phosphate solvent was first reported by Love (1960). It has also been performed by Brahms (unpublished work). In our hands chromatography from pyrophosphate solution gives results similar to those depicted in Figure 1; however, the ATP ion is to be preferred because pyrophosphate binds tightly to myosin and is a powerful inhibitor of ATPase.

As already implied, the nucleotides contaminating myosin obtained in chromatography from an ATP-solvent were totally eliminated (see Fig. 5) by passing through a DEAE-Sephadex column equilibrated with 0.2 M KCl, 5 mM Tris-HCl, *p*H 8.0 (in which solvent myosin is soluble even in the absence of ATP). After a column's worth of fluid had filtered through and 83% of the nucleotide-free protein had been recovered, the nucleotides were recovered by eluting with alkaline solution.

DISCUSSION

We feel that the experiments described establish a practical method for the chromatographic purification of myosin; the method is based on the Brahms idea that in the presence of a myosin-adsorbed polyanion (*e.g.*, ATP) myosin is solubilized at an ionic strength low enough to permit its adsorption on DEAE-cellulose. The present investigation shows that a standard preparation of myosin is probably a slowly associating system, so that chromatography reveals "monomers" and a distribution of "*n*-mers." It seems to us likely that the "monomers" in solutions of *ca.* 0.2 M KCl described by Brahms (1959) and Perry (1960) simply filtered through the column, whereas a fraction (perhaps the major fraction) of "*n*-mers" remained truly adsorbed until the KCl reached a sufficiently high concentration.[2] If so, the α-component of Brahms (1959) corresponds to the "peak material" in the present work, and the β-component of Brahms to a portion of the "tail material." Thus the combination of filtration and chromatography in the older work may effect approximately the same fractionation as the present true chromatography, but would fail to eliminate "leading" components detected in the present work.

In other ways, the present results do not agree with those of earlier workers. Our "tail material" does not exhibit an enhanced ATPase activity as suggested by Brahms, and nowhere have we found the microheterogeneity reported by Perry. We suggest that the latter might actually arise from errors in estimating the *myosin* concentration because OD_{280} is subject either to scattering errors or to errors arising from contaminating proteins.

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The Use of Gel Filtration in the Isolation and Purification of Beef Insulin

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A method employing gel filtration for the purification of beef insulin is described. A crude extract (prepared by acid-alcohol treatment of pancreatic tissue followed by ether-alcohol precipitation) is subjected to gel filtration on Sephadex G-50, with ammonium bicarbonate as the solvent. The insulin-containing fraction is lyophilized and crystallized twice. The protein which is obtained is free of nonincorporated radioactive amino acid contamination and has the correct amino acid composition. By performic acid oxidation and a simple countercurrent extraction, the A and B chains may be isolated in pure form.

A convenient procedure for the isolation of insulin from small quantities of pancreatic tissue is necessary for *in vitro* studies of insulin biosynthesis. Many methods for insulin purification have been described, nearly all of which rely upon the preparation of a crude extract by acid-alcohol treatment of the tissue followed by ether-alcohol precipitation. Further purification of the extract is then obtained by paper chromatography (Light and Simpson, 1956; Grodsky and Tarver, 1956; Fenton, 1959; Taylor *et al.*, 1961, 1962), immunochemical precipitation (Taylor *et al.*, 1962), fibril formation and crystallization (Pettinga, 1958; Voelker *et al.*, 1962), or column chromatography with urea-

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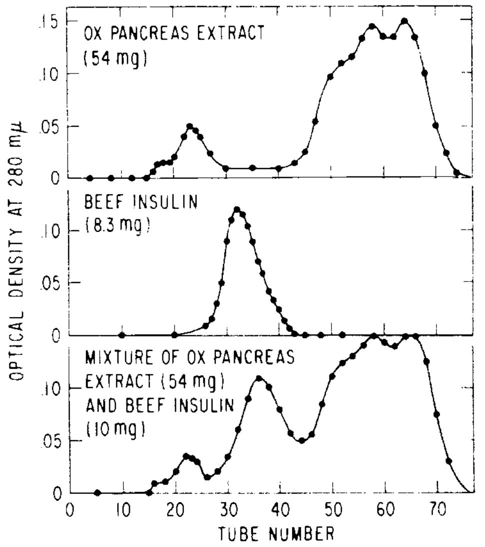

FIG. 1.—Gel filtration patterns of ox pancreas extract (*top*), crystalline beef insulin (*middle*), and a mixture of the two (*bottom*). Each run was on a Sephadex G-50 column with 0.2 M NH_4HCO_3 as the buffer.

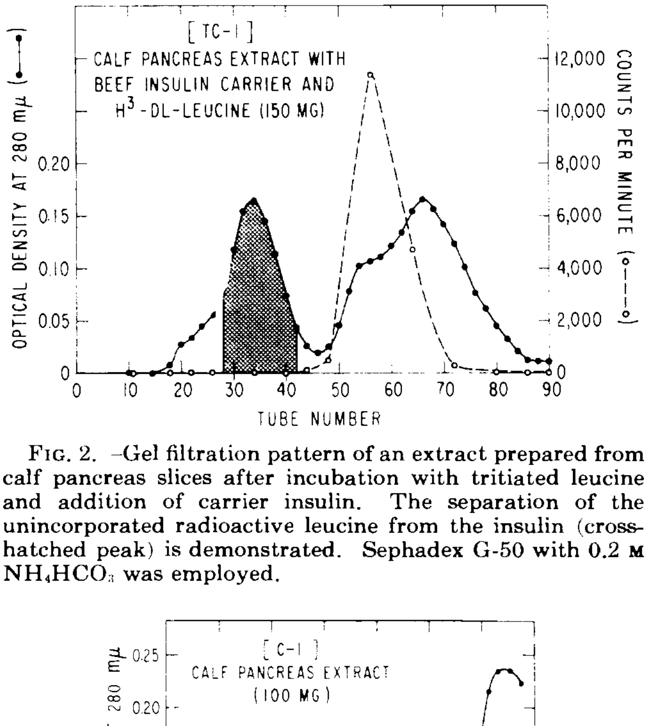

FIG. 2.—Gel filtration pattern of an extract prepared from calf pancreas slices after incubation with tritiated leucine and addition of carrier insulin. The separation of the unincorporated radioactive leucine from the insulin (cross-hatched peak) is demonstrated. Sephadex G-50 with 0.2 M NH_4HCO_3 was employed.

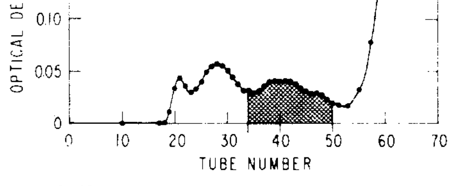

FIG. 3.—Gel filtration of calf pancreas extract on Sephadex G-50 with 0.2 M NH_4HCO_3. Crystalline insulin was prepared from the fractions represented by the cross-hatched area.

containing buffers (Mendiola and Cole, 1960). Other purification procedures have employed multiple precipitations and crystallization (Romans *et al.*, 1940), or countercurrent distribution (Harfenist and Craig, 1952).

All of the methods mentioned involve many, often complicated, operations. However, it appeared likely that several steps could be avoided by the use of dextran gel filtration, an approach which was chosen because it was hoped that the reversible aggregation of insulin to form polymers ranging from dimers to hexamers (Waugh, 1954) might allow for the selection of appropriate conditions for the separation of insulin from other components. By a combination of crude extraction, gel filtration, and crystallization, it was possible to obtain crystalline insulin from as little as 10–20 g of bovine pancreatic tissue.

EXPERIMENTAL PROCEDURE AND RESULTS

1. Preparation of Crude Extract.—The starting materials were either 5–10 g (wet weight) of calf pancreas slices which had been incubated for various periods of time in Krebs phosphate IIA buffer (Krebs, 1950), or fresh frozen whole ox or calf pancreases. The crude extract was prepared by the method of Pettinga (1958), with sulfuric acid–ethanol extraction followed by ethanol-ether precipitation. Approximately 1.5–5 g of air-dried extract per 100 g of pancreas were obtained, the amount depending on the state of the pancreas (*i.e.*, the amount of mincing, the length of incubation, the source of the pancreas, *etc.*) before extraction.

2. Gel Filtration of Crude Extract.—A 2.2 × 85 cm column of medium Sephadex G-50 (Pharmacia, Uppsala) was prepared and equilibrated at 4° with 0.2 M ammonium bicarbonate (*p*H 7.8). Crude extract (50–150 mg) was dissolved in 4 ml of NH_4HCO_3 solution, and a small amount of insoluble material was removed by centrifugation. The supernatant fluid was applied to the Sephadex column and eluted with NH_4HCO_3 solution. Fractions of 3–5 ml (10 minutes per fraction) were collected and the optical density was read at 280 mμ in a Beckman DU spectrophotometer. A representative elution pattern of an ox pancreas extract prepared without any carrier insulin is shown in Figure 1, *top*. The bulk of the material in the extract was found in two multicomponent peaks: a smaller leading one and a larger trailing peak, which were separated by a low "plateau." When crystalline insulin was run under identical conditions, the pattern shown in Figure 1, *middle*, was obtained. It is apparent that the insulin was eluted in the region of the plateau. The elution pattern of an artificial mixture of the ox pancreas extract and crystalline insulin, shown in Figure 1, *bottom*, verified this conclusion. The pattern obtained from a calf slice extract with added carrier (after incubation with tritiated leucine) is shown in Figure 2. In this case there is a large, almost symmetrical peak which corresponds to the insulin. A representative pattern from calf pancreas extract without added carrier is shown in Figure 3.

Figure 2 also presents the results of scintillation counting of the column fractions. Aliquots of 0.5 ml of several fractions were each added to 15 ml Bray's solution and the radioactivity determined in a Nuclear-Chicago liquid scintillation counter. As is clear from the figure, virtually all radioactivity (free H^3 leucine) appears in a peak following the insulin peak.

The column fractions indicated by cross-hatching in Figures 2 and 3 were pooled and lyophilized (to remove water and the volatile NH_4HCO_3), and this material was used for the crystallization of insulin.

3. Crystallization of Insulin.—Crystallizations were carried out by the method of Romans *et al.* (1940) as modified by Voelker *et al.* (1962). The lyophilized material was dissolved in 0.5 M acetic acid and a small amount of insoluble material was removed. Zinc

Table I

Amino Acid Composition of Insulin and Insulin Chain Preparations from Calf Pancreas Extracts

	Composition of Insulin						Composition of A Chain			Composition of B Chain		
		Observed[a]						Observed			Observed	
	Theoretical	XC-1/1 x 2[b] 2× Crystallized	XC-1/3S Sephadex Fraction	XC-1/3 x 1 1× Crystallized	XC-1/3 x 2 2× Crystallized	C-1/x 2 2× Crystallized, No Carrier	Theoretical	LC-1A	TC-1A	Theoretical	LC-1B	TC-1B
Lysine	1	—	—	1.3	—	—	0	0	—	1	0.9	—
Histidine	2	—	—	2.4	—	—	0	0	—	2	1.6	—
Arginine	1	—	—	1.2	—	—	0	0	—	1	0.9	—
Cysteic acid	0	0	0	0	0	0	4	4.0	3.5	2	1.9	1.6
Aspartic acid[a]	3	3.00	3.00	3.00	3.00	3.00	2	2.00	2.00	1	1.00	1.00
Threonine	1	1.0	1.0	0.8	1.0	1.0	0	0	0	1	0.9	1.0
Serine	3	2.8	2.8	2.4	2.6	2.7	2	1.9	2.0	1	1.0	1.1
Glutamic acid	7	7.0	6.9	6.9	7.0	7.0	4	4.0	4.2	3	3.2	2.9
Proline	1	0.8	2.1	1.5	1.3	1.1	0	0	0	1	1.3	1.3
Glycine	4	4.3	3.9	4.0	3.9	3.5	1	1.2	1.3	3	3.0	2.9
Alanine	3	3.1	2.9	3.0	2.9	2.9	1	1.0	1.2	2	1.9	2.0
Half-cystine	6	5.5	10	—	6.2	5.0	0	0	0	0	0	0
Valine	5	3.7	6.2	3.2	3.4	3.5	2	(1–2)	1.6	3	2.6	2.5
Methionine	0	0	0.2	0	0	0	0	0	0	0	0	0
Isoleucine	1	0.5	1.9	0.5	0.5	0.4	1	0.5	0.5	0	0	0
Leucine	6	6.1	6.7	6.9	6.4	6.0	2	1.9	2.2	4	4.1	3.3
Tyrosine	4	4.3	3.9	4.3	4.1	3.5	2	2.4	1.7	2	2.3	1.2
Phenylalanine	3	3.1	3.0	3.3	3.1	2.8	0	0	0	3	2.7	2.7

[a] Calculated by setting the amount of aspartic acid equal to 3 moles/mole insulin, 2 moles/mole A chain, 1 mole/mole B chain. [b] All samples labelled XC, LC, and TC were prepared with insulin carrier.

acetate (0.2 ml of 0.9 mg/ml solution) was added and the *p*H adjusted to 5.9; the final volume was about 4 ml. The crystals obtained after 24–48 hours were separated by centrifugation, and a second crystallization was carried out. The final crystals were dried with absolute ethanol and ether.

When carrier insulin was added at the beginning of the extraction, approximately 60% was recovered after the second crystallization. When the insulin was extracted from whole calf pancreas, approximately 5.5 mg/100 g pancreas was obtained.

4. *Amino Acid Analyses of Insulin Preparations.*—Samples of the various insulin preparations were subjected to hydrolysis, *in vacuo*, in 6 N HCl for 22 hours at 108°, and the hydrolysates were analyzed on the Beckman/Spinco automatic amino acid analyzer. The relative amounts of the various amino acids were calculated in relationship to the aspartic acid content, assuming the latter to be equivalent to 3 moles aspartic acid per mole of protein (Sanger *et al.*, 1955). The results are presented in Table I. All recrystallized samples are characterized by low values for valine and isoleucine (as would be expected with only 24 hours' hydrolysis) but otherwise agree extremely well with published analyses for insulin (Sanger *et al.*, 1955).

6. *Assays of Biological Activity of Insulin Samples.*—Bioassays, by both a mouse convulsion test (40 mice per assay) and by immunoassay, of various samples were carried out by the Eli Lilly Company. The results are shown in Table II.

7. In two cases, recrystallized insulin samples (LC-1 and TC-1) were subjected to performic acid oxidation (Craig *et al.*, 1961) and the A and B chains were separated by countercurrent extraction by a modification of the method of Craig *et al.* Only ten transfers with 5 ml in each phase were employed, and the tubes containing fractions A (tubes 0–3) and B (tubes 4–10) were pooled and lyophilized (after removal of volatile solvents in a rotary evaporator). Paper electrophoresis of each fraction was carried out at *p*H 8.6 in barbital buffer (Voelker *et al.*, 1962), and each fraction was found to be homogeneous and to have the same mobility as purified insulin chains obtained from the Eli Lilly Company (oxidized beef insulin A chain, lot #236-415B-281-1; oxidized B chain, lot #236-415B-287-3). There was no evidence of contamination of one chain by the other. Furthermore, a sample of each fraction was hydrolyzed and amino acid analyses and, in the case of TC-1, scintillation counting were carried out on the hydrolysates. No radioactivity was detectable in either chain, indicating that all free radioactive leucine had been removed by the purification procedure. The results of the amino acid analyses are presented in Table I.

Discussion

By a combination of crude extraction, gel filtration, and two crystallizations, it is possible to obtain crystalline insulin from ox or calf pancreatic tissue. The two chains may then be isolated by performic acid oxidation and a simple ten-transfer countercurrent extraction.

The yield of only approximately 60% obtained after the addition of a large amount of carrier is primarily

Table II

Bioassay of Insulin Preparations from Calf Pancreas Extracts

Preparation	Mouse Assay Units/mg	Immunoassay Units/mg
XC-1/C (crude extract)	1.9	0.9
XC-1/1S (Sephadex fraction)	19.0	8.7
XC-1/x 2 (2 × crystallized)	17.0	12.1
Lilly crystalline insulin	19.6	16.0

the result of the way in which the Sephadex fraction is obtained. In this step, a significant amount of the leading part of the insulin peak is rejected in order to minimize contamination by the front-running peak of the crude extract. Small additional losses are also to be expected in the crystallizations. Gel filtration is quite effective in removing small molecules such as labeled amino acids. This is especially useful when the incorporation of radioactivity into insulin is being measured.

By both amino acid analysis and bioassay, it is apparent that the insulin-containing "Sephadex fractions," although free of most contaminating proteins, are still far from pure. However, after two crystallizations a protein with the correct amino acid composition is obtained. Although there is a discrepancy between the two methods of bioassay employed, it appears possible (on the basis of the immunoassay) that the recrystallized insulin still contains a small amount of nonprotein material (possibly water).[1]

During the course of this work there was a report by Davoren (1962) on the isolation of insulin from a single cat pancreas. His procedure consisted of (1) ethanol-HCl extraction; (2) precipitation of inactive material at *p*H 8.2; (3) precipitation of the crude extract with ethanol and ether; (4) reprecipitation with NaCl; (5) gel filtration on Sephadex G-50 in 1.0 M acetic acid; (6) paper chromatography; and (7) elution of the insulin from the paper. In the gel filtration in acetic acid, the insulin-containing material ran in a trailing peak rather than in a middle fraction as with NH_4HCO_3. Steps (1), (2), and (3) of the Davoren procedure are analogous to steps in the currently described method.

The existence of two quite different methods for use of Sephadex G-50 in the purification of insulin is evidence for the versatility with which gel filtration may be employed with a protein that is capable of undergoing reversible aggregation and disaggregation. It seems reasonable to suppose that similar methods will prove applicable to other proteins which exhibit similar behavior.

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[1] This discrepancy between the assays is not considered to be of great significance since the mouse assay is considered accurate to only about ±40% (Behrens, personal communication, and Irwin, 1943).