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APPENDIX

The Purification of Insulins in Crude Extracts of Rat Pancreas by Two-Dimensional Chromatography and Electrophoresis on Paper

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In most recent methods for the small-scale purification of insulin from pancreas, an initial extraction with an acidified organic solvent is followed by further purification by chromatographic methods. Such separations may be carried out on columns, with a variety of supporting media (Porter, 1953; Mendiola & Cole, 1960; Davoren, 1962; Epstein & Anfinsen, 1963; Smith, 1964), or alternatively by paper chromatography (Light & Simpson, 1956; Grodsky & Tarver, 1956; Taylor, Humbel, Steinke & Renold, 1961). Paper-chromatographic methods offer the advantage of relative simplicity, and an account is given below of attempts to apply a paper-chromatographic technique to crude extracts of rat pancreas. Additional purification after paper chromatography has been carried out by electrophoresis in a second dimension. In this method a sewing technique similar to those used for two-dimensional chromatography (Stöckli, 1954), or for two-dimensional ionophoresis (Richmond &

Hartley, 1959; Naughton & Hagopian, 1962), was used.

Paper chromatography. The crude insulin hydrochloride obtained from rat pancreas, as described in the preceding paper, was dissolved in 0.5 ml. of ethanol-0.8N-HCl (3:1, v/v), and applied to Whatman no. 1 paper as a streak about 15 cm. long. This was chromatographed overnight in butan-2-ol-aq. 1% acetic acid (1:1, v/v), as described by Fenton (1959). Crystalline ox insulin was run alongside as a marker. After staining with bromocresol green, a large amount of material was visible at the origin, and in addition a band was obtained with approximately the same R_f as ox insulin. Alternatively, guide strips were cut from the edges of the chromatogram and stained to locate the insulin. Chemical and biological tests carried out on the main part of the band demonstrated that insulin was present in this zone (Taylor, Smith & Gardner, 1962).

Paper electrophoresis. For further purification the zone believed to contain insulin obtained by paper chromatography was cut out as a strip about 2 cm. wide, and the ends of the strip were sewn on to wicks of a similar width with a sewing machine. The strip was then hung by its shorter mid-line over the nylon-thread support of a vertical electrophoresis tank (Shandon Scientific Co. Ltd., London), with the wicks dipping into 20% (v/v) formic acid. The solvent rose up the paper by capillarity, and insulin together with any contaminating material was washed into a small zone near the mid-line of the strip. The strip was then removed, allowed to dry in air and a rectangle approx. 2 cm. $\times$ 1 cm. containing the insulin cut

from it at the mid-line. This was then sewn on to a sheet of Whatman no. 1 paper approx. 36 cm. $\times$ 18 cm., one of the long sides of the rectangle being sewn along the centre fold of the paper (see Fig. 1). A rectangle of paper of identical area containing ox insulin as a marker was sewn on to the paper in the same manner. The paper was then wetted with 20% formic acid and electrophoresis carried out in the same apparatus for 16 hr. at 6v/cm. in the vertical tank. After electrophoresis the paper was taken out of the tank and dried in air. Proteins were, where necessary, stained with bromocresol green. By this technique, with crude rat insulin two bands (zones 1 and 2 of Fig. 1) were obtained, which stained with this dye, as well as more-basic material (zone X). Crystalline ox insulin treated by the same extraction and chromatographic technique as that used for rat insulin had an unchanged electrophoretic mobility in 20% formic acid.

Chemical identification of insulin. A rectangle of paper corresponding with the areas staining with bromocresol green was cut out and sewn on to a fresh sheet of Whatman no. 1 chromatography paper and sprayed with performic acid (formic acid- H_2O_2 ; 19:1/v/v). After drying in air the paper was placed in a vertical electrophoresis tank containing 20% formic acid and the formic acid allowed to ascend the paper as before. Electrophoresis was then carried out for 16 hr. The paper was then allowed to dry in air and, after neutralization of any residual acid with NH_3 vapour, appropriate areas of the paper were sprayed with aq. 0.03% NN' -diethylpseudocyanine chloride (Taylor *et al.* 1961). Purple spots appeared derived from zones 1 and 2, which were identical in electro-

Fig. 1. Paper-electrophoretic pattern of crude rat insulin. After paper chromatography a rectangle of paper containing the insulin was sewn on to a fresh sheet of paper. A similar rectangle of paper containing ox insulin as a marker was attached in the same manner. Electrophoresis was carried out in 20% (v/v) formic acid for 16 hr. at 6v/cm. Paper was stained after electrophoresis with bromocresol green. 1 and 2, Rat insulins; X, other stainable material.

Table 1. *Response of isolated rat hemidiaphragm to fractions obtained by paper electrophoresis of crude rat insulin*

After chromatography and electrophoresis on paper, protein was eluted from the paper with 0.01 N-HCl and the eluates were diluted 20-fold in bicarbonate-buffered medium containing glucose (2.5 g./l.). Control incubation medium consisted of buffer diluted with an identical amount of 0.01 N-HCl. Effects of eluates on glucose uptake and glycogen synthesis in rat hemidiaphragm were measured. Zones 1 and 2 are as defined by Fig. 1. At least six observations were used to obtain each mean.

Zone examined	Incubation medium	Glucose uptake, mean $\pm$ S.E.M. (mg./g. of wet diaphragm/90 min. incubation)		Glycogen content, mean $\pm$ S.E.M. (mg./g. of wet diaphragm)
		Expt. 1	Expt. 2	
1	(a) Control	2.9 $\pm$ 0.28	5.3 $\pm$ 0.34	3.6 $\pm$ 0.23
	(b) Eluate	7.5 $\pm$ 0.51	7.2 $\pm$ 0.20	5.4 $\pm$ 0.13
	(c) Eluate + 1:20 dilution of antiserum	4.5 $\pm$ 0.45	6.0 $\pm$ 0.27	—
2	(a) Control	3.4 $\pm$ 0.28	4.0 $\pm$ 0.42	3.6 $\pm$ 0.34
	(b) Eluate	6.2 $\pm$ 0.43	7.9 $\pm$ 0.57*	5.1 $\pm$ 0.43
	(c) Eluate + 1:20 dilution of antiserum	3.1 $\pm$ 0.23	5.4 $\pm$ 0.55*	—

* For (b) - (a) and (b) - (c) in each experiment, for each zone examined, the significance of differences between means, P , was < 0.02 , except for values denoted, where $P < 0.05 > 0.02$.

phoretic mobility with A-chain made from either ox or pig insulin. No A-chain was derived from zone X or other areas of the paper.

Biological identification of insulin. Insulin was further identified after elution from paper by its effects in enhancing uptake of glucose and deposition of glycogen in rat hemidiaphragm *in vitro*, by methods already described (Taylor *et al.* 1961). Appropriate areas of paper were cut out and allowed to stand overnight with 1–2 ml. of 0.01N-HCl and tested for biological activity. Effects on isolated rat hemidiaphragm were examined essentially according to Randle (1956); glucose uptake was determined by the use of glucose oxidase (Huggett & Nixon, 1957) and glycogen deposition was determined by the anthrone method (Seifter, Dayton, Novic & Muntwyler, 1950). In some instances the presence of insulin was confirmed by inhibition of effects of eluates on glucose uptake by addition of antiserum, made by injecting guinea pigs with ox insulin (Wright, 1959; Taylor & Randle, 1959). Effects from the two zones on paper corresponding with areas giving A-chain are shown in Table 1.

Since glucose uptake and synthesis of glycogen were markedly enhanced by both extracts, and effects on glucose uptake were abolished by antiserum, it is concluded that both these zones contained insulin. These two insulins are probably identical with those already demonstrated by Smith (1964) to be present in rat pancreas. The B-chains derived from these insulins are known to

differ in their lysine content, though the two A-chains made from them are identical. On account of this difference in charge due to an extra lysine residue in one of them, the two insulins are separable by electrophoresis in 20 % formic acid.

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Metabolism of Polycyclic Compounds

24. THE METABOLISM OF BENZ[a]ANTHRACENE*

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Berenblum & Schoental (1943) investigated the metabolism of benzantracene in rats and mice and detected a compound in the faeces which was identified by methylation and spectroscopic examination as 4-hydroxybenzantracene. Harper (1959*a*) confirmed the presence of this phenol as a metabolite in the faeces of mice and he found evidence for the presence of a second phenol, which was also a metabolite in rabbits (Harper, 1959*b*) and which was claimed to be 2-hydroxybenzantracene.

In the present work it has been shown that benzantracene is converted by rats, rabbits and mice into *N*-acetyl-*S*-(5,6-dihydro-6-hydroxy-5-benzanthracenyl)-L-cysteine, 5,6-dihydro-5,6-dihydroxybenzantracene, 8,9-dihydro-8,9-dihydroxybenzantracene and 10,11-dihydro-10,11-dihydroxybenzantracene, together with smaller amounts of 3,4-dihydro-3,4-dihydroxybenzantracene and possibly 1,2-dihydro-1,2-dihydroxybenzantracene. 4-, 8- and 9-Hydroxybenzantracene and a phenol which is probably 3-hydroxybenzantracene are also formed. 2-Hydroxybenzanthra-

* Part 23: Boyland & Sims (1964).