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Insulin and an Inhibitor of Glucose Uptake, in Protein Fractions of Normal Human Plasma

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Experiments in which the proteins of normal human serum or ox serum were fractionated by zone electrophoresis on columns of treated cellulose have suggested that insulin in serum migrates with fractions containing α_1 -, β - and γ -globulins (Randle & Taylor, 1958; Taylor & Randle, 1959). Further subfractionation of the β - and γ -globulins suggested that insulin was associated predominantly with β - rather than γ -globulins and it seemed possible that insulin in γ -globulins is derived from contamination with β -globulins as a result of zone spreading during electrophoresis. In addition, evidence was obtained for the presence of an inhibitor of glucose uptake by isolated rat diaphragm *in vitro*, migrating in the α_2 -globulin fraction. Detection of this inhibitor was made difficult by contamination of the α_2 -globulin fraction with insulin derived possibly from adjacent α_1 - and β -globulins (Randle & Taylor, 1958).

We have now sought to confirm these conclusions by an alternative method of protein fractionation, with ethanol at low temperatures, which effects a much better separation of β - and γ -globulin fractions of normal human serum than zone electrophoresis. In order to demonstrate the presence of

the inhibitor with certainty it was necessary to subfractionate one of the fractions prepared with ethanol, by an improved method of zone electrophoresis. Details of this method of zone electrophoresis are given in the Appendix.

EXPERIMENTAL

Blood samples. Human blood was drawn from an ante-cubital vein of non-fasting subjects and diluted immediately with 0.173 vol. of acid-citrate-dextrose (Lever *et al.* 1951). The diluted plasma was then separated by centrifuging.

Fractionation of plasma proteins. The proteins of diluted plasma were first separated into three fractions with ethanol at -5° by the method of Lever *et al.* (1951). The fractions obtained have been designated according to Lever *et al.* as fraction I + III (mainly α_2 - and β -globulins and fibrinogen), fraction II (γ -globulins) and fraction IV, V and VI (mainly albumin and α -globulins with a little β -globulin). The fractionation was confirmed in most instances by paper electrophoresis of the fractions, employing the vertical-tank method of Flynn & de Mayo (1951) and staining the protein with bromophenol blue by the method of Henry, Golub & Sobel (1957). The protein fractions were dialysed for 30 hr. against distilled water, in Visking tubing, at room temperature and freeze-dried.

Fraction IV, V and VI was subfractionated by zone electrophoresis at pH 8.4, on columns of treated cellulose, 50 cm. long and 2.0 cm. in diameter, (Porath, 1956) with the phosphate-borate buffer of Campbell & Stone (1956). The faster-moving proteins (albumin and α_1 -globulin) were allowed to move off the column during

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electrophoresis into a glass cell attached to the bottom of the column, from which they were swept by a slow stream of buffer into an automatic fraction collector. The design of this cell and its mode of operation are described in a note at the end of this paper. Electrophoresis for about 30 hr. at 750 v and 40 ma sufficed to displace the faster-moving proteins from the column; electrophoresis was then discontinued and the remaining protein was displaced from the column with phosphate-borate buffer. The protein content of each fraction was assayed by measurement of E at 280 $m\mu$ in the Beckman spectrophotometer. The individual fractions were then pooled to yield three main fractions (Fig. 1): albumin + α_1 -globulin, α_2 -globulin and β -globulin, and these were dialysed against distilled water and freeze-dried.

For assay of biological activity, the freeze-dried protein samples were dissolved in bicarbonate-buffered medium (Gey & Gey, 1936) containing glucose (2.5 mg./ml.). The volume of medium was three times the volume of plasma from which the fractions were prepared. Fraction I + III (containing fibrinogen) clotted at this stage; the clot was allowed to form and it was then removed by centrifuging.

Diaphragm. Diaphragm muscle was obtained from male albino Wistar rats, wt. 100–150 g., which had been fasted for 18–24 hr. The procedure for preparation and incubation of diaphragms was that described by Randle (1954).

Assay of biological activity. The uptake of glucose or synthesis of glycogen by hemidiaphragms incubated for 90 min. at 37° in buffer containing a protein fraction (one hemidiaphragm in 1 ml. of medium) was compared with that of hemidiaphragms incubated in buffer alone. Glucose uptake was calculated as milligrams of glucose disappearing from the medium/g. of wet diaphragm/hr. of incubation. Insulin was deemed to be present if a fraction stimulated glucose uptake or glycogen synthesis significantly (cf. Taylor & Randle, 1959). The term inhibitor is used to describe the factor or factors present in those fractions which depressed glucose uptake significantly.

Glucose. This was estimated in samples (0.2 ml.) of incubation medium by Somogyi's (1945) modification of Nelson's method.

Glycogen. This was isolated in crude form from diaphragm

muscle by the method of Walaas & Walaas (1950) and assayed photometrically by reaction with anthrone (Seifter, Dayton, Novic & Muntwyler, 1950).

RESULTS

The effects of protein fractions of normal human plasma on the uptake of glucose by diaphragm are shown in Tables 1 and 3. Fraction I + III (principally α_2 - and β -globulins) consistently stimulated uptake of glucose by diaphragm; this fraction also enhanced synthesis of glycogen by diaphragm. Fraction II (γ -globulins) had no significant effect on glucose uptake. The effect of fraction IV, V and VI (mainly albumin and α -globulins with some β -globulins) was variable and whereas some samples depressed uptake of glucose, other samples either stimulated glucose uptake or were without effect. The reason for this variable effect of fraction IV, V and VI became apparent when subfractions were prepared by zone electrophoresis and tested for biological activity (Fig. 1 and Table 2). The subfraction containing slower-moving albumin and α_1 -globulins stimulated uptake of glucose, whereas subfractions containing α_2 - or β -globulins depressed uptake of glucose. Further, the sum of the effects of the individual subfractions was very similar to the effect of the fraction from which they were prepared (Table 2). The identity of the subfractions was confirmed by paper electrophoresis.

We conclude from these results that fraction I + III contains insulin and that fraction IV, V and VI contains both insulin (present in the albumin- α_1 -globulin subfraction) and an inhibitor of glucose uptake (present in the α_2 - and β -globulin subfractions).

Fig. 1. Zone electrophoresis of fraction IV, V and VI.

Table 1. *Effect of protein fractions of normal human plasma on uptake of glucose by isolated rat diaphragm*

Plasma protein fractions were prepared by the method of Lever *et al.* (1951). For definition of fractions see Experimental section. For differences marked thus * $P < 0.001$; † $P < 0.01 > 0.001$; ‡ $P < 0.05 > 0.01$. For other differences $P > 0.05$. Each difference is derived from the mean of six observations.

Sample	Glucose uptake as difference (mg. of glucose/g. of wet diaphragm/hr.) from control incubated in buffer alone. Mean difference $\pm$ s.e. of difference		
	Fraction I + III	Fraction II	Fraction IV, V and VI
1	1.5 $\pm$ 0.29*	—	0.8 $\pm$ 0.21†
2	1.2 $\pm$ 0.74	0.5 $\pm$ 0.33	-2.1 $\pm$ 0.40*
3	0.1 $\pm$ 0.20	0.4 $\pm$ 0.40	-0.6 $\pm$ 0.24‡
4	1.4 $\pm$ 0.24*	0.1 $\pm$ 0.30	-0.6 $\pm$ 0.38
5	1.8 $\pm$ 0.51†	0.1 $\pm$ 0.51	-0.4 $\pm$ 0.55
6	1.7 $\pm$ 0.45†	0.6 $\pm$ 0.41	-1.1 $\pm$ 0.39‡
Mean	1.3 $\pm$ 0.25†	0.3 $\pm$ 0.3	-0.7 $\pm$ 0.39

Table 2. *Effect of subfractions of fraction IV, V and VI from normal human plasma on the uptake of glucose by isolated rat diaphragm*

Plasma protein fractions were prepared by the method of Lever *et al.* (1951) and subfractionated by zone electrophoresis on columns of treated cellulose (see Experimental section). For definition of fractions see Experimental section and Fig. 1. For differences marked thus * $P < 0.001$; † $P < 0.01 > 0.001$. For other differences $P > 0.05$. Each difference is derived from means of six observations.

Sample	Subfraction	Glucose uptake as difference (mg. of glucose/g. of wet diaphragm/hr.) from control incubated in buffer alone. Mean difference $\pm$ s.e. of difference
1	—	-0.5 ± 0.29
	Albumin + α_1 -globulin	0.1 ± 0.62
	α_2 -Globulin	-0.6 ± 0.70
	β -Globulin	0.1 ± 0.62
2	—	-0.4 ± 0.75
	Albumin + α_1 -globulin	$1.9 \pm 0.48†$
	α_2 -Globulin	$-1.5 \pm 0.39†$
	β -Globulin	$-1.2 \pm 0.35†$
3	—	-0.3 ± 0.31
	Albumin + α_1 -globulin	$1.5 \pm 0.32*$
	α_2 -Globulin	-0.6 ± 0.39
	β -Globulin	$-1.1 \pm 0.34†$
Pooled	—	-0.4 ± 0.31
	Albumin + α_1 -globulin	$1.2 \pm 0.27*$
	α_2 -Globulin	$-0.9 \pm 0.28†$
	β -Globulin	$-0.8 \pm 0.25†$

Table 3. *Effect of protein fraction I + III, from normal human plasma, on the deposition of glycogen by isolated rat diaphragm*

Plasma protein fractions were prepared by the method of Lever *et al.* (1951). For definition of fractions see Experimental section. For differences marked * $P < 0.001$; † $P < 0.01 > 0.001$; ‡ $P < 0.02 > 0.01$. For other differences $P > 0.05$. Each difference is derived from means of six observations.

Sample	Glycogen deposition as difference (mg. of glycogen/g. of wet diaphragm/hr.) from control incubated in buffer alone. Mean difference $\pm$ s.e. of difference
1	$0.9 \pm 0.3†$
2	0.5 ± 0.34
3	$3.0 \pm 0.71†$
Pooled	$1.5 \pm 0.27*$

DISCUSSION

Earlier studies, in which protein fractions were prepared from normal human serum by column electrophoresis, had shown that insulin is predominantly associated with α_1 - and β -globulins but that some insulin was also present in γ -globulins (Randle & Taylor, 1958). In these studies, insulin in the γ -globulin fraction could have been derived from contamination with β -globulins as a result of spreading of zones during elution. In the present

study a more effective separation of serum-protein fractions has been attained by a combination of fractional precipitation and an improved method of column electrophoresis. Under these conditions insulin could not be detected in γ -globulins and the results confirm that in normal human serum insulin is present in fractions containing α_1 -globulins and β -globulins (cf. Beigelman *et al.* 1956; Bolinger, van der Geld & Willebrands, 1959; Beigelman, 1958).

A substance which inhibits uptake of glucose by diaphragm has been identified in α_2 - and β -globulin fractions prepared from normal human serum by a combination of fractional precipitation and zone electrophoresis. In earlier studies with zone electrophoresis alone, evidence was obtained for the presence of such an inhibitor of glucose uptake in α_2 -globulin fractions, but detection of the inhibitor was difficult, possibly because of contamination with insulin from adjacent α_1 - and β -globulins. In the present study much of the insulin was separated from the inhibitor by preliminary fractional precipitation and the inhibitor was then readily separated from the remaining insulin by zone electrophoresis. Interest in this inhibitor lies in its possible relation to growth hormone, for, whereas treatment of the rat with growth hormone leads to diminished uptake of glucose by diaphragm, the hormone does not inhibit glucose uptake when added *in vitro* (Park, Brown, Cornblath, Daughaday & Krah, 1952). Preliminary

studies have suggested that the inhibitor is not present in α_2 - or β -globulin fractions prepared from the plasma of hypophysectomized patients but that it is present after treatment with human growth hormone. This inhibitor appears to be different from the insulin antagonist identified by Vallance-Owen, Dennes & Campbell (1958) which was associated with albumin fractions of human serum and which did not depress uptake of glucose in the absence of added insulin. Baird & Bornstein (1958) have noted inhibition of glucose uptake by diaphragm with residues from human plasma after extraction with acid-ethanol. We do not know whether our inhibitor is similar to that described by Baird & Bornstein.

The present studies again emphasize that assays of insulin in plasma based on the effects of unfractionated plasma on glucose uptake of diaphragm are of little value since such samples are likely to contain both insulin and inhibitors of glucose uptake (cf. Randle, 1954).

SUMMARY

1. Protein fractions have been prepared from normal human plasma by fractional precipitation with ethanol and an improved method of zone electrophoresis on columns of treated cellulose, and their effect on the uptake of glucose by isolated rat diaphragm has been investigated.

2. Insulin was detected in a protein fraction containing slower-moving albumin and α_1 -globulins and in one containing α_2 - and β -globulins. No insulin could be detected in γ -globulins.

3. An inhibitor of glucose uptake by isolated diaphragm was identified in a fraction containing α_2 - and β -globulins.

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APPENDIX

A Method of Continuous Elution during Column Electrophoresis

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Porath (1956) has pointed out that zone spreading on elution is a serious disadvantage when protein mixtures are fractionated by column electrophoresis. Porath, Lindner & Jerstedt (1958) have suggested that electrophoresis be carried out in such a way that the more rapidly moving components pass from the column into a cell, from which they are swept by a stream of buffer into an automatic fraction collector. A similar method, which has been in use for some time in this Laboratory, is described in this note.

The general design of the cell is shown in Fig. 1. The cell consists of a short tube (length, 5 cm.; internal diameter, 2 cm.) bearing at its upper end a B24 standard ground-glass joint and closed at its lower end by a single layer of Visking dialysis tube stretched across a glass lip and held in place by a rubber band. Two glass side arms are fitted to the cell, the lower of which allows buffer to flow into the cell, the upper tube serving to conduct the eluate to the fraction collector. A baffle plate, consisting of a single layer of Visking membrane perforated with pin holes, is fixed in position

between the side arms by means of a split polythene ring.

The whole cell (and its appendages) is fitted on the lower end of a conventional glass electrophoresis column (length, 50 cm.; internal diameter, 2 cm.) and immersed in a tank of buffer containing the anode.

During electrophoresis a slow stream of buffer is passed through the cell at a rate sufficient to remove the faster-moving components as they pass into the cell during electrophoresis [in the paper (above) by Vargas, Taylor & Randle the rate was

Fig. 1. Design of cell.

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approx. 16 ml./hr. during removal of the albumin]. As electrophoresis proceeds, with serum, it is necessary to slow the stream of buffer once the albumin has been removed, in order to avoid undue dilution of more slowly migrating components.

When this method was applied to electrophoresis of normal human serum, the resolution of faster-moving components was greatly improved, so that, for example, a distinct pre-albumin peak was obtained which was clearly demarcated from the albumin peak in almost all instances.

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Studies on Vitamin E

5. LIPID PEROXIDATION IN DIALURIC ACID-INDUCED HAEMOLYSIS OF VITAMIN E-DEFICIENT ERYTHROCYTES*

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Dialuric acid-induced haemolysis of erythrocytes from vitamin E-deficient rats was discovered by Rose & György (1950, 1952), who postulated that the haemolytic agent was an intermediate in the reversible oxidation-reduction system of dialuric acid and alloxan but was not hydrogen peroxide itself.

Red pigments are produced when 2-thiobarbituric acid reacts with oxidized lipids (Bernheim, Bernheim & Wilbur, 1947) or, more probably, with their aldehydic breakdown products (Glavind & Hartmann, 1951), and the formation of both red and yellow pigments from non-lipids has been discussed by Patton & Kurtz (1951) and by Landucci, Pouradier & Duranté (1955). The thiobarbituric acid reaction was used by Horwitt, Harvey, Duncan & Wilson (1956) in a study of hydrogen peroxide-induced haemolysis of erythrocytes from humans on a diet low in vitamin E, and by Tappel & Zalkin (1959, 1960) to demonstrate the presence of lipid peroxides in mitochondria and

microsomes from vitamin E-deficient rats. Carpenter, Kitabchi, McCay & Caputto (1959) also used it to show that peroxide formation in liver homogenates from vitamin E-deficient rats was paralleled by decreased synthesis of ascorbic acid.

The protective effects of tocopherols and metal ions against dialuric acid-induced haemolysis have been discussed by Bunyan, Green, Edwin & Diplock (1960). This paper deals with the results of fuller investigations into the haemolysis of vitamin E-deficient erythrocytes and describes the use of the 2-thiobarbituric acid reaction to study the relation between lipid peroxidation and the haemolytic phenomenon.

EXPERIMENTAL

Animals. Deficient animals were 3- to 5-month-old male rats of the Norwegian hooded strain which had received the vitamin E-free diet since they were 13 days old. Control rats were males from the stock colony.

Vitamin E-free diet, buffer pH 7.4 and dialuric acid solution. These were as described by Bunyan *et al.* (1960);

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