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Science Papers and Discussions—*Continued*

INSULIN AND RELATED TOPICS

PART I. SOME OBSERVATIONS ON THE CAUSATION OF DERMAL REACTIONS BY INSULIN

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THE literature on dermal reactions following injections of insulin has recently been reviewed by Paley[1], who has made an analysis of the role played by accessory factors in the causation of these phenomena. His results showed that the local effects of insulin therapy which were occasionally encountered were not due to the *p*H of the solution, to the protamine sulphate, or to the presence of cresol B.P. (or pure *o*-cresol) employed as preservative. Paley concluded, on this evidence, that the main factor responsible for the skin-flare reaction was an endogenous substance closely associated with insulin, but failed to comment on the possible character of this contaminant.

Early preparations of insulin were undoubtedly admixed with extraneous material of protein/polypeptide character. Since then major improvements in manufacture, including the introduction of crystallisation techniques in 1935-36, have led to the gradual elimination of biologically inert material. The preparation of a complex polypeptide completely free from impurities, however, is a matter of considerable experimental difficulty, which is further complicated in the case of insulin, by the absence of reliable physico-chemical tests for hypoglycæmic activity. Biological potency still forms the main criterion of purity, and the nature of the minor polypeptide impurities, which may exist in crystalline samples of insulin, has still to be determined.

Nevertheless, some progress in this direction has recently been reported by Sutherland, Cori, and co-workers[2,3,4] in the United States of America, who have successfully established the presence of a hyperglycæmic glycogenolytic factor in certain crystalline insulins of high biological potency. Deactivation of these by heating with alkali or with cysteine gave products producing hyperglycæmia on injection into rabbits. Clinical examination of such preparations gave similar results[5] which were accompanied, in one set of studies, by dermal reactions at the site of injection[6]. These may have been caused by products formed during inactivation of the insulin, by endogenous impurities, or by the hyperglycæmic glycogenolytic factor itself. We have, therefore, sought to establish the presence of the latter material in three samples of insulin

(M.N.981.S.D.317.S.D.318)* known to give rise to cutaneous reactions, hoping thereby to obtain evidence on the part played, if any, by this factor in the causation of these undesirable clinical phenomena. In addition, we have carried out parallel studies on a works batch of AB insulin (B.D.H. 2068), and on a sample of insulin known to contain the factor (N. 4840) for which we are indebted to Prof. F. G. Young, F.R.S.

Determination of the hyperglycæmic glycogenolytic factor was effected employing the "liver slice" technique developed by Sutherland *et al.* (*loc. cit.*). For this purpose rabbit liver slices were incubated aerobically with fractions containing the factor, and the output of glucose compared with that of parallel controls.

Experimental Methods and Results

The liver slice technique employed was a slight modification of that developed by Sutherland *et al.*

An adult rabbit, fed *ad libitum* on carrots some days prior to sacrifice to ensure adequate glycogen content in the liver, was bled as completely as possible under barbiturate anæsthesia. The liver was then rapidly excised and placed in cold isotonic sodium chloride solution. After about 15 minutes (longer standing proved inadvisable) a section of the liver was cut off and held firmly on a specially made block between cold perspex surfaces. Thin slices, approximately 0·5 mm. in thickness, but depending upon the condition of the liver, were then cut, the top slice being rejected. Each slice was divided into a number of sub-slices of approximately equal size, according to the number of comparisons to be made (6 in the present instance, made up of 5 insulin samples and one blank determination), and the sub-slices placed on a marked area of a filter paper moistened with buffer solution in an ice-cold Petri dish. When enough slices had been cut, according to the desired number of replicates, the sub-slices corresponding to each main slice were weighed on a torsion balance to the nearest mg. and adjusted in weight to 50 to 85 mg. with a deviation of not more than 15 to 20 per cent. As each sub-slice was weighed it was placed in a marked 25-ml. conical flask kept ice-cold and containing either 3 ml. of chloride phosphate buffer of *p*H 7·4 or 2 ml. of the same buffer plus 1 ml. of insulin solution containing 0·15 mg. of the crystalline material and adjusted to *p*H 7·4 with chloride phosphate buffer. Slices from the same lobe of the liver were always employed in a particular set of determinations. Rapid manipulation was essential, slices being cut, weighed and placed in the flasks within 30 to 40 minutes of removal of the liver from the animal.

The flasks, when ready for incubation, were clipped on to a holder adapted to fit the shaker of a Warburg apparatus, and shaken at 110 oscillations per minute with full access of air at 37°C. for 30 minutes. They were then removed and placed on trays of melting ice. 2 ml. aliquots of solution were rapidly withdrawn from each flask for glucose estimation, the operation being complete within a few minutes. Each

* Obtained through the courtesy of Dr. B. Garfoth.

aliquot was treated with barium hydroxide solution (1 ml. of 0·3 N) and zinc sulphate solution (1 ml. of 5 per cent.) and the tubes centrifuged. 1 ml. of the clear supernatant fluid was taken from each centrifuge tube and placed in a Folin sugar tube, to which was further added 1 ml. of the Somogyi copper reagent[7]. The tubes were placed in boiling water for exactly 10 minutes, cooled, the colour developed with the Nelson arsenomolybdate reagent[8], and the volume made up to 25 ml. The glucose concentrations were determined by comparison in a Spekker absorptiometer with standard glucose solutions which had been similarly treated, and the results calculated as mg. of glucose produced per g. of liver.

4 sets of experiments, each on a different rabbit liver, were performed in the present instance. The experimental slices were cut in such a way that 6 sub-slices of each individual slice were obtained. The 6 sub-slices were then assigned to flasks which represented, in turn, a " blank " without added insulin, insulin I (No. 4840), insulin II (B.D.H. 2068), and the skin flare samples insulin III (MN 981), insulin IV (SD 317), and insulin V (SD 318). 4 slices were used in each experiment, thus giving 4 replicates.

The first set of experiments gave such exceptionally high outputs of glucose, from all the liver slices, that it was considered advisable to ignore it altogether. The results from the other three sets of experiments are shown in Tables I and II. Examination of Table I reveals the variations so characteristic of this technique. Nevertheless, by grouping individual replicates and summing columns of like treatments (i.e., types of insulins), as in Table II, certain conclusions become evident. It is clear, for example, that apart from insulin I, the additions of insulins

TABLE I

GLUCOSE OUTPUT IN MG./G. LIVER IN 30 MINUTES

Liver	Slice	Insulin						Slice Totals	Liver Totals
		Blank	I	II	III	IV	V		
1... ...	1	8·1	12·7	9·4	9·8	8·8	9·1	57·9	
	2	10·4	11·4	9·7	10·5	8·3	7·4	57·7	
	3	7·8	9·9	9·1	9·8	9·6	8·8	55·0	
	4	9·3	12·5	9·3	9·8	9·8	10·7	61·3	231·9
2... ...	1	8·3	11·2	7·6	9·5	7·9	8·6	53·1	
	2	9·2	9·8	8·8	8·3	9·9	8·4	54·4	
	3	7·6	9·4	7·6	8·4	8·3	9·0	50·3	
	4	7·1	11·2	8·6	8·5	8·7	8·2	52·3	210·1
3... ...	1	9·1	14·3	12·1	10·9	10·9	10·5	67·8	
	2	9·3	13·3	10·1	10·6	9·9	10·3	63·5	
	3	11·1	13·7	11·3	10·5	11·7	11·0	69·3	
	4	10·8	13·6	10·2	12·1	11·0	11·6	69·3	269·9
TOTALS ...	—	108·1	143·0	113·8	118·7	114·7	113·6	—	
								GRAND TOTAL ...	711·9

to the liver slices produced no marked increase in glucose output. Thus by the method of analysis of variance, after eliminating the highly significant variance between different livers, it was found that the variance due to insulins was significant at the 1 per cent. level of significance. That this variance was almost entirely due to insulin I was shown by a fresh analysis of the data omitting this insulin, when the variance between livers was no longer significant even at the 5 per cent. level. Insulins III, IV and V, as well as insulin II, thus yield a glucose output from liver slices not significantly different from the output of glucose from contiguous slices incubated in the absence of insulin. These four samples of insulin are thus shown to be free from the hyperglycæmic glycogenolytic factor within the limitations imposed by this experimental technique.

TABLE II

GLUCOSE OUTPUT IN MG./G. OF LIVER IN 30 MINUTES
SUMS OF REPLICATES

Liver	Type of Insulin Added						Totals for Individual Livers
	Blank	I	II	III	IV	V	
1	35·6	46·5	37·5	39·9	36·4	36·0	231·9
2	32·2	41·6	32·6	34·7	34·8	34·2	210·1
3	40·3	54·9	43·7	44·1	43·5	43·4	269·9
Totals for Treatments ...	108·1	143·0	113·8	118·7	114·7	113·6	—
						GRAND TOTAL ...	711·9
Percentage increase compared with blank...	—	32·2	5·3	9·8	6·1	5·1	—

SUMMARY AND CONCLUSIONS

1. Examination by the liver slice technique of three samples of insulin known to produce dermal irritability on injection has failed to reveal the presence in them of the hyperglycæmic glycogenolytic factor.

2. It, therefore, appears that this factor is not implicated in the causation of dermal reactions to insulin.

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