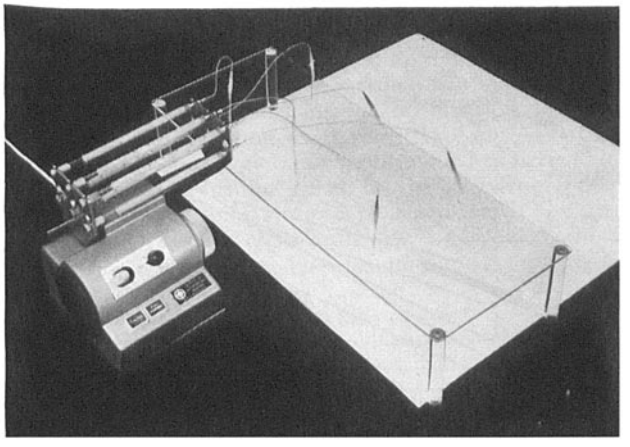
blasenfrei mit einer geeigneten Flüssigkeit (s. u.) zu füllen. Dieses Lösungsmittel wird zunächst eingesaugt, und erst dann wird die Glaskanüle so hoch mit der Probe gefüllt, dass nach Abschluss des Auftragens noch einige mm^3 in der Kapillare verbleiben. Dadurch wird vermieden, dass austretendes Lösungsmittel eine unerwünschte Vortrennung des Substanzgemisches bewirkt. Die Wahl der geeigneten Füllflüssigkeit wird ebenso wie die Auftragegeschwindigkeit von der Art der zu trennenden Substanzen bestimmt. Selbstverständlich sollen die betreffenden Verbindungen in dieser Flüssigkeit praktisch unlöslich sein. Für die Fraktionierung von Dicarbonsäuren, Aminosäuren, Zuckern und Zuckerphosphaten verwenden wir mit gutem Erfolg eine Kohlenwasserstoff-Fraktion («Siedegrenzenbenzin Nr. 4» der Firma Fluka A.G., Buchs, Schweiz). Auf dem Papier 2043 a Mgl der Firma Schleicher & Schüll, Dassel (Deutschland), erhält man hinreichend kleine Startflecke, wenn man von wässrigen Lösungen 20 mm^3/h aufträgt. Die Wahl eines organischen Lösungsmittels als Sperrflüssigkeit zwingt jedoch in vielen Fällen zum Auswechseln der zum Dauerinfusionsgerät gehörenden Spritzen mit Metallkonus und Teflondichtung. Da die Kunststoffdichtung durch Benzin verquillt, arbeiten wir mit Spezialspritzen[1], deren Glaskonus unmittelbar auf die Glaswandung aufgekittet wurde.



Summary. A method for the simultaneous automatic preparation of 6 paper chromatograms is described. The simple apparatus facilitates the handling, especially of temperature sensitive and radioactive solutions.

H. METZNER und HELGA VOLCSIK

Lehrstuhl für chemische Pflanzenphysiologie der Universität Tübingen (Deutschland), 20. Oktober 1963.

[1] Hersteller: Firma B. Braun, Melsungen, Deutschland.

A Diaphragm Bioassay for the Measurement of Total 'Insulin-Like Activity' and of 'Antigenic Insulin' in Serum

The state of insulin in the blood has been studied by a number of authors and several hypotheses published. Some authors, for example ANTONIADES[1], claim that insulin exists in two forms, that is 'free' and 'bound'. SAMAAN and FRASER[2] and FROESCH[3] suggest that there are two forms of 'Insulin-like activity'. BERSON and YALOW[4] think that there is only one form of insulin.

These discrepancies may, in part, be caused by differences between methods but may be the reflection of a real difference in the behaviour of insulin under the conditions of measurement. The most striking difference is that the values of 'insulin-like activity' detected by *in vitro* bioassay agree neither with the values obtained by other bioassays nor with the values obtained by the radioimmunoassays of YALOW and BERSON[5] or of HALES and RANDLE[6].

The assay reported has been designed as a bridge assay to measure insulin which is both biologically and immunologically active. The serum insulin values are in the same order as those obtained by the radio-immunoassay of HALES and RANDLE.

The total 'insulin-like activity' (ILA) of a sample is measured in terms of the effect the sample has on an insulin-sensitive metameter. A value, in insulin units per unit volume, is allocated to the sample by comparison with a curve of metameter against standard insulin. The total ILA of a serum sample is the sum of three components: the activity due to insulin, the insulin-like activity due to substances other than insulin and the anti-insulin effect of insulin inhibitors.

In the assay presented the total ILA is determined in whole serum and a second determination of ILA carried out after incubation with a mixture of insulin antibodies and anti-γ-globulin serum. The ILA measured in this second determination is the residual ILA. The difference between the total and the residual ILA is caused by the binding of insulin to the antibody-anti-γ-globulin complex; the insulin is thus prevented from acting on the diaphragm. The insulin measured by the difference between the two ILA's is the antigenic insulin (AI).

The insulin-sensitive tissue is the isolated mouse quarter diaphragm and the metameter the incorporation of glucose 1-C14 into the glycogen fractions of the diaphragms.

Materials and Methods. Kreb's bicarbonate buffer containing 2.5 mg of glucose and 2.0 mg of gelatine per ml is used throughout the experiments. The buffer is prepared each day and is gassed with 95% oxygen-5% carbon dioxide before use. Mouse diaphragms are prepared according to WARDLOW and MOLONEY[7] and were used in halves in the early experiments and are used in quarters for

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serum measurements. Insulin standards of beef insulin[8] are prepared each day by dilution, in Kreb's buffer, of a stock solution of 1 mg/ml.

Insulin antibodies are produced in the guinea-pig. The anti-γ-globulin serum is produced in the rabbit by injections of guinea-pig γ-globulin. The antibodies and the anti-γ-globulin serum are incubated together before being added to the serum samples. In the early stages of the assay the mixture of antibodies and anti-γ-globulins was added directly to the serum samples. The location of an insulin inhibitor in the anti-γ-globulin serum (see results section) led to the adoption of a different system. After overnight incubation, the mixture of antibodies and anti-γ-globulins is centrifuged and the precipitated antibody-anti-γ-globulin complex is resuspended in Kreb's buffer. The resuspended complex is then added to the serum samples.

Blood samples are obtained by venipuncture, the sera prepared and stored at $-20°C$ until use. The blood sugar and the serum glucose are measured by the glucose oxidase method. Before incubation the glucose in the samples is adjusted to 2.5 mg/ml; where the blood sugar is higher than 2.5 mg/ml (for example in a diabetic serum) the glucose levels of the sera and of the insulin standards are adjusted to the highest level in the sera.

Twelve incubation flasks, each containing four tissues, are used in each assay. Four standards are used: 12.5, 25, 100 and 500 μU of insulin/ml. The total and residual ILA's are measured in four serum samples. Each flask receives 0.1 ml of a solution of 1-C14 glucose before incubation. After gassing with a mixture of 95% oxygen-5% carbon dioxide the flasks are incubated for $1\frac{1}{2}$ h at 37°C.

After incubation the tissues are washed, blotted, weighed and digested separately in 30% KOH. The glycogen fraction of each tissue is prepared and dissolved in 1 ml of 5% R.B.S.25 (a commercial detergent) for the determination of radioactivity. The samples are totally transferred to counting vials containing 800 mg of anthracene and counted in a Packard Tri-Carb Liquid Scintillation Counter. After use the anthracene is washed with hot water, dried and re-used.

Establishment of the standard curve and estimation of the ILA. The recorded counts per min for each sample are converted into c.p.m./g wet weight/h of incubation. The regression curve for the standards is calculated by the method of least squares, treating the result for each quarter as a separate observation. In Figure 1 are displayed the regression curves obtained over a period of five weeks for the dose range 12.5 to 500 μU of insulin/ml. The slopes of the curves show little variation though there is a marked difference in values at the lowest dose of insulin. The value of λ (the index of precision) is calculated for each curve[9]: the initial values were in the region of 0.25 and current values lie between 0.1 and 0.05.

Results. Preliminary experiments: Half diaphragms were incubated in Kreb's buffer containing gelatine, glucose, glucose 1-C14 and known concentrations of insulin. The following measurements were made after incubation: (a) the disappearance of glucose from the medium, (b) the disappearance of glucose 1-C14 from the medium, (c) the total C14 incorporated into the tissue, (d) the formation of glycogen in the tissue, (e) the amount of C14 in the glycogen fraction.

Figure 2 shows these measurements plotted as percentages of the control values obtained after incubation in buffer alone. The disappearance of glucose (either by counting the C14 loss or by the method of glucose oxidase) and the total incorporation of C14 into the tissue do not give very steep response curves. Both the formation of glycogen and the incorporation of C14 into the glycogen fraction give steep response curves; as the curve of C14 in glycogen against insulin is the steeper of the two, the measurement of C14 incorporation into the glycogen was adopted as the metameter for the assay.

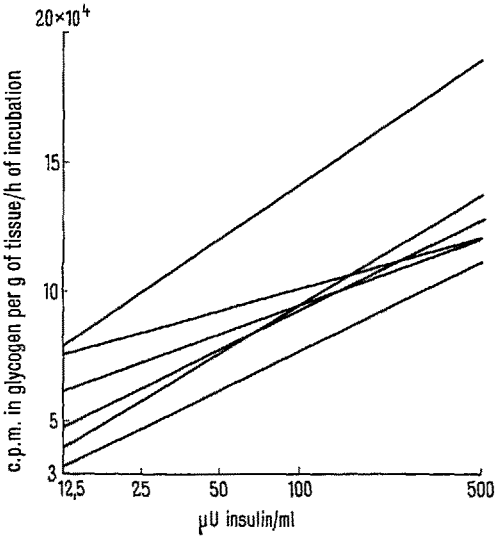

Fig. 1. Standard regression curves calculated from the results obtained over a five week period. Four standard insulin concentrations, of 12.5, 25, 100 and 500 μU/ml, are used. The c.p.m./g wet weight of tissue/h of incubation are plotted against the log of the insulin concentration.

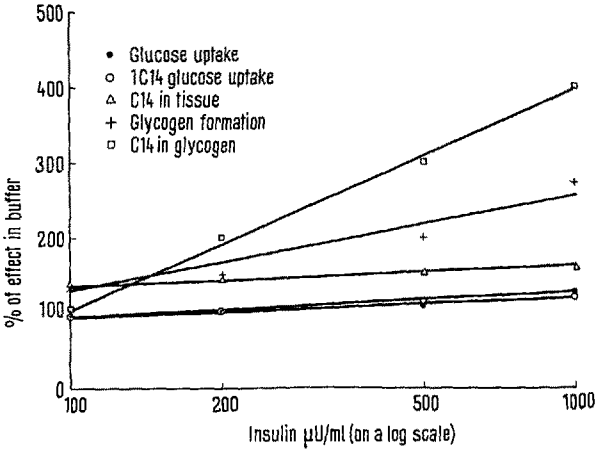

Fig. 2. Comparison of the effect of insulin concentration on five metameters. The value of each metameter obtained after incubation in Kreb's buffer alone is taken as 100%. The metameters are expressed as percentages of the control values. Four estimates of each metameter are made at 100, 200, 500 and 1000 μU of insulin/ml. The mean value at each insulin level is plotted against the log of the insulin concentration.

[8] Burroughs Welcome are thanked for their gift of crystalline beef insulin.

[9] C. T. BLISS, *The Statistics of Bioassay* (Academic Press, 1952).

Early serum ILA measurements were carried out using mouse hemidiaphragms and serum diluted four times in Kreb's buffer. It was found, however, that, on diluting whole serum, the recovered ILA rises rapidly above the expected value in the dilution range of 1/4 to 1/20. Subsequent measurements were carried out in whole serum, whereupon the ILA values were found to be in the same order of magnitude as the immunoinsulin values. Mouse quarter diaphragms were then adopted as standard tissue since 1 ml of serum is required as incubation sample, whereas the more bulky hemi-diaphragms require an incubation volume of 2 ml.

In Table I are presented the values of the ILA in the same serum after the addition of varying amounts and concentrations of anti-insulin serum. These results indicate that the antibodies alone, which can neutralize 500 μU/ml of crystalline beef insulin, do not give the same degree of neutralization as the antibody-anti-γ-globulin complex. The anti-γ-globulin serum used has been found to have an anti-insulin effect in the absence of other serums. The precipitation and resuspension of the antibody-anti-γ-globulin complex yields a suspension of almost the same insulin binding power as the original mixture (95%).

Serum measurements: Oral glucose tolerance tests have been carried out on twenty hospitalized subjects. No attempt was made to select the cases. Blood samples were taken before glucose ingestion and 30, 60, 120 and 180 min after.

The mean values for the total ILA, the residual ILA and the AI are presented in Table II.

Figure 3 shows the results obtained with non-diabetic patients and Figure 4 the results obtained with two diabetics. The number of cases examined is too small to estimate the fasting insulin in the normal and diabetic subject. In the diabetics examined the fasting total ILA and the fasting AI were both higher than corresponding values in the normal subjects. In both diabetics the curves of ILA and of AI rose higher and stayed higher than in the normal subjects.

The immunoinsulin has not been measured in all the sera determined by this bioassay. To date the comparison of the AI and the immunoinsulin (measured by the method of HALES and RANDLE) shows that the AI forms between 40 and 140% of the immunoinsulin. The mean percentage is 55%.

The residual ILA as measured here forms only a small percentage (between 10 and 40%) of the total ILA.

Discussion. The measurement of the incorporation of glucose C14 into the glycogen fraction of isolated mouse quarter diaphragms is the basis of a sensitive and precise assay for serum ILA in the range of 10 μU to 500 μU of insulin per ml. The values of the λ so far obtained, lying between 0.25 and 0.05, are small enough to make the

Table I. ILA values obtained in the same serum after the addition of differing concentrations of anti-insulin serum

Additions	ILA measured in μU/ml of serum
Serum alone	81
Serum + antibodies 1/100	74
Serum + antibodies 1/100 + anti-γ-globulins	60
Serum + undiluted antibodies + anti-γ-globulins	45
Antigenic insulin (81–45)	36

The immunoinsulin in the same serum was 37 μU/ml. The figures for the residual ILAs are corrected for the ILA of 10 μU/ml detected in the anti-γ-globulin serum.

Table II. Twenty oral glucose tolerance tests. Mean values of the ILA determined

Time of sample				
0 min	30 min	60 min	120 min	180 min
85–9–76	99–44–55	480–60–420	225–55–170	45–5–40

All values are in μU of insulin/ml of serum. The figures are arranged in the following order: total ILA – residual ILA – AI.

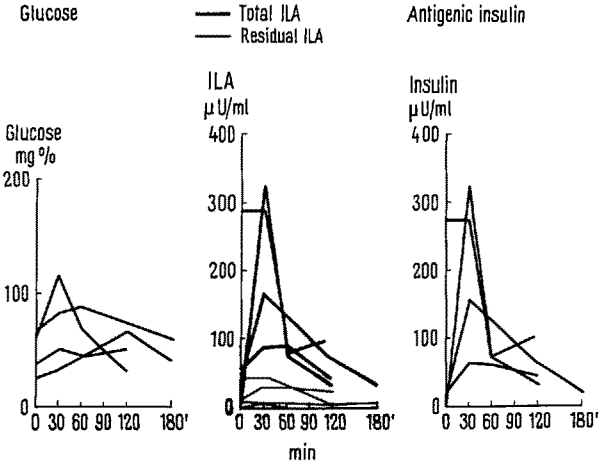

Fig. 3. Blood glucose, total ILA, residual ILA, and AI determined during oral glucose tolerance tests in non-diabetic subjects.

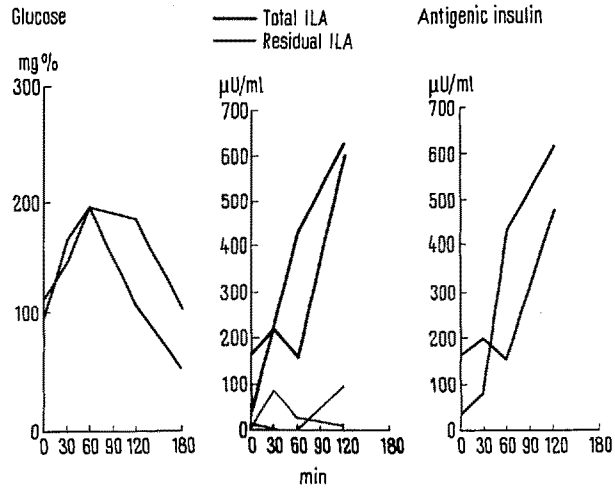

Fig. 4. Blood glucose, total ILA, residual ILA, and AI determined during oral glucose tolerance tests in two diabetics.

assay suitable for the detection of small variations in ILA and it is hoped that, during further development, the precision will be improved.

The use of insulin antibodies to separate the total ILA into antigenic and non-antigenic fractions is not an innovation as this system is already in use in the assays of FROESCH[3] and SAMAAN and FRASER[2]. Antibodies are used by WARDLOW and MOLONEY[7] to improve the precision of the diaphragm assay. The introduction of anti-γ-globulin serum into the system to improve the binding of insulin decreases the possibility that some of the sample insulin binds to the tissue used in the assay rather than to the antibodies. This improved binding may explain the low percentage of residual ILA found in whole serum.

The AI, determined by a system which is specific for insulin, can be considered as the insulin in the serum which is both biologically and antigenically active. It is not certain that the total ILA measured in the whole serum is a true measurement of the *in vivo* effect of the serum. The finding that the AI is only 55% of the immunoinsulin, indicates that the serums so far measured have contained a component which reduces the *in vitro* biological effect of serum insulin. This is supported by the finding of an anti-insulin effect of the anti-γ-globulin serum used and by the observation that there is not always a 100% recovery of human insulin added to human serum. If an anti-insulin exists in the human serum there is no conclusive evidence that this factor will exert the same effect *in vivo* as *in vitro*; until such time as the question of anti-insulins is clarified the AI measured may best be considered a minimum estimate of the circulating insulin rather than an absolute estimate.

The precision of this assay combined with the antigenic specificity afforded by the use of insulin antibodies and anti-γ-globulin serum make it suitable for the investigation of the state of human insulin as well as for the routine measurement of serum ILA and AI.

Résumé. Description d'une méthode de détermination de l'insuline antigénique utilisant le diaphragme de souris avec et sans addition d'anticorps anti-insuline préalablement précipités par de l'anti-γ-globuline.

A. J. MOODY and J.-P. FELBER

Laboratoire de Biochimie de la Clinique Médicale Universitaire, Lausanne (Suisse), le 27 novembre 1963.

Technik der Kanülierung des Confluens sinuum beim Kaninchen für Versuche mit partiellem extrakorporellem Kreislauf

Der extrakorporelle Blutkreislauf bei Jung- oder Kleintieren, Foeten, Neugeborenen und Kleinkindern hat in den letzten Jahren sehr an Interesse gewonnen, sei es zur Oxygenierung eines Teils des zirkulierenden Blutes, sei es zur Hämodialyse[1–9].

Im Rahmen unserer Untersuchungen über die humorale Vermittlung der Schlaf- und Wachzustände ist es uns daran gelegen, venöses Hirnblut zu erhalten. Da beim Kaninchen die Venae jugulares internae sehr wenig ausgebildet sind, haben wir versucht, das Blut aus dem Confluens sinuum zu erhalten. Gleichzeitig interessierte uns im Zusammenhang mit den klinisch-physiologischen Forschungen der Universitätsfrauenklinik Basel die Frage, ob sich der leicht zugängliche Confluens sinuum als Blutentnahmestelle für einen veno-arteriellen «Bypass» bei Neugeborenen mit pulmonaler Insuffizienz eignen könnte.

Bei rund 40 Kaninchen eröffneten wir den Confluens sinuum und führten dessen Blut mit Hilfe einer Spezialkanüle dem extrakorporellen Kreislauf zu.

Technik. Der Confluens sinuum des Kaninchens liegt unter der Spitze des Os interparietale[10] (Figur 1). Zur Eröffnung dieses venösen Blutleiters gehen wir wie folgt vor: (1) Im Bereich zwischen den Ohren Längsschnitt der Haut bis auf den Knochen (Länge ca. 3 cm). (2) Präparation des Os interparietale durch seitliches Abschieben des Periostes (Figur 1). (3) Planfeilen des Os interparietale bis keine Unebenheiten mehr vorhanden sind. (4) Aufsetzen einer trepanartigen Bohrerführung aus rostfreiem Stahl auf die in Figur 1 eingezeichnete Stelle (schwarzer Punkt). (5) Anbohren des Knochens. Am besten eignet sich ein von einem Elektromotor angetriebener Hohlbohrer (Aussendurchmesser 3 mm). (6) Entfernen der Bohrerführung. Kontrolle der angebohrten Stelle, wobei die beiden vorderen Suturen des Os interparietale tangential angeschnitten sein sollen (vgl. Figur 1). (7) Unter möglichst geringem Druck senkrechtes Durchbohren des Knochens und der Wand des Confluens sinuum (Dura mater) ohne abzusetzen. Dabei spürt man zwei Veränderungen des Bohrwiderstandes: die eine beim Übergang zwischen Tabula externa und interna, die andere beim Durchbruch durch die Tabula interna. Daraufhin soll der Bohrer nicht sofort hochgezogen werden, damit die Dura sicher ausgestanzt und der Sinus auf der ganzen Fläche eröffnet wird. Unter diesen Umständen haftet das kleine Scheibchen aus harter Hirnhaut am entfernten Knochenstück, welches sich in der Höhlung des Bohrers befindet. (8) Mit Hilfe

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