concentrated to 6.2 times the antibody content of the original low-titre pooled serum from which they were derived, were injected through another tail vein. The same volume (0.5 ml.) was administered to each animal. Further bleedings were made at 30 min. intervals after injection, with the animals re-anaesthetised for each bleeding.

(In addition to these results, two further animals were injected with 0.5 ml. and 1.0 ml. of antibody preparation, but only bled one hour after injection, from the heart. The blood was collected in heparinised tubes, and the plasma was found to have blood glucose concentrations of 256 and 396 mg./100 ml. respectively.)

antiserum to investigate the time course of the disappearance of antibodies to insulin, as it was considered interesting to briefly repeat the results of Wright, Rivera-Calimlim and Malaisse (1966) using the present method of antibody measurement. In these experiments, which are presented in Table 25, the largest volume of serum administered (1 ml.) was calculated to be slightly in excess of the content of pancreatic insulin of the rats used, as serum 8 had a neutralising capacity of 85 µg./ml. (page 47) and the average pancreatic insulin content was found to be 74 µg./rat (page 204).

All animals receiving antibody exhibited a hyperglycemic response of at least 40% by half an hour after injection, and showed an apparent disappearance of antibodies from the blood. The concentration declined to approximately 12% of the initial (calculated) value, but did not apparently fall further, in agreement with the results of Wright et al. (1966). It is likely that the non-neutralised antibody represents antibody which cross-reacts to antigenic sites present in ox insulin, which are absent from rat insulin.

There was an increase in the magnitude and duration of the hyperglycemic response with the injection of increasing amounts of anti-insulin serum. In the animal in which the largest amount of antibody was injected (1 ml.), the hyperglycemia appeared to be disproportionally slow to decline, and the antibody content of the serum was still changing slowly between two and two and a half hours after administration of AIS. However, in animals receiving less antibody, the content had fallen to static levels by one or one and a half hours. It may be that the decrease in hyperglycemia was relatively slow in animal no. 1 because the pancreatic insulin reserves were

Table 25. BLOOD GLUCOSE VALUES AND THE TIME COURSE OF ANTIBODY NEUTRALISATION IN RATS INJECTED WITH ANTI-INSULIN SERUM.

Rat number	1	2	3	4	5
Material injected	serum 8	serum 8	serum 8	serum 8	saline
Volume injected (ml.)	1.0	0.5	0.25	0.1	0.5
Time (hours) after injection	Blood glucose values (mg./100 ml.) (average of duplicate determinations)				
0	98	109	101	123	122
0.5	143	228	140	200	105
1.0	269	308	290	133	104
1.5	391	228	225	108	93
2.0	383	132	100	96	112
2.5	333	120	98	102	126
Heart	356	141	104	152	-
Time (hours) after injection	Apparent concentration of insulin antibodies relative to serum 8 (%)				
0 (calc.)	6.67	3.33	1.67	0.67	-
0.5	5.18	1.02	0.99	0.16	-
1.0	2.72	0.41	0.29	0.09	-
1.5	1.46	0.31	0.21	0.09	-
2.0	1.09	0.29	0.20	0.09	-
2.5	0.85	0.27	0.19	0.08	-
Heart	0.86	0.28	0.21	0.08	-

Male Sprague-Dawley rats (280-305 g.) were anaesthetised with Nembutal interperitoneally and bled from a tail vein. Five minutes later, various amounts of A.I.S. 8 were injected into another tail vein. The control rat was injected with saline. Further bleedings from the tail were made at 30 minute intervals up to two and a half hours, and finally, from the heart also.

Serum was prepared from blood obtained at each bleeding, and assayed for insulin antibodies over a range of six two-fold dilutions from 1 in 250 to 1 in 10,000, in comparison with serum 8. The dilution curve of serum 8 is presented in Fig. 1.

The values for the antibody content of each rat serum at zero time were calculated by assuming that the injected antibody was initially distributed in a serum volume of 15 ml.

completely depleted by the administration of the maximal quantity of antibodies and the remaining antibody could only be neutralised by the relatively slow process of the synthesis of new insulin.

It will be noticed that glucose levels of heart blood were found to be higher than those from the tail vein, as was also found in the glucagon injection experiments. The reason for this discrepancy is unknown in either case. The difference is not considered to invalidate the results, as glucose values of tail blood of animals injected with insulin antibody fell to approximately pre-injection levels by two and a half hours (except for animal no. 1, which had elevated heart levels also) suggesting that the tail circulation was relatively unimpaired. This conclusion is supported by the results of the measurement of serum antibody levels. In both the glucagon and insulin antibody injection experiments the concentration of antibodies in heart blood was the same as that in tail blood taken at the same time. Antibody levels from the tail of an animal in which there was perhaps a failure of injection technique (animal no. 3 of the glucagon antibody injection experiment) were not elevated above those in the heart, suggesting that there was no leakage of antibodies from the intravascular space of the tail (where the injected material was presumably administered) to the cut end. Measurements from tail blood, therefore, quantitatively reflect those from blood in the general circulation.

D. DISCUSSION.

The results of the experiments reported in this section show that the administration of insulin antiserum intravenously to fed rats caused a transient acute hyperglycemia which had a time course of one or two hours, and was associated with the neutralisation of antibodies present in the serum over the same time course, as reported by other investigators.

Injection of glucagon antibodies, on the other hand, did not cause an obvious change in blood glucose levels over the same period, and there was no apparent disappearance of antibodies from the serum of injected animals.

These latter results conflict with those of Braun (1966) who claimed that injection of pooled anti-glucagon serum caused a depressed blood sugar level for at least two hours. The time course of this effect was not measured in fed rats, although with force-fed rats this author noted hypoglycemia only after five hours.

On the other hand, the present results are consistent with those of Grey, McGuigan and Kipnis (1970) who have administered glucagon antiserum intravenously to both fed and forty eight hour starved adrenalectomised rats. These authors reported that there was no change in the blood sugar level of the fed animals, although there was a steady decrease in blood sugar to approximately 60% of the initial level over a period of one and a half hours following injection in the forty eight hour starved group. These authors considered that the hypoglycemia observed in starvation was caused by inhibition of glucagon-stimulated gluconeogenesis.

There is considerable evidence that the main

physiological function of glucagon is to stimulate glucose output by the liver. As it has been established that one of the ways in which this is achieved is through stimulation of hepatic glycogenolysis, glucagon depletion should induce hypoglycemia over the time period when hepatic glycogenolysis would normally be at a maximum.

It has been shown that the liver glycogen reserves of rats are completely depleted after twenty four hours of starvation (Guest, 1941). The animals have been found to show a moderate decrease in blood glucose level between 6.00 a.m. and 12.00 a.m. as part of their circadian rhythm (Friedman and Walker, 1969). This is probably due to the cessation of feeding in the early hours of the morning. Therefore a brief period of starvation (four to five hours) from mid-morning should induce a situation where active mobilisation of glycogen is maintaining blood glucose levels. The fact that there was no decrease in blood glucose when glucagon antibodies were injected after a period of brief starvation could indicate that glucagon stimulation of glycogenolysis is of minor significance in the initial phase of starvation of the rat. However, there are alternative explanations.

A very plausible explanation for the apparent failure of glucagon antibodies to cause hypoglycemia is that there was insufficient time for glucagon to be neutralised after leaving the pancreas before entering the liver.

An approximate calculation from the kinetic data of Fig. 19 of the rate at which glucagon reacts with antibody at the concentrations that were injected into rats in this study (assuming that the rate of reaction was proportional to the concentration of antibody administered) suggests that it would take twenty

seconds to half neutralise a 1.0 ng./ml. concentration of glucagon at 4^{o}. If the rate of reaction of antibody with glucagon doubles for a 10^{o} rise in temperature, at 37^{o} the half neutralisation time would be approximately two seconds.

This time interval is approximately the same as the time that one might expect blood to take to travel from the pancreas to the liver via the portal circulation in a rat. Hence it is conceivable that there is insufficient time for glucagon leaving the pancreas to be extensively neutralised by antibody present in the circulation before it arrives at its target organ (the liver).

Ketterer, Eisentraut and Unger (1967) have shown that 1 µg. of glucagon injected into the portal blood supply of dogs elevates blood glucose concentrations without elevating arterial glucagon values to a degree sufficient to be detected by immunoassay. This suggests that most of the glucagon secreted by the pancreas is destroyed in the circulation, and that levels which will cause changes in blood glucose by stimulating glycogenolysis are only present in the portal blood supply as a direct result of secretion by the pancreas. The residual amounts found in the peripheral circulation may not be significant in regulating phosphorylase activity in liver.

If glucagon is only partially neutralised during the time taken to travel from the pancreas to the liver by the amounts of antibody administered, there may be little or no effect on blood glucose levels, as approximately the same quantities of free glucagon will reach the liver as when no antibody is administered.

A second possible explanation is that depletion of blood glucagon levels by antiserum was off-set by an increase in the circulation of catecholamines caused

by the experimental procedure. This explanation would account for the difference in response obtained in the present study and that obtained by Grey et al. (1970) who used adrenalectomised rats. These were not used in this present investigation because hypoglycemia often spontaneously accompanies adrenalectomy in starved animals (Long, Katzin and Fry, 1940).

No apparent increase in tail blood glucose concentration was found in control animals in either insulin or glucagon antibody injection experiments, suggesting that the experimental procedure did not cause a stimulation of glycogenolysis, and therefore that removal of the adrenals was unneccessary. The animals were anaesthetised with Nembutal throughout the experiments, and it has been shown (Guest, 1941) that Nembutal causes minimal stimulation of glycogenolysis in the rat. There appears to be some doubt whether epinephrine in physiological quantities contributes significantly to hyperglycemia. Sokal, Sarcione and Henderson (1964), using perfused rat liver, have shown that normal blood concentrations of glucagon will stimulate glycogenolysis but that, to achieve the same stimulation with epinephrine, it is necessary to use greater amounts than known to occur in the blood naturally.

A third possible explanation for the lack of metabolic effect of glucagon antibodies on blood glucose levels is that insufficient antibody was administered. In the study of Grey et al. (1970) the binding capacity of the antiglucagon serum used for injection was calculated to be 450 ng. glucagon equivalents per ml. This figure was an underestimate of the true binding capacity, however, as it was derived by assuming that the affinity constant of the serum was infinite. When the binding capacity

of the purified antibodies used in the present study was calculated on the same basis (Table 26) a value of 2-5 µg. glucagon equivalents per ml. was obtained. It is, therefore, probable that the binding capacity of the concentrated antibodies used in the present study was considerably higher than that of the serum used by Grey et al. (1970).

A fourth possible explanation is that the affinity of glucagon binding sites in the liver was very much higher than the affinity of the administered antibodies so that glucagon antibodies were simply acting as a carrier for glucagon, which diffused onto receptor sites as soon as these were encountered. No attempts to measure the affinity of glucagon receptor sites for the hormone have been reported in the literature, but one may speculate that the affinity is in all probability extremely high, as the concentration of glucagon which will cause a measureable increase in glucose output from the liver is very low (Sokal et al., 1964).

In conclusion, there are several possible explanations for the lack of effect of purified glucagon antibodies on blood glucose levels of the rat, most of which cannot at present be distinguished experimentally.

Table 26. THE TITRE OF INJECTED GLUCAGON ANTIBODIES, CALCULATED BY THE METHOD OF GREY ET AL. (1970).

Expt. no.	Serum dilution	Specific binding (%)	Binding capacity- µg. glucagon per ml. serum
I	1 in 10,000	24.7	2.5
	1 in 20,000	16.4	3.3
	1 in 50,000	9.1	4.5
II	1 in 10,000	17.7	1.8
	1 in 20,000	11.2	2.2
	1 in 50,000	5.1	2.6
	1 in 100,000	4.4	4.4

In two separate experiments, performed several months apart, the concentrated glucagon antibody preparation used in the experiments of Table 22 was diluted as indicated, and equal volumes (75 µl.) of the diluted samples incubated with the same volume of iodinated glucagon (1.01 ng./ml., uncorrected for damaged hormone), for two days. The tubes were then ethanol precipitated and counted.

CHAPTER 5.

THE MEASUREMENT OF PANCREATIC INSULIN AND GLUCAGON.

A. INTRODUCTION.

The initial aim of the present study was to develop a method of glucagon immunoassay which would be sufficiently sensitive to measure glucagon secretion from isolated islets of Langerhans, and to develop a double antibody precipitation technique in order to measure serum glucagon.

However, the assay developed and described above was not sufficiently sensitive to achieve these aims, but was of sufficient sensitivity to assay glucagon in extracts of rat and mouse pancreases over a wide range of dilutions.

For many years, active programmes of research have been pursued in this Department on obese hyperglycemic mice and on experimental diabetes in rats, produced by treatment with Streptozotocin. Therefore it was decided that it would be of interest to apply the glucagon immunoassay technique to measure the pancreatic glucagon content of genetically obese mice and their lean littermates, and of normal and Streptozotocin-treated rats. The insulin content of the pancreases was also assayed.

No measurements by immunoassay of pancreatic glucagon levels in these two groups of animals have been reported in the literature, although there have been numerous studies on the pancreatic insulin levels. These studies will be discussed below.

1. Measurements in obese mice (ob/ob) and lean littermates.

The American strain of obese hyperglycemic mice (ob/ob), from which the strain of animals used in this study were derived, was first discovered as a new mutant by Ingalls, Dickie and Snell (1950), and was found to be homozygous for a recessive mutant gene. The characteristic biochemical features of the obesity have been reviewed by Hellman (1965). A vast literature exists on the hormonal control of carbohydrate and lipid metabolism in the ob/ob mouse, and in this section, discussion is restricted to the work relevant to the pancreatic hormones insulin and glucagon.

A characteristic feature of the ob/ob mouse is the hyperinsulinemic condition in the fed state. Insulin levels may become elevated to twenty times those of lean mice but are reduced by restricting the food intake (Stauffacher, Lambert, Vecchio and Renold, 1967; Abraham, Dade, Elliott and Hems, 1971). Westman (1968) has shown that elevated blood insulin is amongst the earliest signs of developing obesity and occurs at approximately the same age (one month) at which hyperglycemia and the accumulation of excess fat resulting in overweight are first observed.

Extensive studies on the histology of the obese mouse pancreas have been made. It has been shown that there is increased number and hyperplasia of the islets of Langerhans (Bleisch, Mayer and Dickie, 1952; Gepts, Christophe and Mayer, 1960; Hellman, Brolin, Hellerstrom and Hellman, 1961). The average volume as well as the number of islets have been shown to be increased in comparison with lean littermates (Hellman, 1965).

The increased volume of islet tissue in obese animals has been shown to be correlated with an increase in the total insulin present in the pancreas, which in older mice has been estimated as ten to twenty times that of control animals (Wrenshall, Andrus and Mayer, 1955). More detailed studies have shown that the insulin content of the obese mouse pancreas varies with the age of the animal, being lower in very young animals and elevated in old animals. Thus Stauffacher et al. (1967) have shown that obese mice of five months of age of both sexes have pancreatic insulin contents which are about five times those of lean littermates, although young obese mice of five weeks showed a decrease in pancreatic insulin.

Genuth (1969) has extended these observations to a wider range of ages, and has shown that there is less pancreatic insulin in obese animals of six weeks of age, identical levels at seven weeks, and thereafter an increase to eleven times the normal levels at forty four weeks. Hyperinsulinemia was shown to develop before there was noticeable hyperplasia, at an age when the pancreatic insulin content was reduced or normal. This suggests that the low pancreatic insulin content of young obese mice is caused by oversecretion, and that with increasing age insulin synthesis appeared to adapt and large amounts of insulin are stored.

Very much less information is available concerning the glucagon content of the obese mouse pancreas, or factors influencing its secretion. Mayer, Andrus and Silides (1953) and Mayer, Silides and Bates (1953) have shown that diethyl dithiocarbamate (an agent which has been reported to selectively destroy α cells of islets of Langerhans (Kadota and Midorikawa, 1951) lowers hyperglycemia in obese mice. They have concluded that obese animals are characterised by oversecretion of an α cell hormone.

No experiments to confirm this suggestion have been made. Indirect evidence for the possibility of elevated glucagon levels comes from the findings in this Department (Elliott, Hems and Beloff-Chain, 1971) that hepatic glycogenolysis is much more rapid in the obese mouse. However, contrary to this, no increase in gluconeogenesis in the fed or starved obese mouse liver was observed by these authors. Schull and Mayer (1956) have reported a three-fold increase in glycogen turnover of the liver of obese mice (measured by the incorporation of uniformly-labelled ^{14}C-glucose) relative to control animals, and Schull, Ashmore and Mayer (1956) have shown a six-fold increase in total liver phosphorylase activity, although part of the explanation for this was that liver weights were three times as high in the obese as in normal animals.

There is evidence from histological studies that there may be a very limited hyperplasia of the α cells in the pancreases of obese animals. Thus Wrenshall, Andrus and Mayer (1955) have observed a frequency of α cells of 7% in islets from obese animals, compared with 30% in controls. This represents a slight increase in the total number of α cells. Gepts, Christophe and Mayer (1960) have extended these studies and have shown that hypertrophy of obese hyperglycemic mouse islets was due primarily to an increase in the number of β cells, but there was also a limited increase (approximately three times) in the number of α cells. Hellman (1961) has shown that α_1 cells, which are probably not involved in glucagon release, but perhaps secrete a substance which inhibits the release of insulin (Hellman, 1970), are increased in number in obese animals to half the extent of β cells. This author has found that the frequency of α_2 cells in obese mice is approximately the same as the frequency found for both α cell types by Gepts *et al.* (1960) and has concluded that there is a very low proportion of α_2 cells (i.e. glucagon secreting

cells) in the obese mouse pancreas.

The conflict in the results of different histological studies and metabolic experiments as to whether there is an over-secretion of glucagon in obese animals can only be resolved by the measurement of blood glucagon values in these animals. This has not yet been reported.

Measurement of pancreatic glucagon levels by immunoassay may, however, provide some information as to whether there is increased secretion of glucagon in obese mice because increased secretion may be associated with increased pancreatic storage, as has been shown for insulin in these animals.

2. Measurements in Streptozotocin-diabetic rats.

Streptozotocin is a cytotoxic agent, causing a permanant diabetic condition in various species of animals, including rats and dogs (Rakieten, Rakieten and Nadkarni, 1963), monkeys (Pitkin and Renolds, 1970) and mice and guinea pigs (Brodsky and Logothetopoulos, 1969).

Junod, Lambert, Orci, Pictet, Gonet and Renold (1967) have reported that the drug is specific for β cells of the islets of Langerhans, and causes no damage to the α cells or to the exocrine pancreas of rats. These results are in accord with those of Brodsky and Logothetopoulos (1969) working with mice and guinea pigs. These authors have shown that Streptozotocin has no effect on the α cells in either species. Arison, Ciaccio, Glitzer, Cassaro and Pruss (1967) on the other hand have suggested that very large doses cause changes in both the exocrine pancreas and the α cells of rats. Each of these studies was based upon histological examination of pancreatic tissue. No attempt was made to measure the glucagon content of the islets of the treated animals. Katsilambros, Rahman, Hinz, Fubganger, Schroder,

Staub and Pfeiffer (1970) have, however, measured blood glucagon concentrations, and have shown serum immunoreactive glucagon to be elevated from 0.14 ng./ml. to 4.17 ng./ml. in starved rats treated with Streptozotocin (65 mg./Kg.) forty eight hours previously.

As Streptozotocin in the doses used in these experiments did not cause histologically identifiable damage to αcells, the increased rate of glucagon secretion may have been a metabolic response to low blood insulin or high blood glucose levels in these animals. In which case it is likely that over-secretion continues into the chronic phase of Streptozotocin-diabetes. If this is so, measurement of the pancreatic glucagon contents of animals which have been made diabetic with Streptozotocin some considerable time previously will provide evidence as to whether oversecretion of glucagon is accompanied by a change in the amount stored in the pancreas.

B. METHODS OF EXTRACTION.

1. Mouse pancreases.

Care was taken during dissection to ensure that as much of the total pancreas was removed as possible, especially the part attached to the small intestine. The pancreases were collected in small weighed plastic tubes, immediately frozen in dry ice, and stored at -25° for periods not exceeding a month.

The pancreases were extracted by the method of Kenny (1955) by homogenising the whole organ in acid ethanol (2 ml.)*. The suspensions were left to stand at 4° overnight and were then centrifuged at 4,000 g for ten minutes. The precipitates were re-extracted with 1.6 ml., and finally 1.2 ml. of acid alcohol.

The pooled supernatants (ca. 4 ml.) were neutralised to pH 7.5 with concentrated ammonia, using indicator paper. The precipitate which formed was centrifuged and discarded. The volume of the total supernatant was found by weighing, taking the density of neutralised acid ethanol to be 0.8775.

Aliquots of the supernatants (1.0 ml.) were pipetted into polyethylene tubes, and a mixture of eighty five parts (by volume) alcohol plus one hundred and forty parts (by volume) ether (4.5 ml.) was added to each. The tubes were capped, mixed and left to stand overnight. The resulting precipitates which contained insulin and glucagon were recovered by centrifugation. The supernatants were discarded.

To recover glucagon, 0.2 M glycine buffer, pH 9.0,

*(750 ml. absolute alcohol + 250 ml. water + 15 ml. concentrated HCl, cooled to 4° before use).

containing 2% albumin (0.5 ml.) was added to each tube and the dried precipitate thoroughly broken up and suspended using a 'Vortex' homogeniser/mixer. The suspension was allowed to stand at room temperature for five hours to allow all the glucagon present to dissolve. Any insoluble material was then centrifuged off. To recover insulin the dried precipitates were dissolved in 0.8% phenol in 0.03 M HCl (0.5 ml.) at 4^{o}. The extracts were diluted with radioimmunoassay buffer to concentrations suitable for assay which was carried out shortly afterwards.

2. Rat pancreases.

Rat pancreases were extracted in a similar way, but all volumes were increased two and a half times relative to those used for mouse extracts.

C. PANCREATIC INSULIN ASSAYS.

Each extract was assayed at four or more serial, approximately two-fold dilutions, and the insulin content was compared with an extract of a pancreas of the same species chosen as a 'standard', which was assayed over a very large range of dilutions. 'Standards' were always chosen from among control animals.

This procedure was necessary because the cross-reactivity of both mouse and rat insulin was different from that of the ox insulin that was available as 'standard'. This is illustrated by Fig. 29, which presents mouse and ox assay curves plotted on a log. scale. The figure shows that the curves were not superimposable over most of the dilution range. This result differs from that of Stauffacher et al. (1967) who reported that a standard curve obtained with pork insulin was similar to that obtained with serial dilutions of a mouse pancreatic extract. Fig. 30 presents an equivalent curve to that of Fig. 29 for rat and ox insulin which indicates that these were not superimposable either.

The technique of comparing extracts with a 'standard' extract gives hormone concentrations in relative rather than absolute units. It is possible to convert from relative to absolute measurements if it is assumed that rat and mouse insulin bind to antibody in equivalent amounts to ox insulin at low concentrations, and have lower cross-reactivity only at higher concentrations. The curves can then be 'fitted', as has been done in both Figs. 29 and 30.

It is not claimed that this method of obtaining absolute insulin contents is accurate, or gives results which would correspond to bioassays. The justification

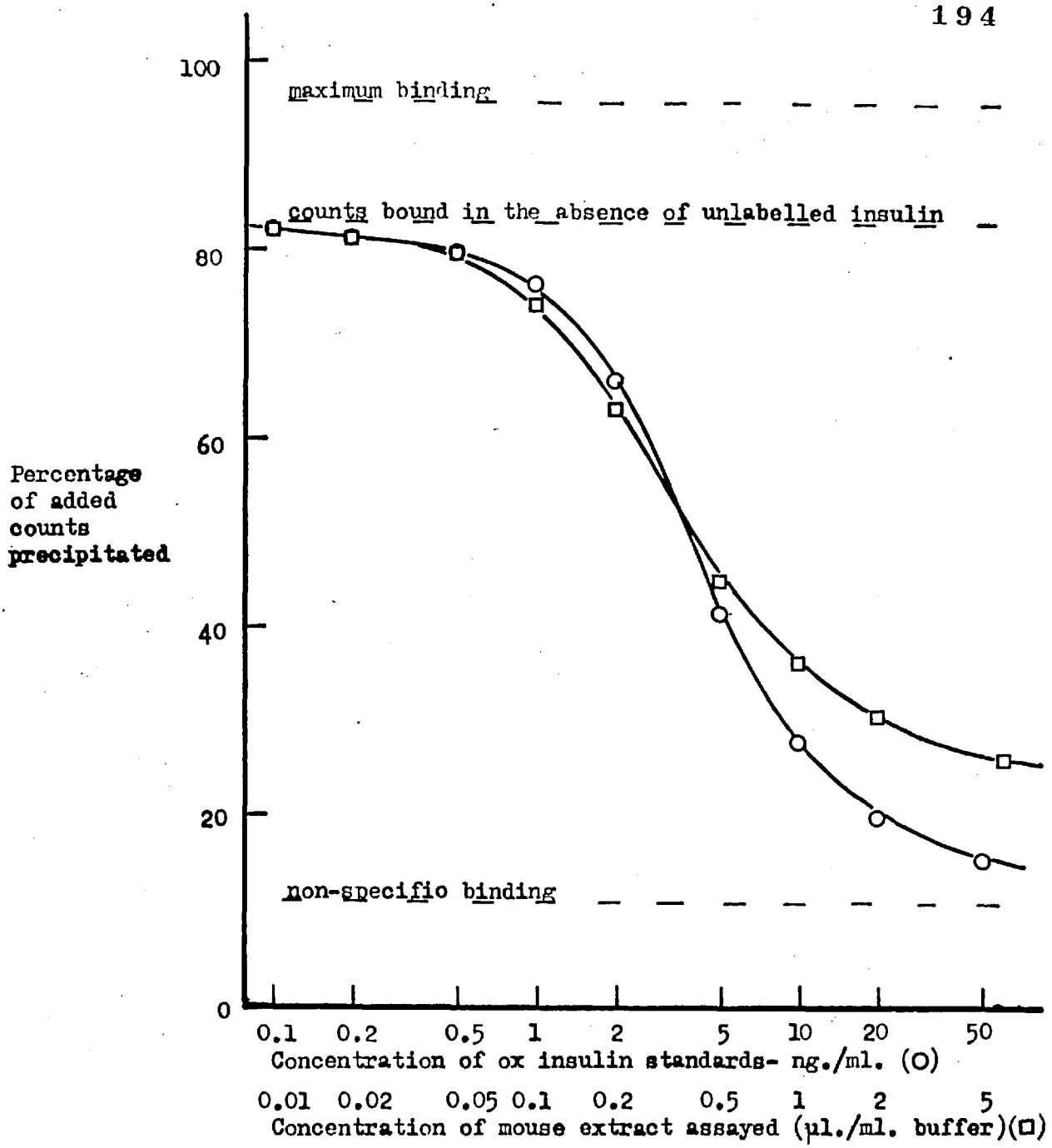


Fig. 29. THE CROSS-REACTIVITY OF A MOUSE PANCREATIC EXTRACT WITH GUINEA PIG ANTIBODIES TO OX INSULIN.

A pancreas from a lean mouse was extracted with acid-ethanol, and an aliquot (approx. 25%) of the neutralised extract was precipitated with alcohol-ether. The precipitate was resolubilised in 0.8% phenol in 0.03 M HCl (0.5 ml.). Various dilutions of this solution in veronal assay buffer, and a range of ox insulin standard solutions, (50 µl.), were incubated in replicates of five with ^{125}I-insulin (50 µl.: 2.5 ng./ml.), and with serum 8 (diluted 1 in 30,000) (50 µl.). After incubating for two days at 4°, the tubes were ethanol precipitated and counted.

(—O—O—) ox insulin standard curve
(—□—□—) mouse pancreas extract dilution curve

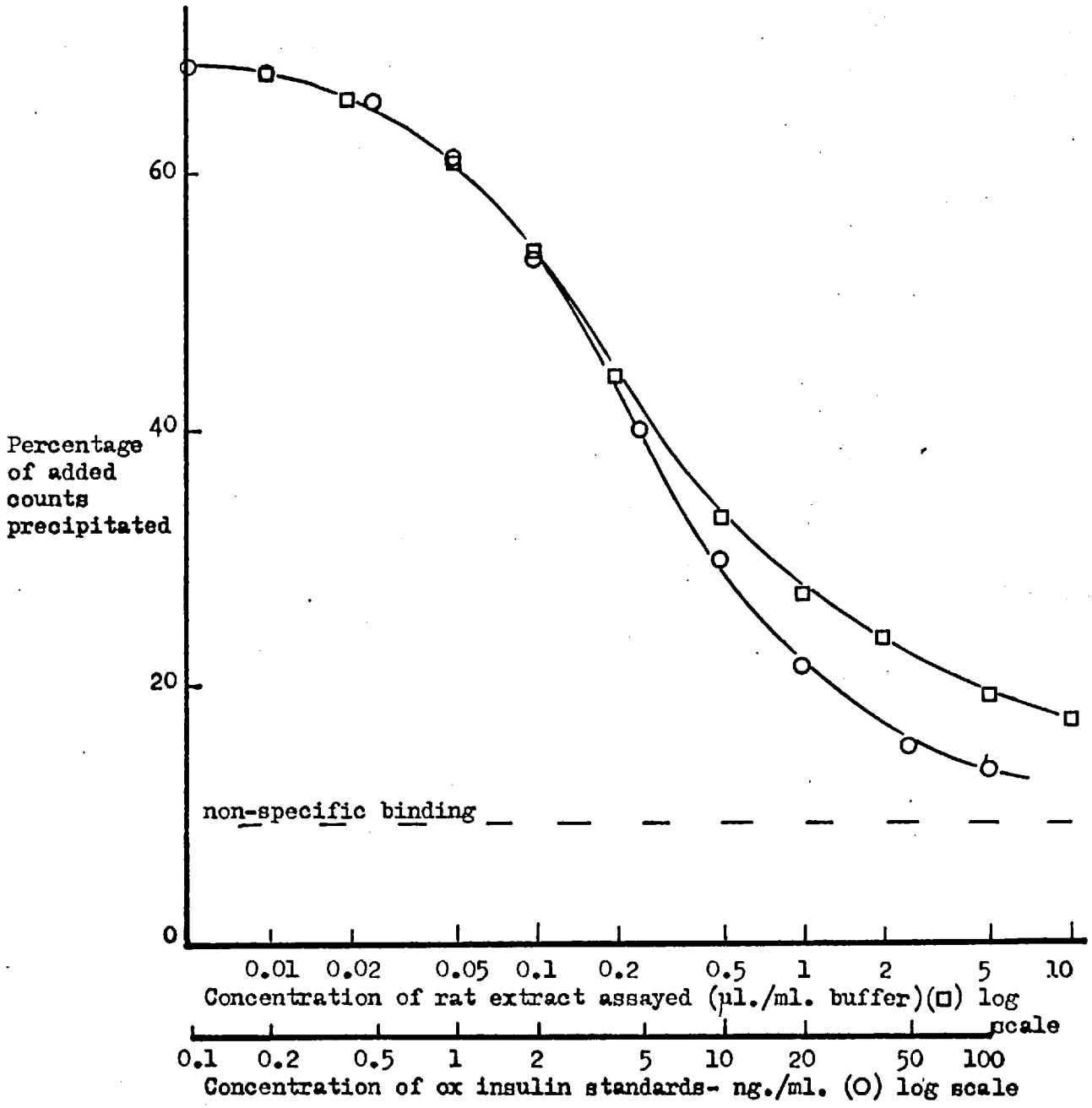


Fig. 30. THE CROSS-REACTIVITY OF A RAT PANCREATIC EXTRACT WITH GUINEA PIG ANTIBODIES TO OX INSULIN.

A pancreas from a male Sprague-Dawley rat was extracted with acid-ethanol, and an aliquot (approx. 8%) of the neutralised extract was precipitated with alcohol-ether. The precipitate was resolubilised in 0.8% w/v phenol in 0.03 M HCl (0.5 ml.). This solution was assayed at various dilutions against a range of ox insulin standard solutions, as in the analogous experiment with a mouse extract, presented in Fig. 29. The experimental conditions in the two assays were identical. Maximum binding was 85.4% of the added counts in each case.

(-□-□-) rat pancreas extract dilution curve
(-O-O-) ox insulin standard curve

for presenting the results in absolute units is that it is thereby possible to make an approximate comparison with the results of other workers.

D. PANCREATIC GLUCAGON ASSAYS.

Mouse and rat glucagon appear to cross-react with beef-pork glucagon antiserum to the same extent as beef-pork glucagon.

There was very little difference between the shape of a beef-pork standard curve and that in which serial dilutions of a mouse pancreatic extract were assayed. As can be seen from Fig. 31, the curves only differed slightly at the highest concentrations of hormone. This was also true for pancreatic extracts from rats as can be seen from the curves presented in Fig. 32. It was not, therefore, considered necessary to use 'standard' extracts, and the counts of unknown rat and mouse samples were related directly to a beef-pork standard curve.

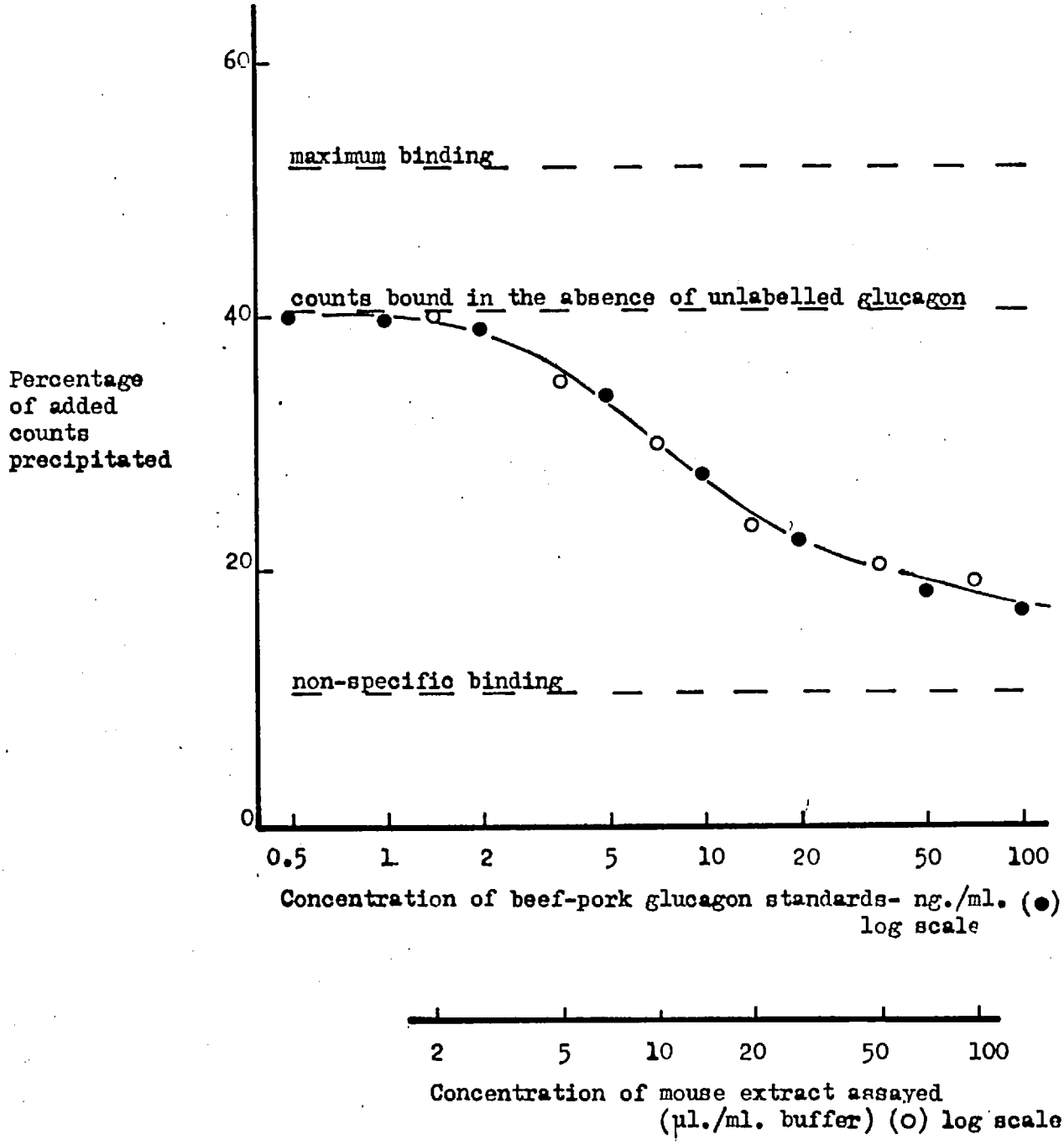


Fig. 31. THE CROSS-REACTIVITY OF MOUSE PANCREATIC EXTRACT WITH RABBIT ANTIBODIES TO BEEF-PORK GLUCAGON.

Two pancreases from lean mice were extracted with acid-ethanol, and an aliquot of the neutralised extract (approx. 25%) was precipitated with alcohol-ether. The precipitate was resolubilised in 0.2 M glycine buffer, pH 9.0 (0.5 ml.). Various dilutions of this solution in veronal buffer, and a range of standard beef-pork glucagon solutions (50 µl.) were incubated in replicates of four with ^{125}I-glucagon (50 µl.: 1.2 ng./ml.), and with serum 4B (diluted 1 in 400) (50 µl.). After incubating for three days at 4°, the tubes were ethanol precipitated and counted.

(—●—●—) beef-pork glucagon standard curve
(—o—o—) mouse pancreas extract dilution curve

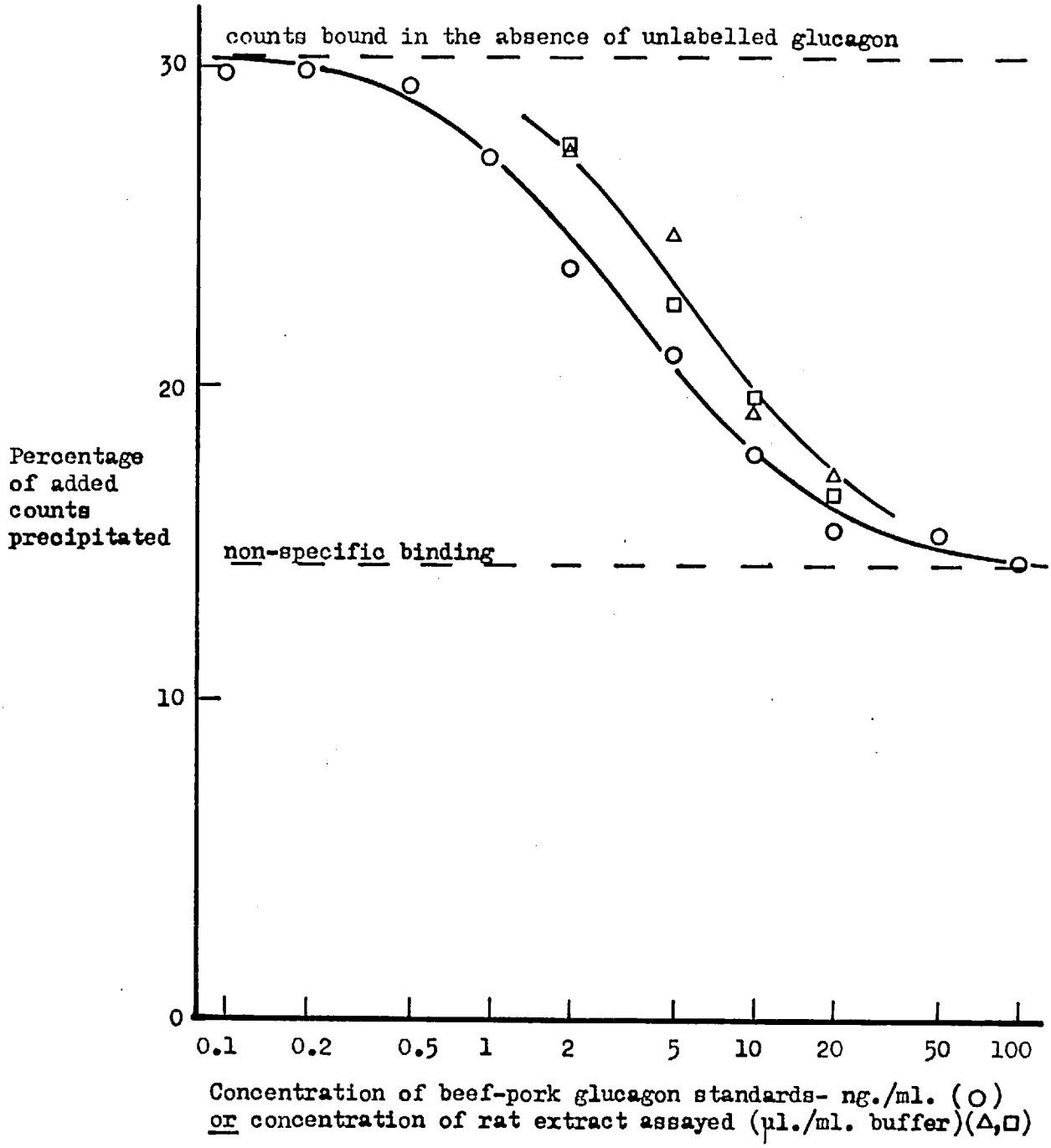


Fig. 32. THE CROSS-REACTIVITY OF AN EXTRACT FROM THE PANCREAS OF A STREPTOZOTOCIN-TREATED RAT, AND A CONTROL RAT, WITH RABBIT ANTIBODIES TO BEEF-PORK GLUCAGON.

Pancreases from Streptozotocin-treated and untreated Sprague-Dawley rats were extracted with acid ethanol, and aliquots of each neutralised extract (approx. 8% in each case) were precipitated with alcohol-ether. The precipitates were resolubilised in 0.2 M glycine buffer, pH 9.0 (0.5 ml.). These solutions were assayed at several dilutions against a range of beef-pork glucagon standard solutions, as in the analogous experiment with mouse extract, presented in Fig. 31. ^{125}I-glucagon was pipetted at a concentration of 1.0 ng./ml., and the antiserum was diluted 1 in 500. Otherwise conditions were identical to those for the assay of mouse extract. Maximum binding was 47.5% of the added counts.

(—O—O—) beef-pork standard curve
(—□—□—) Streptozotocin-treated rat pancreas extract dilution curve
(—Δ—Δ—) control rat pancreas extract dilution curve

E. RESULTS.

1. Obese mice and lean littermates.

Tables 27 and 28 present the results of the measurement of glucagon and insulin contents of the pancreases of obese hyperglycemic mice of the genetic type ob/ob, in comparison with lean animals.

At the time when these experiments were carried out obese mice were in short supply, and only animals of the age groups used were available for these experiments. Littermates which had not developed the obese syndrome were used as the control animals. Approximately two thirds of these would be expected to carry the obese gene recessively.

The interpretation of the data for three to four month animals is complicated because there was a large and statistically significant difference between the average weights of the pancreases of the two groups, the obese animals having pancreases over twice the weight of lean animals (Table 27). This was thought to be caused by the presence of fat in the obese pancreases, which was not apparent in the one year old animals. In fact, obese animals of that age had a lower pancreatic weight than comparative lean animals, the difference being significant at $p = 5\%$ (Table 28).

For reasons which will be mentioned in the Discussion, the results will be considered in terms of the weight of hormone extracted per whole pancreas, rather than in relative terms, although the data expressed in weight of hormone extracted per gram of tissue is also presented to enable comparison with the results of other workers.

The amount of insulin stored in the pancreases of

Table 27. PANCREATIC INSULIN AND GLUCAGON CONTENTS OF 3-4 MONTH OLD OBESE FEMALE HYPERGLYCEMIC MICE IN COMPARISON WITH LEAN LITTERMATES.

	Female lean littermates* (6)	Female ob/ob* (4)
Pancreas weight (mg.)	176 ± 52	428 ± 124
Insulin extracted (μg.)	26 ± 7	119 ± 58
Glucagon extracted (μg.)	0.61 ± 0.12	1.00 ± 0.08
Insulin extracted (μg./g. wet weight pancreas)	138 ± 50	300 ± 18
Glucagon extracted (μg./g. wet weight pancreas)	3.2 ± 0.9	2.5 ± 0.6
$\frac{\text{Insulin}}{\text{Glucagon}}$	42 ± 7	118 ± 53

*Values are presented as the mean, ± S.D., with the number of animals given in parenthesis.

The pancreases were acid-ethanol extracted, and the extracts assayed for insulin and glucagon respectively at four two-fold dilutions over the range 1 in 500 to 1 in 5,000 (insulin assay), and 1 in 100 to 1 in 1,000 (glucagon assay). These dilutions were chosen so that the hormone content of the diluted samples was within the range of concentrations that could be assayed with high relative precision in each case.

Table 28. PANCREATIC INSULIN AND GLUCAGON CONTENTS OF APPROXIMATELY ONE YEAR OLD OBESE HYPERGLYCEMIC FEMALE MICE, AND MALE AND FEMALE LEAN LITTERMATES.

	Male lean littermates (6)	Female lean littermates (6)	Female ob/ob (7)
Pancreas weight (g.)	0.33 ± 0.05	0.37 ± 0.06	0.28 ± 0.06
Insulin extracted (µg.)	56 ± 22	75 ± 18	760 ± 421
Glucagon extracted (µg.)	0.97 ± 0.35	1.42 ± 0.35	2.38 ± 0.52
Insulin extracted (µg./g. wet weight pancreas)	170 ± 70	232 ± 72	2783 ± 1676
Glucagon extracted (µg./g. wet weight pancreas)	3.0 ± 1.3	3.9 ± 0.7	8.7 ± 2.2
Insulin / Glucagon	57 ± 14	54 ± 13	319 ± 175

Values are presented as the mean, ± S.D., with the number of animals given in parenthesis.

The pancreases were acid ethanol extracted, and the extracts assayed for insulin and glucagon at four two-fold dilutions over the range 1 in 500 to 1 in 5,000 (insulin assay) and 1 in 100 to 1 in 1,000 (glucagon assay). These dilutions were chosen so that the hormone content of the diluted extracts was within the range of concentration that could be assayed with high relative precision in each case.

obese mice was found to be much larger than in lean controls, being approximately five times as large in the three to four month old group, and ten times as great as in pancreases from lean mice in the one year old group. This data is entirely in accord with that of previous investigators.

The glucagon content of the pancreases of obese animals was not increased to the same extent as the insulin content of these animals, and the amount stored was found to be about twice that in the pancreases of lean animals at both ages studied. The difference between animals of three to four months was significant at the 1% level: that between animals of one year of age at the 5% level (Student's t test).

The larger relative increase in insulin in comparison with glucagon in the pancreases of obese mice is reflected in the insulin/glucagon ratios, also presented in Tables 27 and 28. The ratio was three times as large in the obese animals in comparison with lean animals in the three to four month old group, and six times as large in the older group.

2. Streptozotocin-diabetic and normal rats.

Table 29 presents the results of the measurement of glucagon and insulin in pancreases and blood glucose in five male Sprague-Dawley rats, and in eleven rats of the same strain which had been treated with Streptozotocin (65 mg./kg.) one and a half to three months previously.

There was a highly significant difference between the blood glucose levels and pancreatic insulin contents of Streptozotocin-treated and control animals, but only a small difference in the extractable glucagon content, which was not significant at the 5% level. This was independent of whether the results were expressed on a

Table 29. PANCREATIC INSULIN AND GLUCAGON CONTENTS OF STREPTOZOTOCIN-DIABETIC AND NORMAL RATS.

	Streptozotocin treated rats (10)	Untreated rats (6)
Blood glucose (mg./100 ml.)	475 ± 92	124 ± 23
Pancreas weight (g.)	0.89 ± 0.28	1.07 ± 0.14
Insulin extracted (µg.)	2.8 ± 0.8	73.8 ± 20.5
Glucagon extracted (µg.)	4.9 ± 3.5	3.8 ± 0.6
Insulin extracted (µg./g. wet weight pancreas)	3.5 ± 1.3	69.6 ± 20.4
Glucagon extracted (µg./g. wet weight pancreas)	5.7 ± 2.7	3.6 ± 0.8
$\frac{\text{Insulin}}{\text{Glucagon}}$	0.76 ± 0.39	18.1 ± 6.8

Ten male Sprague Dawley rats weighing between 290 and 310 g. were injected in the tail vein with Streptozotocin (19.5 mg.) dissolved in acidified saline. Between two and four months later, the animals were bled from the heart under ether anaesthetic, and the pancreases were removed. Those from animals with blood glucose values above 350 mg./100 ml. (all but one animal) were acid-ethanol extracted, and the extracts assayed for insulin and glucagon at four two-fold dilutions over the range 1 in 500 to 1 in 5,000 (insulin assay) and 1 in 50 to 1 in 500 (glucagon assay). With the exception of the insulin extracts from Streptozotocin treated pancreases, the hormone content of the diluted extracts was within the range of concentration that could be assayed with high relative precision.

per g. wet weight of pancreas basis or on an absolute basis. There was no difference between pancreas weights in the two groups.

These results suggest that Streptozotocin is specific for the β cells of the pancreas, and that the reported increase in blood glucagon levels in Streptozotocin-treated rats cannot probably be ascribed to hyperplasia of the ∝ cells which might be expected to result in higher pancreatic glucagon levels.

F. DISCUSSION.

1. Pancreatic insulin and glucagon measurements in obese and lean mice.

There is some difficulty in comparing values for the insulin and glucagon contents of mouse pancreases because, as previously mentioned, there was a difference in the amount of tissue taken from obese and lean animals. This difference was noticeable in the three to four month old group, but was not evident in older mice.

It has been previously noted by Wrenshall, Andrus and Mayer (1955), who state that "Because of this difference (in excess weight due to fat) between obese and control animals, results expressed as units of insulin per g. of pancreas or per unit body weight are difficult to interpret. Hence it was considered that the most meaningful way of expressing the results..... is in units extracted per whole pancreas, and per mg. of nitrogen in pancreas". The results will be compared, therefore, on an absolute basis, in terms of weight of hormone extracted per whole pancreas, rather than in relative terms.

In the present work, both male and female animals were used, and no sex difference in the lean mice as regards insulin and glucagon contents of the pancreases were observed. Wrenshall et al. (1955) have previously reported that the obese-hyperglycemic syndrome appears to be essentially identical in both sexes. However, as all the obese animals used in this study were female, the data from these animals will be compared with that of female control animals only.

The values that were obtained for pancreatic insulin measurements reported above were in close

agreement with the results of other workers. Thus Genuth (1969) obtained values of 429 µg./g.* and 4,880 µg./g.* for the insulin contents of forty-four week-old Bar Harbour lean and obese mice comparing with values of 232 and 2,783 ng./g. for one-year-old female lean and obese animals obtained in this work. Stauffacher et al. (1967) obtained values of 27.5 µg./pancreas and 138.3 µg./pancreas for the insulin contents of five-month-old, fed, female Bar Harbour lean and obese animals respectively, in comparison with the values of 26 and 119 µg./g. for the contents of three to four month animals in this work.

There was also consistency in values for the pancreatic glucagon content of untreated rats obtained in this study and obtained by other investigators, suggesting that the extraction technique and ethanol precipitation assay method for insulin and glucagon were reliable.

There is considerable evidence that the hyperinsulinemia of obese mice is caused by raised insulin secretion. This is associated with hyperplasia of the β cells, and, as the results of this study confirm, increased storage of insulin in the pancreas. The stimulus for increased secretion has not been established and very little is understood about the balance between insulin synthesis, storage and secretion.

From the results reported here one can conclude

*These values, like others quoted from published work, are calculated from results given in Units of insulin by assuming a conversion factor of 24 Units/mg.

that the glucagon content of the pancreases from obese mice was slightly elevated, both in young and older age groups. The differences were, however, much less than for insulin, which was increased five times in three to four month animals, and ten-fold in the older age group.

It is not possible to say whether there is an analogous relationship of the synthesis, storage and secretion of glucagon to that of insulin; nothing whatever is known of the relationship between the rate of glucagon secretion and the amount stored in the pancreas. It is, however, possible that there is chronic over-secretion of glucagon in the obese mouse, which depletes pancreatic reserves, and that the response to this includes increase in the number of glucagon-secreting cells and in the amount of glucagon stored in the pancreas as well as increased synthesis.

2. Pancreatic insulin and glucagon measurements in Streptozotocin-treated and normal rats.

As was to be anticipated, the insulin content of the pancreases of Streptozotocin-treated rats was extremely reduced. The glucagon contents remained constant, or were perhaps slightly lower in Streptozotocin-treated rats than in controls.

One group of investigators (Katsilambros et al. 1970) have reported that Streptozotocin-treated rats oversecrete glucagon, as blood immunoreactive levels in these animals (4171 pg./ml.) are elevated above those in untreated rats (140 pg./ml.). It is not clear whether this rise was entirely of pancreatic origin, as the cross-reactivity of the anti-glucagon serum used for gut glucagon-like activity was not stated. However, the control levels of serum glucagon obtained

by these authors were similar to the mean human value obtained by Aguilar-Parada, Eisentraut and Unger (1969) using a serum which only cross-reacted with pancreatic glucagon. This suggests that Katsilambros et al. (1970) also used antiserum which was relatively specific for pancreatic glucagon, and the increased quantities of glucagon found to be present in the serum after administration of Streptozotocin may therefore have mainly originated from the pancreas, rather than the gut.

If this interpretation of the results of these authors is correct, the increased secretion must be due to increased turnover of glucagon in the pancreas, as the pancreatic concentration of glucagon was not found to be elevated in the present study, suggesting that there is probably no increase in α_2 cell number, or the amount of glucagon stored.

The values obtained for the pancreatic glucagon content of untreated rats are consistent with those obtained by Edwards, Howell, and Taylor (1970), who found a content of 9 µg./g. wet weight of pancreas compared with a value of 5.7 µg./g. in this study.

Both the results of this study, and those of Edwards et al. (1970) give higher values than those of Buchanan, Vance and Williams (1969), who found pancreatic glucagon content of 0.28 µg./g. in the rat.

CONCLUSIONS

The studies presented in this thesis originated from attempts to develop an assay for glucagon of sufficient sensitivity to enable the quantities released from individual isolated islets of Langerhans to be measured. To achieve this an immunoassay was developed in which antibody-bound hormone was precipitated with ethanol. This separation method could be applied with equal facility to the measurement of both insulin and glucagon, and was found to be very convenient and accurate, showing that the assay was potentially of high precision.
It could be used to measure different concentrations of antibody, as well as antigen. The only disadvantage of the method was that it could not be used to measure the glucagon or insulin content of undiluted sera because of the high non-specific binding in media containing large concentrations of protein.

Whereas the insulin assay was of sufficient sensitivity to measure release from isolated islets of Langerhans, the glucagon assay, using an identical method, was of low sensitivity and could only be used to measure concentrations greater than 1 ng./ml. The sensitivity was, therefore, limited by the affinity of the sera raised, and various experimental approaches were used in attempts to improve it.

The first approach was to alter the conditions of incubation. The sensitivity was not found to be usefully increased by varying the pH of the assay medium, by preincubation of unlabelled hormone with antibody, by changing the nature of the buffer present in the medium, or by changing the nature and concentration of the

protein added to the assay buffer. However, it was found that the assay was of higher affinity at low temperatures, suggesting that there would be useful increase in sensitivity if it were possible to assay at low temperatures.

Attempts were therefore made to immunoassay at temperatures below the freezing poont of water, using glycerol and alcohol to prevent freezing. It was found that these solvents had the effect of lowering the equilibrium of the reaction, so that there was no increase in sensitivity.

The second approach was to change the immunisation procedure in various ways to raise sera of high affinity. During the course of the work, a large number of rabbits, some twenty guinea pigs, two chickens and two goats were immunised. Rabbits were found to give the best response. Goats gave sera of approximately the same affinity, but lower titre, while guinea pigs gave a very weak response, and chickens apparently gave no antibodies at all. In these studies, glucagon covalently conjugated to synthetic copolymer was administered subcutaneously or intradermally as an emulsion in Freund's adjuvant.

In further experiments with rabbits, different immunogen mixtures were used. There was no apparent difference in response whether glucagon was administered alone or coupled to protein, and glucagon copolymer emulsified in pertussis-silicone oil adjuvant was no more effective than Freund's adjuvant in eliciting antibodies of high affinity.

The interpretation of these various experiments was made very difficult by the large range of response to each procedure, and the only clear finding was that rabbits were by far the best animal to use in raising sera to glucagon. Despite the immunisation of

hundreds of animals over the course of this work, no sera were obtained which enabled measurements of glucagon concentrations of less than 1 ng./ml.

The failure of attempts to produce high affinity antibodies by immunisation suggested a third approach, in which attempts were made to purify high affinity antibodies from low affinity sera. Initially experiments were performed with the objective of separating different classes of antibodies by Sephadex filtration, as it was thought that these would be of different equilibrium constant. However, both insulin and glucagon sera were shown to consist almost entirely of material with a similar molecular weight to IgG, and no separation was achieved.

Techniques were then developed to purify antibodies using specific immunoadsorbents of glucagon coupled to two cellulose derivatives, diazocellulose and bromoacetyl cellulose. It was found that a crucial aspect of the preparation of these immunoadsorbents was to wash all uncoupled glucagon from the products using phenol/acetic acid/water. It was shown that the washed immunoadsorbents could be used to recover antibodies from an excess of serum, but that the antibodies separated by this method were of only slightly higher affinity than the sera from which they were derived. The method was not, therefore, suitable for the preparation of high affinity antibodies. Several possible explanations for the difficulties encountered in purifying high affinity antibodies were investigated, and it was concluded from these experiments that either the immunoadsorbents were partially non-selective for high affinity antibodies, or, alternatively, that these were not present in sufficient quantity to be effectively purified.

In addition to these experiments, the effects of

purified antibodies on blood glucose regulation were investigated. Insulin antibodies were shown to elicit hyperglycemia in excess of 200 mg. per 100 ml., and of about one hours duration, in rats intravenously injected with a dose which would approximately neutralise the total content of pancreatic insulin. Purified glucagon antibodies did not, however, cause any measureable drop in blood glucose in rats injected with amounts equivalent to the pancreatic glucagon content, and there only appeared to be a slow disappearance of injected glucagon antibodies from serum in contrast to insulin antibodies. It was established that an excess of antibodies was administered. Therefore glucagon antibodies failed to elicit hypoglycemia either because the kinetics or affinity of their reaction with glucagon did not cause blood glucagon levels to be lowered sufficiently, or because glucagon was of minor importance in stimulating the release of glucose under the conditions of these experiments.

In addition to these studies, a limited series of investigations were performed using the immuno-assay developed in this work, in which the levels of glucagon and insulin were measured in the pancreases of obese and lean mice and in Streptozotocin-diabetic and normal rats. The various values for pancreatic hormone levels reported in these studies were consistent with values obtained by other investigators in those cases in which the relevant measurements have been reported, suggesting that the assay methods were reliable.

There was elevation of the glucagon content of the pancreas of obese mice relative to lean litter mates, although when the increases were expressed in a per g. pancreas basis, they were only significant in the older of the two groups of animals assayed, i.e. those of one year. The observed increases were much lower than the equivalent increases for insulin. As no known correlation is established between the glucagon content of the pancreas and the rate of glucagon secretion, it was not possible to make deductions about the secretion of glucagon in obese mice from the data which were obtained.

The studies of the pancreatic insulin and glucagon content of normal rats established that the amount of antiserum administered in antibody injection experiments was in slight excess of the pancreatic content of these hormones.

The pancreatic glucagon levels of Streptozotocin-diabetic rats were approximately equal to those of untreated animals, suggesting that Streptozotocin had a specific action on the β cell, and was not toxic for α_2 cells, which secrete glucagon. In contrast, the insulin contents of the same pancreases, as have been previously reported, were extremely low.

FUTURE STUDIES SUGGESTED BY THIS WORK.

A. IMMUNORADIOMETRIC ASSAY.

Miles and Hales (1969) have developed a radio-immunoassay method for measuring insulin which depends on the competition of iodinated antibody for free and immunoadsorbent-bound hormone.

It would be of interest to develop a similar method for glucagon, as iodinated glucagon is unstable in comparison with iodinated antibody.

B. RAISING ANTISERA TO GLUCAGON.

It is possible that it may be easier to raise high affinity antibodies to glucagon in a species other than guinea pig or rabbit, and it would be of interest to study the immune response in a wide variety of different vertebrates, including amphibians, birds and reptiles as well as other mammals.

C. IMMUNOADSORBENTS.

The immunoadsorbents prepared in this study release small, but significant quantities of free hormone during elution. It would be of value to prepare glucagon conjugates of each of the different types of immunoadsorbent described in the literature to discover whether there is one which gives no release of antigen during elution. It would then be possible to carry out unambiguous experiments designed to discover whether neutralised high affinity glucagon antibodies are present in antisera.

D. THE RESPONSE TO INJECTED GLUCAGON ANTIBODY.

In this study, the effect of intravenous administration of antiglucagon serum on blood glucose levels in 4 - 5 hour starved rats was investigated. The effect of antiglucagon serum on other metabolic parameters which might be influenced by 'glucagon deficiency', such as the level of serum free fatty acids in 48 hour starved rats, or the level of serum insulin in fed rats, should also be studied. It would in addition be interesting to measure the rate of glucagon antibody neutralisation in fed and 48 hour starved rats.

E. THE MEASUREMENT OF GLUCAGON IN ISOLATED ISLETS OF LANGERHANS FROM OBESE-HPPERGLYCEMIC MICE AND STREPTOZOTOCIN-DIABETIC RATS.

A study of the rate of secretion of glucagon from isolated islets from obese hyperglycemic mice and Streptozotocin-diabetic rats would clarify whether glucagon secretion is altered in either of these types of experimental animal. It would be predicted from values quoted in the literature that sufficient glucagon would be released from thirty or more isolated islets incubated in 1 ml. of buffer for accurate measurement using the glucagon immunoassay developed during this work.

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A P P E N D I X.

MATERIALS USED IN THIS STUDY.

General laboratory chemicals were of the highest grade commercially available, generally A.R. grade, but occasionally G.P.R. grade. All water used in the experiments described in this thesis was glass-distilled.

Synthetic copolymer was prepared by the method of Arnon, Sela, Rachaman and Shapiro (1967) by Professor Michael Sela of the Department of Chemical Immunology, Weizmann Institute, Israel. It was found by amino acid analysis to contain L tyrosine, L glutamate, L alanine and L asparate in the molar ratio 1: 5.0: 4.4: 0.06, and was of average molecular weight 35,900 (approach to equilibrium method).

Glucagon was a generous gift from Dr. Mary Root, Eli Lilly and Co., Indianapolis, U.S.A. It was stated to be about 90% pure, and of insulin content 3.3 to 12 mU./mg. according to the method of assay.

Insulin was purchased as pure crystalline beef insulin of biological potency 23.5 I.U./mg. from Burroughs Wellcome and Co., London.

'Ethyl C.D.I.' (1 ethyl-3-(3-dimethyl aminopropyl) carbodi-imide hydrochloride) was purchased from Ralph N. Emmanuel Ltd., London.

m-nitrobenzyl alcohol was purchased from Eastman Organic Chemicals, Rochester, New York, U.S.A.

Thiomersal (sodium thiomersalate) and Paraformaldehyde were purchased from B.D.H. Chemicals Ltd., Dorset.

Powdered Cellulose was of the Whatman Column Chromedia grade C.F.1.

Adsorbant Cotton Wool was of Boots B.P.C. quality.

Sephadex of all grades was purchased from Pharmacia.

Freunds Complete adjuvant was usually purchased complete from Difco Laboratories, but was occasionally

prepared from the constituents.

Silicone Oil base was obtained from Croda Ltd.

Bovine plasma albumin fraction V was purchased as the crystalline grade from Armour Pharmaceutical Company Ltd., Eastbourne.

Trasylol was a most generous gift from Bayer Products Co. It was supplied as a sterile solution of 10,000 K.I.U./ml. in saline.