Chromatography of Guinea-pig Native Insulin

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(Received 27 September 1956)

Moloney & Coval (1955) have shown that insulin can be extracted from guinea-pig pancreas which is not neutralized by anti-insulin induced in the guinea pig by injection of ox or pig insulin. Such extracted insulin, arbitrarily called 'native' insulin, resembled endogenous insulin in respect of non-neutralization by anti-insulin induced by injecting ox or pig insulin into guinea pigs. Guinea-pig native insulin was found to differ from 'altered' insulin (obtained from one batch of guinea-pig pancreas that had been stored in the cold for 6 months), which was neutralized by such anti-insulin.

We are reporting here properties of guinea-pig native insulin and of certain other extracted insulins, as revealed by chromatography and by reaction to anti-insulin.

MATERIALS AND METHODS

Pancreas were obtained from animals as soon as possible after death. Guinea-pig and rabbit pancreas were quick-frozen within 30 sec. of the death of the animal. Sheep and horse pancreas were frozen approximately 20 min. after the animals were slaughtered.

Crude extracts of insulin were prepared by methods which were based on the first steps of a salt-alcohol procedure (Romans, 1954) or of an acid-alcohol procedure (Romans, Scott & Fisher, 1940). Frozen glands were ground with solid CO₂ in a power grinder before being extracted. Approximate estimates of potencies were obtained by mouse-convulsion assay (Culhane, Marks, Scott & Trevan, 1929).

It was found that better yields of guinea-pig insulin were obtained by acid-alcohol extraction than by salt-alcohol extraction; the latter procedure had been used by Moloney & Coval (1955). Extracts of guinea-pig insulin were prepared as follows: to each 100 g. of ground frozen pancreas were added 250 ml. of 92% (v/v) ethanol plus HCl to pH 2.2-2.6 and the mixture was stirred at room temperature for 1 hr. Solids were separated by centrifuging, 220 ml. of 70% (v/v) ethanol was added, the solution was adjusted to pH 2.2-2.6 and the mixture again stirred for 1 hr. After centrifuging, the two supernatants were combined. To each 100 ml. of the combined alcoholic extracts were added 200 ml. of ethyl ether and 60 ml. of HCl-water (pH 2.6). The mixture was shaken in a separating funnel and the aqueous layer removed. The ether layer was re-extracted with 60 ml. of fresh acid water. The combined aqueous layers were adjusted to pH 3.5, concentrated to one-eighth to one-third of original volume in a Flash-Evaporator (Laboratory Glass Supply Co., New York), and then dialysed against HCl-water (pH 2.6) at 1-5° and finally freeze-dried.

Anti-insulin sera were obtained from the guinea pig, rabbit, sheep and horse by inoculation with crystalline ox or pig insulin emulsified in Freund's adjuvant (Moloney & Goldsmith, 1957).

A reversed-phase method of partition chromatography of insulin which was described by Porter (1953) was used. Most of the work involved the use of Porter's system 3 and modifications of it. Modified systems were designated 3A, 3B, etc.

Fractions of column effluent were collected and optical densities at 277 m μ were read on a Hilger Uvispek spectrophotometer. In some cases fractions were also tested qualitatively for insulin by the mouse-convulsion procedure (Culhane *et al.* 1929) after dialysis and concentration by pervaporation.

RESULTS

Thirty-five lots of guinea-pig insulin were prepared from approximately 3600 guinea pigs. Several lots of insulin from four species other than the guinea pig were also prepared. Yields expressed in units/g. of pancreas were approximately 3.5 for pig insulin, 3.0 for horse, 1.5 for sheep, 0.3 for rabbit and 0.5 for guinea pig.

Neutralization tests on mice (Moloney & Coval, 1955) were carried out with mixtures of anti-insulins and insulins. The tests showed that no preparation of guinea-pig insulin was neutralized by anti-insulin produced in the guinea pig, horse, sheep or rabbit.

Preparations of crude insulin from the rabbit, sheep, horse, ox and pig as well as crystalline ox, pig, sheep and monkey insulins were neutralized by anti-insulin from the guinea pig, rabbit, sheep and horse.

Chromatography of insulins

It was found that phase relationships in the solvent systems described by Porter (1953) were extremely sensitive to temperature changes. Separation of the organic phase occurred if the temperature of the more aqueous phase rose slightly above that of equilibration. For this reason chromatographic analyses were carried out at 35° in an incubator, the temperature of which was maintained to within $\pm 0.1^\circ$, and spectrophotometric readings were made at temperatures 21-28°.

The chromatography of crystalline insulin with Porter's system 3 produced results which were similar to those described by that author. However,

chromatographic analyses which were carried out on crude extracts of pig or ox insulin resulted in poor separation of insulin from impurity. Fig. 1 is an example of such an analysis. The difference between these results and those of Porter is due probably to differences in the composition of the phases in system 3 at the temperatures employed, namely 35° and 20° respectively.

It was found that the distribution ratio of insulin could be raised in favour of the more organic phase by increasing the pH of the system. The pH range 2.8–5.2 was investigated. A total of twenty-six solvent systems, which were modifications of system 3, were prepared and eight of these were used for chromatography. Progressively better separations of insulin from impurity were obtained as the pH of the system was raised.

Fig. 2 illustrates the excellent separation of insulin from impurity that was obtained with system 3Y (pH 5.2). However, since insulin is not very soluble in the more aqueous phase of this system, only very small amounts could be processed by the columns which were used.

Attempts were made to purify crude guinea-pig insulin by the use of Porter's system 3 (temp. 35°). The resulting optical-density curve showed only one

large peak in the position normally occupied by impurity. This is shown in Fig. 3. The results of biological assay of each fraction are also shown. There was an active insulin peak at about 20 ml. of effluent and a trace of activity at about 45 ml. of effluent.

Guinea-pig insulin was then chromatographed in system 3Y. The results are shown in Fig. 4. These results were quite similar to those obtained with guinea-pig insulin in system 3 and were considerably different from those obtained with ox insulin in system 3Y (Fig. 2).

The difference in the chromatographic behaviour of ox and guinea-pig insulins was clearly demonstrated by the results of the chromatography of a mixture of these two insulins in system 3Y (Fig. 5). The guinea-pig insulin appeared in the first two peaks, neither of which was neutralized by anti-insulin serum. The ox insulin appeared in the third peak, which was neutralized by anti-insulin serum.

The chromatography in system 3Y of crude extracts of pig, rabbit, sheep and horse insulins showed that each of these insulins produced a single slow-running peak, as did ox insulin. The elution volumes for these insulins varied markedly but were in the same range as the elution volumes for ox insulin (Figs. 2, 5).

Fig. 1. Chromatography of crude pig-insulin extract. System 3: 200 ml. of ethyl Cellosolve (2-ethoxyethanol); 100 ml. of butyl Cellosolve (2-butoxyethanol); 720 ml. of 1.885M- NaH_2PO_4 ; H_3PO_4 to pH 2.8. Column: 6 g. of silane-treated kieselguhr in tube 1.2 cm. internal diameter.

Fig. 2. Chromatography of crude ox-insulin extract. System 3Y: 200 ml. of ethyl Cellosolve; 100 ml. of butyl Cellosolve; 720 ml. of 1.885M- NaH_2PO_4 ; NaOH to pH 5.2. Column was as described in Fig. 1.

Fig. 3. Chromatography of crude guinea-pig-insulin extract. Solvent system and column were as described in Fig. 1. Shaded areas represent the insulin content of the effluent as determined by biological assay.

Fig. 4. Chromatography of crude guinea-pig-insulin extract. Solvent system and column were as described in Fig. 2.

Fig. 5. Chromatography of mixed crude guinea-pig and crystalline ox insulins. Solvent system and column were as described in Fig. 2.

DISCUSSION

The highest yields of guinea-pig insulin were in the order of 0.5 unit/g. of pancreas. These yields were considerably lower than yields which were obtained from pancreas of the other species, except the rabbit. Because of low yields on extraction and losses during concentration and purification, only comparatively small amounts of guinea-pig insulin were obtained from large numbers of guinea pigs.

In the chromatography of ox, pig, sheep and horse insulins under apparently the same conditions, there were disturbing variations in elution volumes, the cause of which was not determined. There are two factors which may have caused these variations, namely differences in the packing of columns and alterations in pH. The system which was used (3Y) was quite sensitive to pH change; for example, a shift in pH of 0.1 unit changed the distribution ratio of ox insulin in the order of 15%. However, variation in elution volumes does not invalidate the conclusion that native guinea-pig insulin is different from extracted ox insulin, since a mixture of the two can be separated by chromatography. Moreover, since pig, rabbit, sheep and horse insulins showed chromatograms similar to those of ox insulin, it is probable that these also could be separated from guinea-pig insulin by chromatography.

SUMMARY

1. Guinea-pig insulin was separated by reversed-phase chromatography into two fractions, neither of which was neutralized by anti-insulin produced in the guinea pig or by anti-insulin produced in the rabbit, sheep or horse by injection of ox or pig insulin.
2. Ox insulin and guinea-pig insulin in mixed solution were separated by chromatography.
3. The hypoglycaemic activity of ox, pig, rabbit, sheep and horse insulins was neutralized by anti-insulin produced in the guinea pig, rabbit, sheep and horse by injection of ox or pig insulin.

We are grateful to Dr A. M. Fisher for a sample of crystalline monkey insulin, to M. Gross Abattoir for sheep pancreas, to Dr Ballard's Animal Foods Ltd. for horse pancreas and to Mr D. Cordwell for technical assistance. We acknowledge with thanks financial help given by the J. P. Bickell Foundation.

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