STUDIES ON THE BIOSYNTHESIS OF INSULIN

I. THE PAPER CHROMATOGRAPHIC ISOLATION OF ^{14}C-LABELED INSULIN FROM CALF PANCREAS SLICES*

by

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INTRODUCTION

Progress in the elucidation of the mechanisms involved in the biosynthesis of proteins has been retarded in part by our ignorance of protein structure. However, the recent determination by SANGER and co-workers[1] of the complete amino acid sequence of insulin makes this substance a promising one for the study of the pathways of protein synthesis. For example, the possibility of testing specific compounds of appropriate structure as possible intermediates in protein synthesis can be realized. Moreover, the study of the synthesis of insulin *per se* is of great interest from the point of view of the homeostatic mechanisms involved in blood sugar control and of the etiology of diabetes mellitus.

For such studies on insulin synthesis, it is of great advantage to have available an *in vitro* system. In a brief note, PETTINGA AND RICE have reported[2] that insulin labeled with ^{35}S in the cystine residue is produced when beef pancreas slices are incubated with methionine-^{35}S. The properties of a somewhat modified system (calf pancreas slices and leucine-1-^{14}C) are described in this communication. In addition, a method for the routine isolation of the labeled insulin from the incubated slices is described and evidence is presented relating to the purity of the isolated insulin. The method involves separation of the insulin from other tissue proteins by preparative paper chromatography and is based on a paper chromatographic procedure described previously for the identification of insulin in crude pancreas fractions[3]. Concurrently with this work, GRODSKY AND TARVER have independently developed a paper chromatographic method for the quantitative estimation and the isolation of insulin from fetal pancreas slices[4].

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MATERIALS AND METHODS

DL-Leucine-1-^{14}C (specific activity, approximately $1 \cdot 10^9$ c.p.m. per mM) was obtained from the Isotopes Specialties Co., Glendale, California, and was checked for purity by paper chromatography. The position of all the observable radioactivity was found to be coincident with the position of the leucine.

The samples of crystalline zinc insulin were commercial preparations*.

Munktell No. 20/150 electrophoresis paper was secured from E. H. Sargent and Co., Chicago, (Catalogue No. S-1886o).

Adult beef and calf pancreas was obtained a short time after the kill, 20–30 minutes in the case of the former, 10–15 minutes in the case of the latter**.

Solution A. 100 ml 95% ethanol + 0.9 ml 12N HCl.

Solution B. 31.5 ml H_2O + 111 ml 95% ethanol + 1.0 ml 12N HCl.

Solvent for chromatography. 2-Butanol is equilibrated with an equal volume of 1% acetic acid. The upper phase is used. Properties of this solvent system are described elsewhere[3].

Slicing procedure and incubation media: The pancreas was sliced in the cold room using Stadie blades in an apparatus similar to one already described[5]. The basic incubation medium consisted of either Krebs-phosphate or, in one experiment, Krebs-bicarbonate medium[21]. Where additions were made to the medium, an appropriate amount of NaCl was omitted. In the following sections, medium referred to as Krebs-phosphate-glucose was $2.0 \cdot 10^{-3}M$ in glucose, Krebs-phosphate-succinate was $5.7 \cdot 10^{-3}M$ in succinate, and Krebs-phosphate-glucose-succinate contained these concentrations of both substances.

Preparation of samples for counting

(a) *Tissue proteins*. After incubation of the pancreas slices, the proteins are precipitated and washed with 5% trichloroacetic acid solution, then further washed with alcohol and ether. The dried powder is suspended in acetone and carefully ground in a loose fitting, slowly rotating, glass-homogenizer and plated by filtration on an acetone-washed, previously weighed, filter paper circle (Whatman No. 50) supported on a demountable Buchner type of filter apparatus[7]. After dessication, the circles are counted in a gas flow counter and reweighed. Blank filter paper circles treated this way did not change weight by more than 0.10 mg.

(b) *Insulin*. It was found that plates of much more uniform thickness could be obtained when insulin was plated from suspension in an organic liquid rather than from an aqueous solution. Accordingly, the insulin is precipitated from solution with trichloroacetic acid, final concentration 5%. After thorough drying *in vacuo*, the trichloroacetic acid is removed by three washings in anhydrous ether. The insulin is then plated by the method described above for tissue proteins. The insulin may be recovered quantitatively by immersing the paper circle in a 0.01N HCl solution, and stirring for 30 minutes. The protein in the extract is determined by the colorimetric method of LOWRY *et al.*[8]. Control experiments on blank filter paper circles showed no interference with this method.

When less than 1 mg amounts of insulin are to be plated, the use of the glass homogenizer is avoided and the insulin is ground in a small agate mortar, suspended in acetone, and plated onto a stainless steel planchet. The planchets can be weighed to greater precision than paper circles, and recovery of the small amount of insulin is simpler and more quantitative.

The drying step is necessary after trichloroacetic acid precipitation because insulin was found to be soluble in wet acidified ether.

All radioactivity data presented are corrected for self-absorption employing the curve corresponding to the particular method of plating used. Overall counting precision was about $\pm$ 5%.

EXPERIMENTAL

The paper chromatographic method described here was developed after attempts to use existing methods of insulin isolation proved unsuccessful. It may be of interest to briefly record some of our experiences with these methods.

* We are indebted to Dr. O. K. BEHRENS of the Eli Lilly Company for a generous gift of insulin and to the Boots Drug Company for making insulin available to us at nominal cost.

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The fibril method

It was hoped that a method could be found which would permit the routine isolation of insulin from reasonably small amounts of pancreas slices (maximum of 20 g of slices) without the necessity of adding carrier insulin. The fibril precipitation method of WAUGH *et al.*[9] seemed to be the most promising one especially since, in confirmation of these authors, recoveries of insulin were found to be quantitative when crystalline insulin was used. However, the results were erratic when crude pancreas extracts were used, confirming the experiences of FOSTER *et al.*[10]. For our purposes, an additional disadvantage of the method is the necessity of adding an appreciable amount of insulin seed fibrils which would make hazardous any attempts to calculate the specific radioactivity of the insulin because of the unknown dilution of the isotopic insulin involved. Furthermore, the ease with which the fibril precipitate carries down other peptide material, for example, glucagon[11], was another deterrent to its use.

Column chromatography

A method of high resolving power for insulin which appeared to offer promise is the reversed phase partition chromatography method of PORTER[12]. After incubation in the presence of DL-leucine-1-^{14}C or DL-phenylalanine-3-^{14}C, the adult beef pancreas slices were treated according to the initial steps of the procedure described in the following section, except that carrier insulin was added to the initial acid alcohol extract and after the dialysis step, any precipitated material was redissolved in HCl and reprecipitated at pH 5.3. The precipitate was redissolved in the aqueous phase of the solvent system and added to the column. The effluent fractions corresponding to the insulin peak were combined and dialyzed until free of the aqueous phase. The insulin was then precipitated at its isoelectric point and its specific activity determined. It was then recrystallized several times and its specific activity measured after each recrystallization. The results of two representative experiments, given in Table I, show that the isolated insulin, although it had a potency equivalent to that of pure insulin as reported by PORTER[12], was not pure by isotope criteria. After further purification, a large decrease in specific activity was noted. Paper chromatography of the material from the column showed an immobile component present in very small

TABLE I

PURIFICATION OF INSULIN AFTER PARTITION CHROMATOGRAPHY

15 g wet weight of adult beef pancreas slices were incubated in Krebs-bicarbonate medium, $2.0 \cdot 10^{-3} M$ in glucose, containing $2.4 \cdot 10^{7}$ c.p.m. of DL-leucine-1-^{14}C. Gas phase, 95% O_2–5% CO_2. Incubation time, 2 hours. 70 mg of carrier insulin were added to the initial acid-alcohol extract. The insulin was crystallized according to the method of ROMANS, SCOTT AND FISHER[13].

		Expt. 1	*Expt. 2*
		c.p.m. per mg protein	
(1)	pH 5.3 precipitate after column	15	7
(2)	(1) reprecipitated at pH 5.3	5.9	7
(3)	(2) crystallized	3.6	4.2
(4)	(3) precipitated at pH 5.3	2.9	—
(5)	(3) recrystallized	—	3.9
(6)	(5) recrystallized	—	3.6
(7)	(6) recrystallized	—	2.4
(8)	(4) and (7) after paper chromatography	2.3	2.9

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amounts but possessing considerable radioactivity. This contaminant cannot be the free labeled amino acid but appears to be protein (or peptide) in nature. Although with further purification, constant specific activity could be achieved, the procedure was extremely tedious, even for a single sample, and was unsuitable as a method for running multiple samples. Furthermore, it became evident that the extremely low specific activities obtained for insulin made the addition of carrier insulin inadvisable.

Paper chromatography

The method which finally proved to be successful was the separation of insulin from other tissue proteins by preparative paper chromatography after preliminary extraction and dialysis of the crude extract. In addition, it was found that superior results are obtained when calf pancreas rather than adult beef pancreas is used. The procedure which follows is based on a starting weight of 20 g of pancreas slices. The initial procedure, up to the alcohol-ether precipitation step, is a modification of the method of ROMANS, SCOTT, and FISHER[13] and of PETTINGA AND RICE*[2].

After incubation of the slices, the flasks were chilled, the contents were homogenized for 30 seconds in a Waring blendor, and the homogenate was lyophilized in a 2 liter flask. The material was removed dry from the flask with a spatula and tranferred to a centrifuge tube. The flask was then washed with 60 ml of solution A which is also transferred to the centrifuge tube. The lyophilized material is then extracted for 1 hour at 30° with stirring or shaking. The material is centrifuged and the supernatant fluid is stored in the cold. The precipitate is reextracted with 40 ml of solution B under similar conditions. The second extraction is repeated for 30 minutes and all supernatant fluids are combined. The pH is adjusted to 5.3–5.6 with 5 *N* NaOH and 2 volumes of ethanol and 4 volumes of ether are added. The material is allowed to stand in the deep freeze (— 20°) for several hours or overnight if possible. As much as possible of the supernatant fluid is drawn off without disturbing the precipitate. The remainder of the material is centrifuged for 5 minutes at about 2000 × *g* and the supernatant fluid is discarded. After the ether is blown off the precipitate, the latter is extracted for 10 minutes at 30° with 5 ml of 0.05 *N* HCl. Should 2 liquid phases result because of the presence of ether, 1 or 2 ml of ethanol are added. The extraction procedure is repeated twice with about half the volume of HCl. The supernatant fluids are pooled and dialyzed overnight against distilled water using size 24/32 Visking casing. When leucine-1-^{14}C was used in the incubation medium, 20 mg of unlabeled DL-leucine were added to the dialysis sac for washing out purposes. At the completion of the dialysis, the material is removed from the sac and is taken to dryness in a rotary evaporator**[14] at 30° using octyl alcohol, if necessary, to prevent foaming. The dried material is taken up in a small volume of 0.05 *N* HCl and applied as a streak along the width of a 20 × 30 cm sheet of washed Munktell paper. The other end of the paper is serrated. The material is then chromatographed in descending fashion using the butanol-acetic acid solvent mentioned earlier. The chromatogram is run for about 16 hours, and the solvent is allowed to drip off the paper. The insulin band moves from 6 to 10 cm under these conditions, separating cleanly from the large mass of protein which remains at the origin. The insulin band is

* We wish to thank Dr. C. W. PETTINGA of the Eli Lilly Co. for sending unpublished details of his procedure and for a generous gift of crystalline hyperglycemic-glycogenolytic factor.

** Available from the Laboratory Glass Supply Co., New York 31, N.Y.

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located by cutting a 5 mm wide center pilot strip from the chromatogram and spraying it with dye according to the method of KUNKEL AND TISELIUS[6]. Paper strips containing the insulin band were cut from the remainder of the sheet and sewn together with the aid of a sewing machine. A pointed piece of filter paper is sewn onto one end and a paper flap onto the other. The insulin is then eluted with 0.01 *N* HCl by a technique similar to that used for descending paper chromatography. The eluate is permitted to drip into a beaker and is evaporated to dryness using the rotary evaporator. The insulin is again taken up in small volume of 0.01 *N* HCl and rechromatographed using the same solvent, but on a narrower sheet of paper (about 13 cm wide). The development may be stopped after six or seven hours. Spraying a pilot center strip shows the strong insulin band at an R_F of about 0.5, a faint trace of protein at the origin, and occasionally a weak, but distinct band at R_F 0.25. The identity of the latter is being investigated. It is essential to use the Munktell paper in the first chromatography step for clean separation of the insulin. The type of paper used for the second step is not critical.

The insulin is then eluted, precipitated, and washed with 5% trichloroacetic acid and taken to dryness at 35° *in vacuo* over $CaCl_2$. The precipitate is then washed three times with ether and allowed to dry in air. It is then ground in acetone and plated for counting according to the procedure described in a previous section.

The yield of insulin from 20 g of slices varied from 2 to 4 mg in different experiments. It is possible to isolate considerably less insulin than this amount, should it be desired to use less than 20 g of tissue. In fact, in several experiments, less than 100 μg of insulin were isolated from several g of pancreas.

Discussion of individual steps in the isolation procedure

a. *Alcohol-ether precipitation.* Paper chromatography of the supernatant fluid from the alcohol-ether precipitation showed that appreciable amounts of insulin remain unprecipitated when the pH prior to the addition of the two solvents is less than 4.2. At pH 5.3, no insulin whatsoever could be detected in the supernatant fluid.

b. *Dialysis.* Another cause of low insulin yields was traced to the dialysis step. The following results were confirmed using several samples of each size of casing purchased at different times from different sources. When insulin was dialyzed for 16 hours in Visking casing, size 20/32, about 50% disappeared from the inside of the sac and could be quantitatively recovered from the outside solution; under certain conditions, the loss of insulin was as high as 92%. Collodion sacs, prepared by the method of GUTFREUND[9] could be used but their preparation is extremely tedious. A reinvestigation of Visking casing showed that almost quantitative recovery of insulin could be achieved if size 24/32 or 28/32 casing was used. It is possible that the evident decrease in pore size of the larger diameter casing is somehow connected with its greater wall thickness.

c. *Preparation of the chromatography paper.* In attempting to establish the purity of the insulin isolated by means of paper chromatography, it was noticed that the total weight of the dried insulin sample after elution from unwashed Munktell paper did not correspond to the amount of protein present, as assayed by the method of LOWRY *et al.*[8]. Further investigation showed that material of amino acid and peptide nature originating in the paper caused large positive errors in the Lowry determination. The insulin may be freed of most of this material by prewashing the paper for 48

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hours with 0.01 *N* HCl by a method similar to that of descending paper chromatography. The eluted insulin then gives a protein assay which is too low for its weight. Control experiments still indicated contamination by material present in the paper, probably peptide in nature. The material gives a negative protein test when measured by the Lowry method, but upon acid hydrolysis and removal of ammonia, gives a very strong ninhydrin test. The results of attempts to eliminate this material have been only partly successful. Prewashing the paper with 0.01 *N* HCl for 5 days followed by water for one day reduces the amount of material eluted each day to a very low value. However, treating this washed paper with the chromatography solvent (made up with redistilled 2-butanol), drying the paper, and eluting with 0.01 *N* HCl again greatly increases the amount of solid material eluted. Experiments showed that the developing solvent contained essentially no non-volatile material. Prewashing the paper with the developing solvent and then with 0.01 *N* HCl is not effective, nor is washing the paper with stronger HCl. The material is a solid, non-dialyzable under ordinary conditions. It coprecipitates with insulin upon isoelectric or trichloroacetic acid precipitation of the latter although the material itself is soluble under these conditions. Boundary electrophoresis at pH 2.7 failed to separate it appreciably from insulin. Dialysis using the size casing (20/32) which permits insulin diffusion also permits this material to diffuse through the sac. The material from the paper does not interfere with the Lowry determination provided that the paper is washed sufficiently so as to eliminate the material which is Lowry positive. The substance does interfere with radioactivity determinations because its presence makes self absorption corrections difficult but the interference is small. Thus far, all attempts to completely remove the material form the paper or to free the insulin from it have failed. Experiments are now in progress testing the extensive washing procedures of CONNELL *et al.*[15] who encountered a similar difficulty. The amounts of material that contaminate the insulin can, however, be appreciably reduced by cutting the paper close to the insulin band for elution, and eluting with a minimal amount of 0.01 *N* HCl.

After the 6 day period of washing the paper it must be allowed to dry for at least three or four hours in a stream of air or preferably in a warm oven. The R_F of crystalline insulin drops from its normal value of about 0.45–0.50 to an R_F of 0.18 when the filter paper has not been allowed to dry sufficiently. Under these circumstances insulin does not separate from the crude proteins in the first chromatography step.

Paper electrophoresis of insulin: The separation of the hyperglycemic-glycogenolytic factor from insulin

In order to have an additional and independent test for the identification and for the homogeneity of insulin, the method of paper electrophoresis was investigated. Table II gives the results of the electrophoresis of crystalline insulin at various pH values. The most discrete spots were obtained when about 100 volts were used although satisfactory spots were obtained at higher potential differences. For preparative purposes, the 5.8 *N* acetic acid solvent of SLUYTERMAN[17] was used since the acetic acid can conveniently be removed by evaporation. All runs were made at 2°.

After these experiments were completed, some crystalline hyperglycemic-glycogenolytic factor (HGF) was made available to us. It was of interest to determine whether or not this substance was present in the insulin isolated by the paper chro-

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TABLE II

PAPER ELECTROPHORESIS OF CRYSTALLINE INSULIN

Size of insulin sample, 200 μg in 0.01 N HCl spotted on Whatman No. 3 filter paper. Potential difference, 110 V; 3.4 V per cm. The symbol + indicates migration toward the anode. The preparation of the buffers is described by MILLER *et al.*[16].

pH	*Buffer*	*Migration per 15 hours*
		cm from origin
2.0	Glycine-NaCl	—8
2.5	Glycine-NaCl	—7.7
3.0	Glycine-NaCl	—5.4
3.5	Glycine-NaCl	—2.3
7.0	Phosphate-NaCl	+1.5
7.5	Phosphate-NaCl	+3.3
8.0	Veronal-NaCl	+4.3
8.5	Veronal-NaCl	+5.1

matographic method. It was found that crystalline HGF and crystalline insulin were not separable by paper chromatography under the conditions used for insulin isolation. However, a clear separation of this substance could be achieved by paper electrophoresis for 24 hours at 2° on Whatman No. 1 paper using 0.03 M sodium phosphate buffer, pH 7.5 at about 6.7 volts per cm of paper. Under these conditions no movement or diffusion of HGF occurs whereas insulin migrates toward the anode. The position of HGF is ascertained with the same spray used for insulin. It is expected that this method will prove valuable for the detection of small amounts of HGF in crystalline insulin samples.

RESULTS

Properties of the pancreas slice system

Prior to studies on the synthesis of insulin itself, a few general properties of the pancreas slice system were investigated in order to find suitable incubation conditions. It was also of interest to discover whether the behavior of pancreas slices with respect to amino acid incorporation into protein resembled that of other more thoroughly investigated tissues such as liver.

The effect of different media and different weights of tissue on various properties of the pancreas slice were studied. It is evident from Table III that the severe pH drop during incubation makes it inadvisable to use more than a ratio of 100 mg of slices per ml of medium. Even at this ratio, a considerable decrease in pH occurs unless succinate is added to the Krebs-phosphate medium. The addition of both succinate and glucose appears to result in a somewhat higher Q_{O_2} than with succinate alone. Leucine-1-^{14}C incorporation into protein appears to be substantially the same in all the media tried. The decrease in incorporation with an increase in the amount of slices probably results from either isotope dilution or exhaustion of the labeled amino acid.

The results of experiments on the effect of aging of the tissue on leucine-1-^{14}C incorporation (Table IV) indicate the desirability of permitting as short a period of time as possible to elapse between the sacrifice of the animal and the slicing step.

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TABLE III

THE INFLUENCE OF WEIGHT OF SLICES AND COMPOSITION OF MEDIUM ON THE DECREASE IN pH, THE OXYGEN UPTAKE, AND THE EXTENT OF LEUCINE-1-^{14}C INCORPORATION DURING INCUBATION*

Tissue, adult beef pancreas slices. Incubation time, 2 hours at 38°. Gas phase, 100% O_2. Initial pH, 7.40. Counts per ml medium, $3.0 \cdot 10^5$ c.p.m. of DL-leucine-1-^{14}C. Incorporation measured in total protein of the slice.

mg of slices per ml medium	*Medium*											
	Krebs-phosphate			*Krebs-phosphate-glucose*			*Krebs-phosphate-succinate*			*Krebs-phosphate-glucose-succinate*		
	*pH decrease***	*S.A.****	Q_{O_2}	*pH decrease*	*S.A.*	Q_{O_2}	*pH decrease*	*S.A.*	Q_{O_2}	*pH decrease*	*S.A.*	Q_{O_2}
100	0.6	1600		0.7	1585		0.1	1550		0.05	1685	
200	0.6	900		0.7	955		0.5	820		0.5	900	
300	0.6	750	4.1	0.7	800	4.2	0.6	645	5.0	0.6	560	5.8

* Tissues from different animals were used for the 3 different measurements.
** Expressed in pH units.
*** Specific activity, c.p.m. per mg of tissue slice protein.

TABLE IV

EFFECT OF AGING ON THE INCORPORATION OF LEUCINE-1-^{14}C INTO THE PROTEINS OF PANCREAS SLICES

Each flask contained 200 mg wet weight of adult beef pancreas slices, 2.0 ml of Krebs-phosphate-succinate medium, and $6.0 \cdot 10^5$ c.p.m. of DL-leucine-1-^{14}C. Gas phase, 100% O_2. Incubation time: experiment 1, 2 hours; experiment 2, 3 hours. Temperature, 38°.

Conditions	*Specific activity c.p.m. per mg protein*
Control	934
Intact pancreas aged at 38° for 45 minutes before slicing	388
Slices preincubated 20 minutes at 4° in oxygenated medium	812

TABLE V

EFFECT OF 2,4-DINITROPHENOL ON RESPIRATION AND DL-LEUCINE-1-^{14}C INCORPORATION BY BEEF PANCREAS SLICES

Each flask contained 200 mg wet weight slices, 2.0 ml Krebs-phosphate-succinate medium, the appropriate amount of 2,4-dinitrophenol, and $6.0 \cdot 10^5$ c.p.m. of DL-leucine-1-^{14}C, specific activity, $1.0 \cdot 10^9$ c.p.m. per mM. Incubation time, 2 hours at 38°; atmosphere, 100% O_2. Dinitrophenol given as final concentration. Q_{O_2} of control, 3.5. Specific activity of control, 760 c.p.m. per mg protein.

2,4-Dinitrophenol concentration [M]	Q_{O_2} *Per cent of control*	*Specific activity Per cent of control*
None	100	100
$1.0 \cdot 10^{-4}$	120	25
$2.0 \cdot 10^{-4}$	122	6
$4.0 \cdot 10^{-4}$	104	2
$5.0 \cdot 10^{-4}$	97	1

Table V shows that leucine-1-^{14}C incorporation is almost completely inhibited in the absence of oxygen or in the presence of 2,4-dinitrophenol at a concentration which does not inhibit respiration. These results are substantially the same as those obtained with other tissues[18].

Experiments on insulin. Purity of the isolated insulin

Several lines of evidence attest to the degree of purity of the insulin preparation with respect to freedom of contamination by other constituents of the pancreas or by the labeled leucine. In the discussion that follows, the purity is referred to the

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protein content rather than to the total solids of the preparation since in most cases the insulin was contaminated with some material originating in the filter paper.

The insulin travels as a single band or spot (as determined by the bromphenol blue stain[6]) when chromatographed on paper. The potency of the insulin in a test involving 40 mice was, within the experimental error of the method[19], at least equal to that of Eli Lilly crystalline zinc insulin. The potency determination must be considered only approximate, however, since facilities were not available to permit a full scale assay requiring several hundred mice. When subjected to paper electrophoresis at various pH values, including the conditions under which the hyperglycemicglycogenolytic factor is easily detectable (see a previous section), only one spot, corresponding to the position of insulin, was observed.

While the preceding methods would be expected to detect any appreciable amount of contamination by other proteins and might, for ordinary purposes, serve as tentative evidence for protein purity, more sensitive methods are needed to establish the isotopic purity of insulin. For example, an extremely small amount of a protein of very high specific radioactivity might be present in the insulin preparation, and although separating from the insulin during paper chromatography or electrophoresis, might be present in too small an amount to be detectable by the bromphenol blue staining procedure. Further, some proteins and polypeptides possessing few basic groups might not be stained by this reagent. The A chain of insulin is an example of this*. Accordingly, the more sensitive method of purification to constant specific activity was adopted. A sample of insulin isolated by paper chromatography was successively, precipitated at its isoelectric point, rechromatographed, dialyzed in the presence of carrier DL-leucine, and subjected to paper electrophoresis at pH 1.7[17]. The insulin was precipitated with trichloroacetic acid after each step and the specific radioactivity (c.p.m. per mg of protein) was determined.

The results are shown in Table VI. The small change in specific activity after the first chromatography step is not surprising since it is known that the insulin

TABLE VI

THE EFFECT OF PURIFICATION PROCEDURES ON THE SPECIFIC RADIOACTIVITY OF LABELED INSULIN

80 g of calf pancreas slices were incubated in 4 flasks. A total of 800 ml of Krebs-succinate buffer were used containing $1.0 \cdot 10^8$ c.p.m. of DL-leucine-1-^{14}C. Incubation time, 1.5 hours. The specific activity of whole pancreas protein was 490 c.p.m. per mg protein. The specific activity of the insulin fraction was measured after the stated procedure was used.

Procedure	*Specific activity c.p.m. per mg protein*
First paper chromatography	370
Second paper chromatography	402
Dialysis in the presence of unlabeled DL-leucine at pH 2.7 and then at isoelectric point (pH 5.3)	395
Paper electrophoresis at pH 1.7	401

TABLE VII

THE EFFECT OF 2,4-DINITROPHENOL, ANAEROBIOSIS, AND INCUBATION FOR ZERO-TIME, ON THE INCORPORATION OF DL-LEUCINE-1-^{14}C INTO INSULIN

Each flask contained 20 g wet weight of calf pancreas slices in 200 ml of Krebs-succinate buffer containing $1.1 \cdot 10^7$ c.p.m. of DL-leucine-1-^{14}C. Incubation time, 40 minutes.

Conditions	*Specific activity c.p.m. per mg protein*
Control	23
N_2 atmosphere	0.2
2,4-dinitrophenol, $4 \cdot 10^{-4}$ *M*	1
Zero-time incubation	1

* Z. N. CANELLAKIS. Personal communication.

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is contaminated with a trace of protein which remains at the origin in the second chromatogram. However, the specific activity after the second chromatography step remains constant thereafter, throughout all the purification procedures used.

The results in Table VII show that virtually no radioactivity enters the molecule when the incubation is carried out for zero time, or in the presence of 2,4-dinitrophenol or in the absence of oxygen. It may be noted that 2,4-dinitrophenol was used at a concentration at which, in this system, respiration is not inhibited. The lower control value obtained in this experiment as compared with the values given in Table VI may be attributed to the shorter incubation time and to the smaller amount of leucine-1-^{14}C used.

DISCUSSION

The new method described here appears to be suitable for isolating insulin from reasonably small amounts of pancreas in a routine manner. It has proved satisfactory in the hands of several different workers. A good number of samples can be handled simultaneously without much increase in time since all the time-consuming operations (lyophilization, extractions, dialyses, chromatography) can be done in parallel permitting an experiment to be completed in less than a week.

The data presented in this communication suggest that the insulin isolated is free from other constituents of the pancreas or from labelled leucine. However, it cannot be stated that the isolated insulin is completely pure, since it is evident that the filter paper is contributing material to the insulin preparation which is extremely difficult to remove. This problem is receiving attention at present.

The almost complete inhibition of incorporation by anaerobiosis, 2,4-dinitrophenol, or incubation at zero time speaks for the participation of metabolic reactions rather than a simple adsorption process as being responsible for leucine-1-^{14}C incorporation. The latter possibility has been further reduced by the recent work of VAUGHAN AND ANFINSEN[20]. These authors incubated pancreas slices in the presence of labeled glycine and isolated the insulin by standard precipitation procedures using carrier insulin. Following the methods of SANGER[1], the insulin was partially hydrolyzed and a heptapeptide was isolated which was shown to contain labelled glycine.

The virtually complete inhibition of incorporation by anaerobiosis and 2,4-dinitrophenol is indicative of an energy requirement at some step in the incorporation process. Inasmuch as the dinitrophenol is able to inhibit incorporation at levels at which no inhibition of respiration occurs, it would appear that phosphorylation processes are directly or indirectly involved.

The energy requirement involved in leucine incorporation speaks against this process having occurred as a direct result of exchange reactions, for example, reactions similar to transpeptidation. However, the results do not exclude the possibility that energy is required to form an amino acid derivative (leucineamide or leucine peptides for example) which can then take part in non-energy requiring exchange reactions with insulin. The demonstration of net synthesis of insulin in this system and the correlation of the amount of insulin synthesized with the extent of incorporation of radioactivity would aid in the exclusion of these possibilities. Such experiments are now in progress.

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SUMMARY

A paper chromatographic method for the isolation of insulin from calf pancreas slices is presented. The method is reasonably rapid and is particularly adaptable to running multiple samples. If the pancreas slices are incubated in the presence of leucine-1-^{14}C, the insulin becomes labeled. Evidence is presented relating to the purity of the isolated insulin. A method is also presented for the separation of the hyperglycemic-glycogenolytic factor from insulin.

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Addendum: The results of recent experiments indicate that the lack of correspondence between the gravimetric and colorimetric determinations of the isolated insulin results not only from contamination by material in the filter paper but also from the fact that the insulin preparation exists as the trichloroacetate salt. Both the contaminating material and the trichloroacetate ion may be removed by the following procedure which results in an insulin preparation which gives very nearly quantitative values on colorimetric analysis: The trichloroacetic acid precipitated insulin is redissolved in several ml of cold 0.5 *M* $NaHCO_3$, reprecipitated and washed with trichloroacetic acid, suspended in and dialyzed against 0.05 *M* HF and then against water. Because of the low atomic weight of fluorine, any remaining F^- ion bound by the insulin does not appreciably affect its weight.

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