THE DIFFERENCE BETWEEN INSULIN FROM CATTLE AND FROM PIGS

by

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The difference, that might exist between insulin obtained from pig and from cattle pancreas has received little attention. SANGER[1] observed, that the composition of a fraction, obtained after cleavage of the disulfide bridges, differed somewhat for pig and cattle insulin.

In this fraction from cattle insulin, he found much alanine and no threonine; the corresponding fraction from pig insulin contained some threonine and little alanine. This work is open to some criticism as the insulin samples used might not have been altogether pure, and the difference observed therefore might be described to the presence of impurities. The object of our investigation was to compare absolutely pure pig and cattle insulin in order to see whether they actually differ or not. This comparison has been made with the aid of four techniques:

a. the analysis of the amino acids present;

b. the solubility curve determinations;

c. the biological assay;

d. the original method, first used by SANGER for the demonstration of the difference between the two.

Dr SANGER kindly performed the latter demonstration of the difference with the aid of his original technique, thus removing every trace of doubt about their non-identity.

MATERIAL

The samples of pig and cattle insulin crystals, obtained from batches of ordinary comm ercia material were recrystallized until they showed a horizontal solubility curve, as described in a previous paper[2]. The cattle insulin did not give rise to many difficulties.

After only six crystallizations, material giving a horizontal solubility curve at pH 5 in acetate buffer was obtained in 70–80% yield.

The last two crystallizations were made with a slight modification of one of the usual procedures in the following way:

The crystals, obtained from 10 g of insulin after 4 recrystallizations from acetate buffer were suspended in 360 ml 1/15 *M* phosphate buffer pH 7 and 360 ml of acetone. To this suspension were added 15 ml 0.5 *N* KOH, whereupon the insulin dissolved, leaving most of the zinc as phosphate in suspension. After filtration through a folded filter paper, the filtrate was diluted with 1440 ml phosphate buffer, 1300 ml of water and 0.9 ml of zinc acetate solution, containing 25 mg zinc per ml. The pH was adjusted to 5.94 with 0.5 *N* HCl. The solution was left overnight for crystallization at room temperature. The crystals were centrifuged, thoroughly washed with water and dried *in vacuo*. Yield: 6.8 g with a zinc content of 0.51%.

The pig insulin gave far more difficulties and had to be recrystallized 8–9 times to give a pure product in a yield of only 10–20%. Crystallization usually proceeded only very slowly, frequently yielding large crystals, visible to the naked eye. The amount of amorphous material which usually was present could be greatly diminished by a threefold increase of the amount of zinc acetate added.

References p. 338.

This had an unfavourable influence on the zinc content of the final product, which in one instance was 1.56%, in another even 4.1%.

METHODS

a. *Amino acid analysis*

For the quantitative determination of the amino acids, the technique of STEIN AND MOORE[3] has been used with slight modifications to adapt it to our apparatus.

As it is believed that the modifications introduced may bring this beautiful method within the reach of every laboratory, the description given will be rather extended.

The fraction collector, described by the authors was not available, and the apparatus of RANDALL AND MARTIN[4] has been used instead. This collector has several advantages. Any good mechanic can build one, it is independent of an outer source of energy and the fractions are measured by their volume and not by a number of drops. On the other hand, the smallest fractions that could be obtained, were not less than 4 ml, at least with an apparatus as constructed in our workshops. If the fractions are smaller, the collector sticks occasionnally and would have to be under constant supervision. With fractions of 4–5 ml and a properly adjusted apparatus, it could be left working overnight and during the weekends. A first model, with a ply-wood test tube rack, soon became undependable, because the wooden base curbed, thus interfering with the proper functioning of the apparatus. A second one with an aluminium rack was quite satisfactory and remained so. The apparatus should be placed in a quiet room, where no other work is done, free from drafts and out of direct sunlight. Ammonia vapours are to be avoided as they will increase the blanks. A constant temperature room would have many advantages.

As the fractions collected have about 10 times the volume of those obtained by STEIN AND MOORE and no much smaller separating capacity can be tolerated, the size of the starch colum used and consequently the weight of the sample under investigation had to be adjusted to obtain results, comparable to those of STEIN AND MOORE. The following dimensions, weights and volumes were chosen:

Weight sample: 10–30 mg.

Chromatography tube: 38 cm long, 2.0 cm internal diameter with a sintered glass plate

Weight of starch: 55 g, calculated as dry material

Volume of fractions: 4.66 ml

Under these conditions and with the solvent mixture *n*-butanol: *n*-propanol: 0.1 *N* HCl 1:2:1, followed by *n*-propanol: 0.5 *N* HCl 2:1, the whole chromatogram could be collected in 180–190 fractions in the course of 5–7 days. With these mixtures, the separation between the leucines and phenylalanine is not sufficiently distinct to allow their individual determination, nor can valine, methionine and tyrosine be distinguished. But the same holds under the experimental conditions of STEIN AND MOORE, who have twice as many fractions as we obtained. As the fractions were collected in ordinary test tubes and contained about 20 × as much material as those, described in the original work, the errors due to creeping of the solutions along the walls of the tubes were so small that they could be neglected. For the ninhydrin reaction, samples were taken of 0.5 ml. The determinations were made according to MOORE AND STEIN[5] but the methyl-cellosolve was replaced by ethyl-cellosolve, the first one being unobtainable. The photometer used was a Coleman. The solutions were measured in a single pair of tubes and not in a series of matched tubes. The results were fully in accordance with those of MOORE AND STEIN.

The results obtained with butanol:benzylalcohol:water as a developer, recommended for the separation of the group phenylalanine, leucine and *iso*leucine and of the group methionine, tyrosine and valine were disappointing, undoubtedly because the room temperature fluctuated too much. In the solvent reservoir on top of the column, water condensed on the upper wall of the reservoir. The drops occasionally fell back into the bulk of the solvent, but did not dissolve quickly enough not to reach the starch column. Once a drop had been adsorbed by the column the latter was completely useless. The phenomenon was even observed if the composition of the butanol:benzylalcohol:water mixture was changed from 1:1:0.288 to 1:1:0.24. If fluctuations in room temperature were avoided and by using a reservoir with an outflow not at its lowest point, these difficulties were overcome, but the separation obtained was disappointing. The group of the leucines + phenylalanine was divided over 25 fractions, but no differentiation was possible. The tyrosine and valine peak was collected in 40 fractions but here again not more than a fair guess was possible about the single components. It should be stressed that these results probably are not to be considered as contradictory to those of STEIN AND MOORE, because a solvent with less water was used. They only indicate that our simplified but admittedly less perfect technique was not adequate to give a satisfactory solution of the problem in this instance. On the other hand and as pointed out already, the degree of differentiation obtained with the mixture propanol:butanol:0.1 *N* HCl was just as satisfactory as claimed by these authors.

In order to differentiate the two above mentioned groups, their u.v. absorption was explored.

References p. 338.

It was found that in the first group, *i.e.*, the leucines and phenylalanine, owing to the presence of traces of humin, no useful information could be obtained but the differentiation of the valine plus tyrosine peak by means of the extinction at 277 mμ was quite satisfactory. Valine shows a negligible absorption at this wavelength. The molar extinction of tyrosine is quite high. In the solvent used (*n*-butanol:*n*-propanol:0.1 *N* HCl 1:2:1) it was found to be 1570. Equal parts of all fractions of the valine-tyrosine peak were pooled, and the extinction measured in a Beckman spectrophotometer model D.U. Valine was then calculated by substraction of tyrosine from the total value obtained from the ninhydrin determination, taking in account the 86% colour yield of the tyrosine.

Theoretically, the separation of glutamic acid and alanine, and of aspartic acid and threonine should be possible by filtration of the mixtures over acid-washed aluminium oxide. This method of separation of the dicarboxylic amino acids from the others, proposed by TURBA[6] gave satisfactory recoveries with quantities of a few micromoles of the amino acids concerned, though the blanks were quite high. With the actual mixtures obtained from the starch column, the problem how the blanks should be taken in account, becomes insoluble.

These blanks do not arise from the reagent solutions only, but the starch column eluate as such contains already unknown ninhydrin positive substances. On the Al_2O_3 column these apparently do not behave uniformly, making the whole analysis too unreliable to mention the results so obtained in this paper.

The chromatographic procedure was checked by the analysis of a synthetic mixture of amino acids corresponding in composition to an acid hydrolysate of cattle insulin. The results are recorded in Table I. In the analysis of hydrolysates of insulin, the peaks were found just as sharp as in this experimental run, with the exception of the cystine peak, showing a very strong "tail" in the hydrolysate analysis. This made the determination of cystine so uncertain that values could be calculated according to personal judgment and differing by more than 30%. The cystine values in the actual analysis were therefore calculated from the sulphur content of the preparations.

TABLE I

ANALYSIS OF A SYNTHETIC MIXTURE, CORRESPONDING IN COMPOSITION TO A HYDROLYSATE OF INSULIN

Solvent *n*-propanol:*n*-butanol:0.1 *N* HCl 2:1:1 followed by propanol: 0.5 *N* HCl 2:1 at fraction 80. Results expressed in micromoles amino acid. Amounts added corrected for colour yields

Fraction numbers	Amino acids	Added	Found	Error in %
12–16	*Iso*leucine, leucine phenylalanine	30.3	31.0	+2
22–30	Valine, tyrosine	21.6	23.5	+9
49–53	Proline	0.83	0.80	—4
52–62	Glutamic acid, alanine	33.3	32.7	—2
70–83	Threonine, aspartic acid	9.77	9.5	—3
92–97	Serine	8.30	8.27	0
97–103	Glycine	10.5	10.5	0
124–131	Arginine	2.91	2.58	—11
135–140	Lysine	3.80	4.10	+8
143–152	Histidine	4.90	5.12	+4
160–171	Cystine	4.97	5.19	+4

b. *Determination of the solubility curve*

The solubility curves in acetate buffer at pH 5.0 were determined with the method described previously[2]. It was found that, in order to reach equilibrium, pig insulin crystals had to be shaken 48 hours with the solvent instead of 24 hours.

As in the course of this work the desirability became evident to extend these determinations to other media, curves were also measured in a 0.25 *M* phosphate buffer at pH 6.38 in the following way: 10.658 g Na_2HPO_4 and 23.830 g KH_2PO_4 were dissolved in 1 l with water, giving a solution of pH 6.38.

75 mg insulin were shaken with 30 ml of this buffer for 5 hours. From the homogeneous suspension, further dilutions were made of 1, 2, 4, 5 and 8 ml with 25 ml buffer solution. These were shaken for 48 hours to reach equilibrium and subsequently filtered. Filtration often had to be repeated 5–6 times through the same filter in order to get clear solutions. Kjeldahl determinations were made in the filtrates.

References p. 338.

The results with this buffer are somewhat less reproducible than those with the acetate buffer, probably because of the filtration difficulties.

c. *Assay*

The assay on rabbits, as described by DE JONGH *et al.*[7] has been used.

RESULTS

a. *Amino acid analysis*

The insulin samples (50 mg) were hydrolized with 9 ml 25% HCl for 24 hours by boiling under reflux. The hydrolysate is evaporated to dryness *in vacuo*, dissolved in as mall volume of water, and again taken to dryness *in vacuo*. The latter procedure is twice repeated. The residue is then dissolved in 2.5 ml propanol:butanol:0.1 *N* HCl 2:1:1, and 2 ml of this solution is transferred to the column. The chromatogram is developed with the same solvent mixture. After fraction 80, when the peak of threonine and aspartic acid has been collected, the solvent is changed to propanol:0.5 *N* HCl 2:1. The results of these analysis, together with those from the literature, are condensed in Table II. The molecular weight of insulin has been assumed as 12,000, in accordance with CHIBNALL[8]. The value of 11,620, calculated by ACHER, FROMAGEOT, AND JUTISZ[9] is probably more accurate, but its introduction would, certainly under the conditions of these experiments, suggest a greater accuracy than actually has been obtained.

TABLE II

ANALYSIS OF PURE CATTLE AND PIG INSULIN IN MOLES AMINO ACID RESIDUES PER MOLE INSULIN

Assumed M.W. of insulin: 12.000. Comparison with values from the literature

Amino acids	This investigation		TRISTRAM[10]	ACHER[9] *c.s.*	BRAND[11]	VELICK[12] RONZONI
	pig	cow		cow		
Leucine, phenylalanine	21.5	22.4	20.5	21.0	20.7	20.3
Tyrosine	8.3	8.3	8.7	8.0	8.2	8.1
Valine	7.8	9.5	7.8	7.7	9.0	9.5
Proline	2.4	1.7	2.7	2.2	3.0	2.9
Glutamic acid, alanine	20.6	22.0	21.1	21.9	16.6+?	16.3+?
Aspartic acid, threonine	10.5	8.9	8.2	7.3	9.4	3.5+?
Serine	5.4	6.3	6.0	5.9	6.6	6.9
Glycine	7.1	7.9	6.9	7.2	7.3	7.3
Amide N	12.3	12.5	11.6	12.0	14.4	—
Arginine	2.0	2.0	2.1	2.0	2.3	2.3
Lysine	2.2	2.1	2.1	2.2	2.1	2.0
Histidine	3.8	3.2	3.8	3.8	4.1	4.1
Cystine	12.2	12.2	12.5	12.4	11.2	11.2

The sample, investigated by ACHER, FROMAGEOT, AND JUTISZ is of the same batch of pure cattle insulin as the one analysed in our investigation. The high value for valine is somewhat unexpected. Because this value is obtained as the difference of the total value of the peak by the ninhydrin method and the value for tyrosine, calculated from the u.v. adsorption at 277 mμ, the valine analysis may show a greater error than the others. In a sample of another batch of insulin, the still higher figure was obtained of 10.6 μ moles per mole of insulin, but the tyrosine value was exactly the same. It is remarkable that in the experiment with the amino acid mixture, described in the

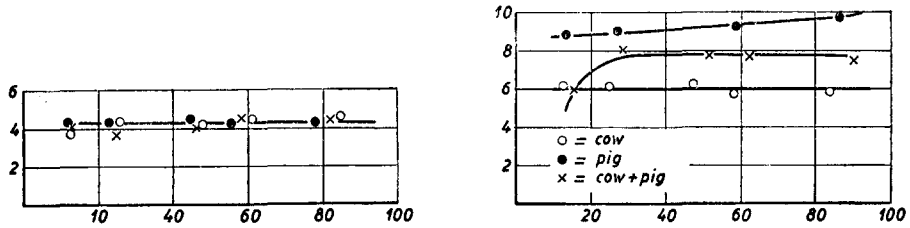
References p. 338.

previous section of this paper, as well as in the analysis of other proteins, the peak for tyrosine and valine is also too high. The values for the colour yields of tyrosine and valine with ninhydrin have been extensively checked and those given by STEIN AND MOORE have been confirmed. The cystine values recorded are not obtained by chromatography but calculated from the sulphur content of the preparations.

In the calculations, the weight of the insulin sample has been corrected for moisture and zinc content.

b. *Solubility curves*

Crystalline cattle insulin with 0.51% Zn and pig insulin with 1.56% Zn both gave a horizontal solubility curve in the acetate buffer solution of pH 5. The experiment was repeated with a mixture of 25 mg pig insulin and 50 mg cattle insulin. A curve was obtained, coinciding with the two first ones (Fig. 1a).



1a 1b

Solubility curves for cattle and for pig insulin and their mixture in 0.1 *M* acetate buffer pH 5 (Fig. 1a) and in 0.25 *M* phosphate buffer pH 6.38 (Fig. 1b)
Ordinate: solubility, expressed in μg N per ml
Abscissae: total amount of insulin per ml also expressed in μg N per ml

The result was confirmed with a sample of pig insulin of a different batch. The investigation was therefore extended to the behavior in phosphate buffers at pH 6.4, accordingly just at the alkaline side of the isoelectric point. The solubility of the insulin increases considerably in the range of from pH 6.5 to 7.0 and, if this range had been chosen, small fluctuations of the pH in the different suspensions would have influenced the results greatly. Furthermore, there is the additional difficulty in this range of securing clear filtrates, a difficulty far worse than at a slightly lower pH. Even at pH 6.4, as chosen for the experiment, the buffer has to be rather concentrated, otherwise clear filtration becomes impossible.

With this buffer, the two solubility curves of cattle and pig insulin differed somewhat and the curve for the pig insulin was no longer exactly horizontal, but showed the presence of a small amount of impurity. However, the curve for a mixture of the two insulins did not show a slope of any significance (Fig. 1b) but indicated by its type that the mixture behaved as a solid solution with the two insulins as the components.

c. *Biological assay*

Pure cow and pig insulin show the same biological activity within the error of the experiment. The activity of the first one has been reported previously as 26.8 $\pm$ 0.8 international units per mg. For pig insulin an activity of 26.1 $\pm$ 1.0 units per mg was found. The results are expressed as the activity on a moisture free basis. The assay was performed by Dr J. HARTKAMP.

References p. 338.

d. *Analysis of peptide chains*

Dr F. SANGER (Cambridge) kindly investigated our samples of cow and pig insulin by isolating the "fractions A" after oxidation. The paper-chromatogram, obtained by him demonstrates quite convincingly that in the cattle insulin fraction threonine is absent, where asalanine is present in much higher concentration than in the pig insulin fraction, which contains some threonine as well. We wish to thank Dr SANGER for this valuable contribution to our investigation.

DISCUSSION

The most satisfactory method to demonstrate the difference between insulin from cattle and from pig is the one originally used by SANGER. The determination of the solubility curves at pH 5 did not allow to draw a conclusion. At pH 6.4 the solubilities of the two types differ slightly and a mixture of the two behaves as a system containing a solid solution as the precipitate. In this respect it resembles the system of α and γ trypsinogen at pH 4.0 in 0.4 saturated ammonium sulphate, described by KUNITZ[13]. It is surprising that the solubility curve of not altogether pure insulin reacts so strongly to the presence of the impurities, whereas it does not to the same extent in mixtures of cattle and pig insulin. The impurities in almost pure insulin must closely resemble it in its properties for otherwise they would have been removed in the course of the purification process. Their presence in amounts of less than 10% can easily be demonstrated by means of the determination of the solubility curve of the sample in question. On the other hand, the presence of over 30% of pig insulin in cattle insulin would pass unnoticed, if this curve were determined at pH 5. At pH 6.4 it would be perceptible but far less conspicuous than the presence of 10% of "natural" impurities.

The results of the amino acid analysis for the cow and pig insulin do not offer very convincing evidence for a difference between the two, as soon as they are compared with the data from the literature. The latter values have been obtained with quite different methods of analysis. If it may be assumed that the results of the analyses of two samples with one and the same method are subject to the same systematic errors, the difference, if any, is still not very clearly demonstrated. The values found for the group glutamic acid and alanine and those for the group threonine and aspartic acid are consistent with the results of SANGER[1] but can hardly be claimed as a strong support for them, because the difference in the "A fractions" might be counterbalanced by differences in the other peptide chains of the molecule.

The value obtained for valine in the analysis of the cattle insulin confirms that of BRAND and of VELICK AND RONZONI, but it should be taken with reserve since, not only in this investigation but also in others, valine values with this technique tend to be slightly too high.

The total nitrogen contents, calculated from these data are 15.3% for pig and 15.6% for cattle insulin. In this calculation, the number of residues has not been rounded off. If the number of valine residues in cattle insulin is taken as 8 instead of 9.5, the calculated nitrogen content would be 15.4%, whereas the actual figure for both insulins is 15.3%, confirming the results of the analysis by ACHER, FROMAGEOT, AND JUTISZ[9].

In the biological assay, cattle and pig insulin show equal activities within the error

References p. 338.

of the experiment. On the other hand, the difference between the two is best demonstrated with the method, originally used by SANGER. No quantitative analysis is necessary to distinguish the one from the other with the aid of this technique.

The difference encountered between cattle and pig insulin in the purification by recrystallization must mainly be due to the properties of the impurities. The low yields cannot be explained on the basis of the solubility of the pure pig insulin crystals. In the impure crystals, the impurities must have a considerable solubilizing effect on the insulin, otherwise the yields would have been much better. This follows also from the solubility curves of the impure crystals, which have not been described in this paper, but which show a solubility many times greater than found for the pure substance.

SUMMARY

Insulin from pig pancreas has been obtained in the pure state according to the solubility criterium. Its over-all amino analysis gives a result not sufficiently differing from that of cattle insulin to warrant the conclusion that the two are different. Their biological activity is equal. The solubilities at pH 6.4 differ slightly.

The solubility curve of a mixture of the two shows that they form a solid solution. The only conspicuous difference is found in the composition of the "A" fractions of SANGER.

RÉSUMÉ

A partir de pancréas de porc il a été isolé de l'insuline, dont la pureté fut démontrée à l'aide de la courbe de solubilité. La différence entre la teneur totale en acides aminés de cette préparation d'insuline d'une part et de l'insuline pure obtenue du pancréas de boeuf ne permet pas de conclure que ces deux préparations soient différentes. De plus leurs activités biologiques sont identiques. Leurs solubilités à pH 6.4 ne sont que faiblement différentes.

La courbe de solubilité d'un mélange de ces deux préparations démontre qu'elles donnent lieu à une solution solide. La seule différence nette fut trouvée en comparant la composition des deux fractions "A" de SANGER.

ZUSAMMENFASSUNG

Aus der Bauchspeicheldrüse von Schweinen ist reines Insulin isoliert worden. Als Kriterium der Reinheit diente die Löslichkeit. Die Aminosäuren-Analyse dieses Insulins ergab Resultate, die nur wenig von einer analogen Analyse von Rinder-Insulin abweichen, so dass man auf Grund dieser Resultate nicht behaupten kann, dass die beiden Insuline verschieden sind. Die biologische Aktivität der beiden Präparate ist gleich und ihre Löslichkeit bei pH 6.4 unterscheidet sich nur wenig.

Die Löslichkeitskurve einer Mischung der beiden Insuline deutet auf Bildung fester Lösungen. Der einzige auffallende Unterschied zwischen den beiden Insulinen wurde in der Zusammensetzung der Fraktion "A" von SANGER gefunden.

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