Decomposition of *l*-α-Phenylethyl-*t*-butyl Peroxide in Thiophenol.—A 500-ml. flask was fitted with a reflux condenser, a gas inlet tube and an outlet leading to two Dry Ice traps backed by a mercury safety valve. The flask, charged with 19.4 g. (0.1 mole) of *l*-α-phenylethyl-*t*-butyl peroxide (n^{20}D 1.4809; α^{24}D −51.0°, *l* 1 dm.) and 381 g. (3.46 moles) of thiophenol [b.p. 58.5–60° (14 mm.), n^{20}D 1.5898] was heated for ten hours at 125 ± 2° while maintaining a nitrogen atmosphere. At the end of this time the Dry Ice traps were empty.

The cold product was poured into 20% aqueous sodium hydroxide solution (*ca.* 4.5 moles) and the mixture was thoroughly extracted with ether. The ether solution was washed with dilute alkali (*ca.* 10%) and then thoroughly with water. After drying over anhydrous magnesium sulfate the ether was removed and the remainder distilled at 2.7 mm. Three fractions weighing 0.98 g., 5.70 g. and 2.39 g. were collected; n^{20}D 1.5280, 1.5294 and 1.5301, respectively. The middle fraction was largely *l*-α-phenylethanol; it had α_D^{25} −10.6° (*l* 1 dm.) and immediately gave a positive test for acetophenone.[30]

The residue from this distillation (17.4 g.) was recrystallized once from 95% ethanol to give 14.3 g. of diphenyl disulfide, m.p. 59–60° and mixed m.p. 59–60° (lit. value 60–61°[34]).

l-α-Phenylethanol was isolated as the acid phthalate ester from fractions 1, 2 and 3. These were combined and mixed with 11.9 g. (0.08 mole) of finely powdered phthalic anhydride and 7.1 g. (0.09 mole) of anhydrous pyridine. The mixture was heated at 75 ± 2° for three hours with occasional shaking. On cooling it was poured into dilute hydrochloric acid layered with ether. The aqueous phase was thoroughly extracted with ether and the combined extracts were washed with water. Following this, the ether solution was carefully extracted with aqueous sodium bicarbonate, dried over sodium sulfate and then used for the isolation of acetophenone (see below).

The bicarbonate solution was acidified and extracted with chloroform. The chloroform solution was dried and then the chloroform was distilled off, the final traces being removed *in vacuo*. The hydrogen phthalate ester was dried to constant weight in a vacuum desiccator, 14.76 g. (55% yield); m.p., begins to shrink *ca.* 82° and melts 101–107.5°. Racemic α-phenylethyl hydrogen phthalate melts at 108° whereas the enantiomorphs melt at 86°.[13]

The acid phthalate thus isolated had $[\alpha]^{23}$D −18.5° in carbon disulfide (*c* 1.70, *l* 0.5 dm.) and $[\alpha]^{23}$D +9.8° in absolute ethanol (*c* 4.02, *l* 2 dm.). The *dl*-acid phthalate does not dissolve completely in carbon disulfide; it was necessary, therefore, to use a large half-angle shadow to get a reading on the somewhat turbid solution. Downer and Kenyon[13] report $[\alpha]$D −65.8° in carbon disulfide; $[\alpha]$D +36.5° in ethanol at "room temperature."

The dry ether solution (see above) gave the 2,4-dinitrophenylhydrazone of acetophenone, m.p.[25] and mixed m.p. 248–249°; yield 2.66 g. (0.009 mole). The rather unlikely possibility that this acetophenone is produced by the action of diphenyl disulfide on α-phenylethanol was not investigated. It is noteworthy that even in the absence of any additive acetophenone is produced (see below).

In a separate experiment it was shown that *t*-butyl alcohol is also formed, n^{20}D 1.3855, 3,5-dinitrobenzoate m.p., 141–142°; lit. values: n^{20}D 1.3878[35]; m.p. of dinitrobenzoate 142°.[36]

That α-phenylethanol is not derived from the reduction of acetophenone by thiophenol is shown by the following experiment. A solution of 6 g. (0.05 mole) of acetophenone and 11 g. (0.1 mole) of thiophenol was held at 125 ± 2° for 12 hours in a nitrogen atmosphere. A 98% recovery of acetophenone (as the 2,4-dinitrophenylhydrazone, m.p. and mixed m.p. 246–247°)[25] was obtained.

Decomposition of α-Phenylethyl-*t*-butyl Peroxide in the Absence of Any Additive.—α-Phenylethyl-*t*-butyl peroxide (n^{20}D 1.4807), 1.34 g. (0.007 mole) was heated in a Pyrex flask for five hours at 125 ± 3°. The product was an opaque yellow-brown liquid having a pronounced odor of acetophenone. It was dissolved in petroleum ether (b.p. 35–37°) and washed with water. After removal of the petroleum ether the residual liquid was converted to the 2,4-dinitrophenylhydrazone; yield 1.02 g. (49%), m.p. 244–245°; m.p. of a mixture with an authentic sample of acetophenone 2,4-dinitrophenylhydrazone 245–246°.

The aqueous wash was saturated with potassium carbonate whereupon a few drops of a second phase appeared; this gave a positive ceric nitrate test.

Acknowledgment.—It is a pleasure to record our indebtedness to Messrs. S. L. Clark and K. L. Nelson of this Department for their assistance.

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(35) H. S. Davis and W. J. Murray, *Ind. Eng. Chem.*, **18**, 844 (1926).

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WEST LAFAYETTE, INDIANA

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Countercurrent Distribution Studies with Insulin

BY ELIZABETH J. HARFENIST AND LYMAN C. CRAIG

RECEIVED JANUARY 25, 1952

A number of systems have been developed which permit the protein insulin to behave almost as an ideal solute as far as countercurrent distribution is concerned. The patterns obtained with different insulins have indicated a major component and varying amounts of minor components, some of which are biologically active. The major components of insulins from different species of animals appear to be identical as far as countercurrent distribution in its present stage of development is able to distinguish.

The recent technical improvements in countercurrent distribution both of a mechanical nature[1] and from the standpoint of the development of systems are now such as to warrant a serious attempt to apply the method to the most difficult problems of biochemistry. Certainly the purification and characterization of proteins fall within this category. An attempt is therefore being made to study proteins along the lines which have yielded valuable information with the higher polypeptides.

Insulin is a well known protein of considerable stability which is soluble in dilute alcohol solutions. It has been the subject of many investigations and often has served as a type substance for protein study. Certain preparations have passed reasonably well the criteria of purity given by electrophoresis, the ultracentrifuge and the solubility method.[2,3] Because of this background and the availability of samples on which such studies al-

(1) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

(2) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(3) (a) B. Sjögren and T. Svedberg, *ibid.*, **53**, 2657 (1931); (b) J. L. Hall, *J. Biol. Chem.*, **139**, 175 (1941); (c) H. Gutfreund and A. G. Ogston, *Biochem. J.*, **40**, 432 (1946); (d) H. Gutfreund, *ibid.*, **42**, 544 (1948).

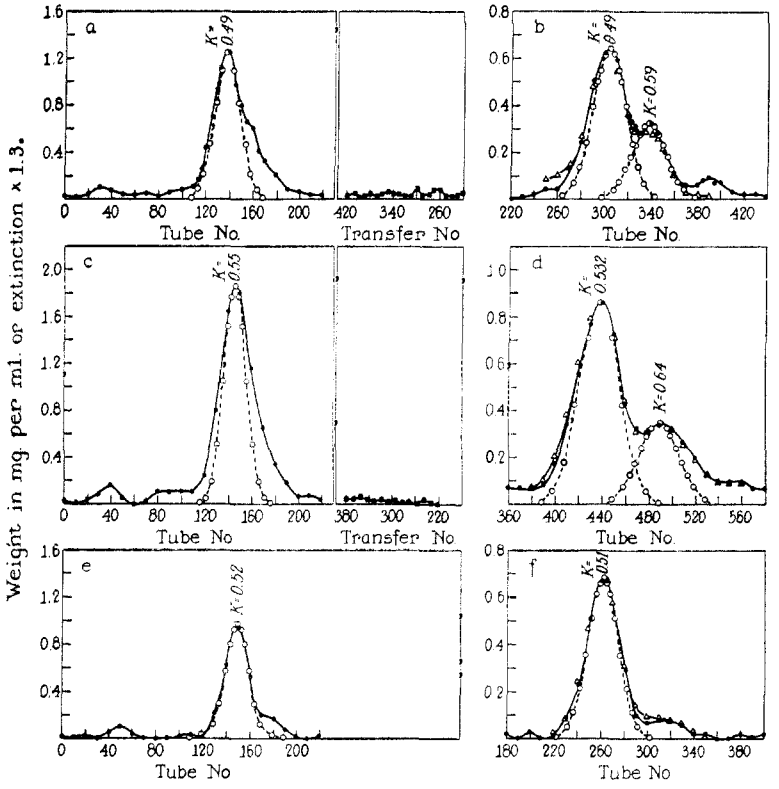

Fig. 1.—Distribution patterns with crystalline Lilly insulin: a, 500 mg. after 424 transfers; b, after 909 transfers; c, one gram after 383 transfers; d, after 1253 transfers; e, 422 mg. of component A after 424 transfers; f, after 770 transfers: ●, weight, lower layer; ■, weight, upper layer; O, theoretical; Δ, extinction × 1.3 at 277 mμ, lower layer.

ready had been made, insulin appeared to be the protein of choice for the initial studies with countercurrent distribution.

It was soon discovered that a partition ratio within a practical range could not be reached with simple systems such as 2-butanol/aqueous acetic or hydrochloric acid since the partition favored the aqueous phase almost exclusively. However, partition ratios within a suitable range could be reached by addition of salt to such systems. Here the salt remained almost entirely in the lower phase. A number of complexing agents also were found capable of increasing the partition toward the alcohol phase. Of these the chloroacetic acids offered the most promise from the standpoint of isolation work subsequent to the distribution. A considerable number of experiments finally led to a choice of the system 2-butanol/1% aqueous dichloroacetic acid (DCA) as the most suitable system.

Experimental

The insulin used for these investigations was obtained from a variety of sources. We are greatly indebted to the manufacturers named below for generous gifts of their valuable material.

The preparations studied most were the following: (1) Beef insulin (Lot T-2344), 5 times recrystallized, obtained from the Eli Lilly Co., Indianapolis: N, 15.52 (Dumas), 15.63 (Kjeldahl); biological activity, 27 u./mg. (2) Amorphous pork insulin (Lot 200-1B-17) obtained from the Eli Lilly Co.: N, 15.18; biological activity, 21 u./mg. (3) Ox insulin (Lot 9011 G), 6 times recrystallized, obtained from the Boots Pure Drug Co., Ltd., Nottingham, England: N, 15.50; biological activity, 22 u./mg. (4) Crystalline sheep insulin (Log PPC 3091), obtained from the Boots Pure Drug Co.: N, 15.81; biological activity, 19 u./mg. (5) Pork insulin, 5 times recrystallized, obtained from Nordisk Insulinlaboratorium, Copenhagen, Denmark: N, 15.56; biological activity, 28 u./mg.

The countercurrent distribution apparatus used for these studies was the 220 tube automatically operated train recently described.[1] All distributions were made in a constant temperature room held at 24°. Weight analyses were made by the technique recently described,[4] except that a longer drying period, 15 minutes on the evaporator at 100° and 20 minutes *in vacuo* (0.2 mm.) at 100°, was used. The residues still contained approximately 23% of solvent from the system, most of which was DCA. All distributions reported in this paper were made as nearly alike as possible. The system used was 2-butanol/1% aqueous DCA in which most of the DCA partitioned into the alcohol phase and the *p*H of the aqueous phase was found with the glass electrode to be 2.7. The patterns shown in Fig. 1 are representative results, and were obtained with Lilly beef insulin (Lot T-2344).

In an experiment in which 0.5 g. of the insulin was scattered initially in 5 tubes, pattern 1a was obtained at 424 transfers. The small amount of material contained in tubes 0 to 110 was withdrawn and the tubes were filled with fresh phases. The apparatus was then set for recycling[1] and permitted to run until 909 transfers had been reached, approximately 1 minute and 35 seconds being required for the phases to separate after equilibration. Analysis of the distribution at this point gave pattern 1b. In addition to weight analysis, a pattern by absorption at a wave length of 277 mμ in the Beckman quartz spectrophotometer was obtained. Two major partly overlapping components are indicated. These could be more completely separated by continuing the distribution further, but if this were done without using a higher charge initially, the precision of the weight analysis would suffer because of dilution of the solutions. The insulin was therefore recovered in two fractions, tubes 280–310 and 330–370.

For recovery, both cuts were treated with ethyl ether to transfer all the protein to the aqueous phase. The upper phases were discarded. The lower phases were then extracted twice with ether to remove most of the DCA and were concentrated under reduced pressure at 10°[5] to appropriate volumes for crystallization (about 50 ml. per 100 mg. of protein). The insulin was then crystallized following the directions of Scott.[6] On standing overnight at 6°, both fractions gave identical, beautifully formed crystals. The fraction from component A, the larger peak of Fig. 1b, gave 161 mg. of crystals while the other, component B, gave 81 mg. The biological activities of the two components were nearly the same, 22 and 26 units per mg., respectively, for A and B. Elementary analysis revealed no differences. A: C, 53.23; H, 6.57; N, 15.32. B: C, 53.40; H, 6.56; N, 15.36.

In order to confirm the presence of two main components and learn the extent of the influence of concentration effects the experiment was repeated using twice the charge (1 g.) of insulin scattered in 7 tubes. This gave pattern c of Fig. 1 at 383 transfers and pattern d after 1253 transfers. The crystalline insulins from two cuts (A, tubes 410–460 and B, tubes 480–530) were recovered as given above.

The crystalline material (422 mg.) from component A was redistributed in exactly the same way. At 424 transfers pattern e of Fig. 1 was obtained, and at 770 transfers

(4) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1326 (1951).

(5) L. C. Craig, J. D. Gregory and W. Hausmann, *ibid.*, **22**, 1426 (1950).

(6) D. A. Scott, *Biochem. J.*, **28**, 1592 (1934).

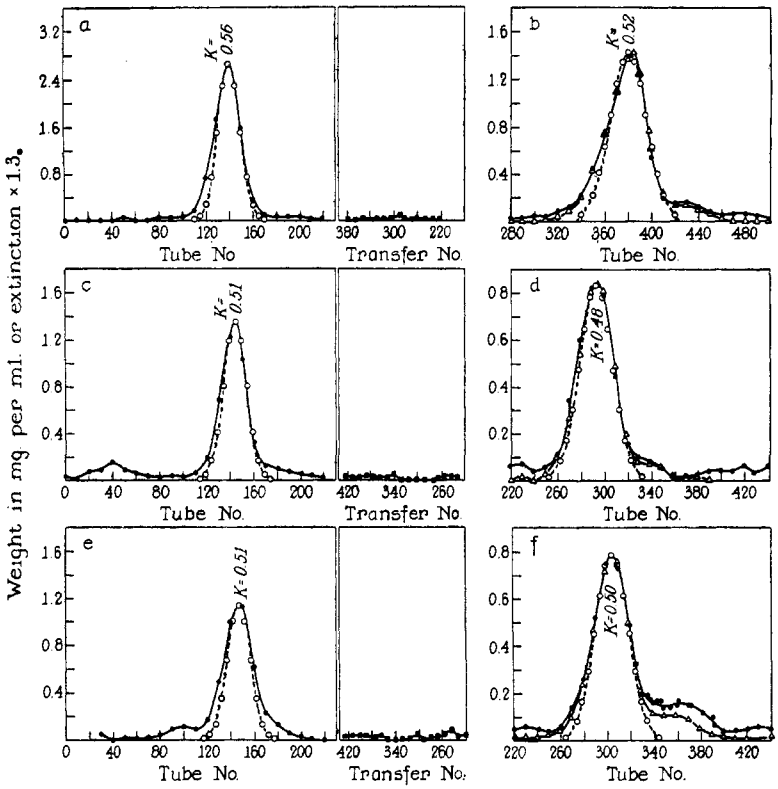

Fig. 2.—Distribution patterns of crystalline insulins from different species: a, one gram Boots ox insulin after 380 transfers; b, after 1118 transfers: c, 500 mg. Danish pork insulin after 425 transfers; d, after 900 transfers; e, 500 mg. Boots sheep insulin after 425 transfers; f, after 901 transfers: the meaning of the symbols is the same as in Fig. 1.

pattern 1f was obtained by the recycling procedure. Crystalline material was recovered from the now much smaller B component as well as from the A component. The biological activities were 23 and 22 units per mg., respectively, for A and B.

Distribution of Boots Ox Insulin.—A 1-g. sample of 6 times recrystallized insulin (Lot 9011 G) was distributed. At first, difficulty with emulsions was encountered and a settling time of 20 minutes was required. However, after 91 transfers analysis showed the emulsifying agent to be separated from the main band and it (total weight 30 mg.) was removed. The distribution then continued normally. At 380 transfers pattern a of Fig. 2 was obtained. The recycling procedure gave pattern b at 1118 transfers. Three cuts, tubes 320–350, tubes 367–404 and tubes 413–446, were taken for recovery of the insulin. Activities were 20, 21 and 22 units per mg., respectively.

Danish Pork Insulin.—A 500-mg. sample of Danish pork insulin was distributed. At 425 transfers pattern c of Fig. 2 was obtained. Recycling of the main band gave pattern d of Fig. 2 after 900 transfers.

Boots Sheep Insulin.—A 500-mg. sample of Boots sheep insulin (Lot PPC 3091) was distributed. At 425 transfers pattern e of Fig. 2 was obtained and by recycling the material in tubes 115 to 220, pattern f was obtained at 901 transfers.

Lilly Amorphous Pork Insulin.—A 500-mg. sample of amorphous pork insulin (Lot 200-1B-17) which had not been subjected to crystallization was distributed. It had been purified to the point of crystallization in the routine process of isolation from the glands. At 411 transfers pattern a of Fig. 3 was obtained. On recycling the material in tubes 110–220 to 900 transfers pattern b was obtained. Crystalline material was recovered from components A and B in the usual way.

Discussion

In considering the possibility of the purification and characterization by countercurrent distribution of such a large molecule as insulin, the primary question to be answered is whether or not a system can be found which will give a favorable partition without irreversibly changing the molecule in some way. Secondly, will the substance behave sufficiently like an ideal solute (in the sense of counter-current distribution) so that a distribution approaching the theoretical can be obtained. Thirdly, will a molecule of such size equilibrate and satisfy a true partition ratio (K) with sufficient rapidity so that each transfer will not require excessive time. Finally, can the experimental details of analysis and recovery of unchanged solute be accomplished. These questions have now all been answered in the affirmative by the experimental work reported in this paper.

In the initial stages of this problem it was found that simple systems such as 2-butanol/aqueous hydrochloric or acetic acid did not give satisfactory K's. The next step was the addition of salt to the system in the hope of salting out the protein from the aqueous phase and thereby gaining a partition more in favor of the alcohol phase. A system made by equilibrating a solution of 1 volume of n-propanol and 3 volumes of 2-butanol with an aqueous solution containing 20% ammonium acetate and 15% acetic acid gave a K of 0.8. The pH of this system was 5.65. A preliminary distribution of 108 transfers indicated behavior approximating the ideal, but technical difficulty in weight analysis was encountered. A second system was made by equilibrating n-propanol–n-butanol (3:1) with a solution containing 10% of ammonium

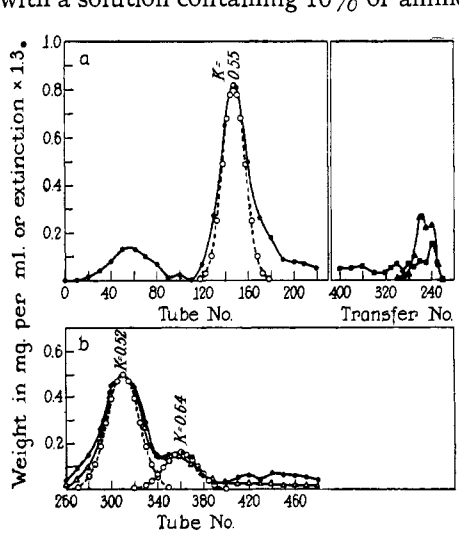

Fig. 3.—Distribution patterns of amorphous Lilly insulin: a, 500 mg. after 411 transfers; b, after 900 transfers: the meaning of the symbols is the same as in Fig. 1: ▲, extinction × 1.3 at 277 mμ, upper layer.

sulfate in 0.05 *N* hydrochloric acid. The *p*H of this system was 2.5. In spite of the high salt content and the low *p*H, insulin gave emulsion difficulties in this system. A distribution of 108 transfers presented a pattern similar to the above system. Here the weight analysis could be made on the upper phases only.

In investigating the effect of trichloroacetic acid on the system 2-butanol/water, it was quickly found that a small concentration of this acid greatly decreased the solubility of insulin in the aqueous phase, at the same time greatly increasing the partition ratio. Trichloroacetic acid itself strongly favors the alcohol phase in this system. The value of the partition ratio of insulin proved to be proportional to the concentration of trichloroacetic acid and by plotting acid concentration against *K*, any desired value of partition ratio could be reached quickly. The system 2-butanol/6.25% aqueous trichloroacetic acid gave a partition ratio of 0.74. In a distribution of 108 transfers in this system a pattern similar to the two mentioned above was obtained. The system was satisfactory from nearly every technical standpoint except capacity and some evidence of transformation. The solubility of insulin in the system was limited.

DCA was found to have the same effect at higher acid concentrations, but here the solubility of the insulin was more favorable and little or no evidence of transformation was seen. The system 2-butanol/1% aqueous DCA, in which the *K* was found to be 0.5, therefore became the system of choice. When the rate of approach to equilibrium was studied by the method previously described,[7] it was found that in this system essential equilibrium could be reached in four or five tips of the tube. Surprisingly enough, little more time was required than for small solutes such as acetic acid. Undoubtedly a difference would be noted if a more precise method were at hand for measuring rates, but such a point is of no practical significance to the present work.

The result of a 909 transfer distribution, Fig. 1, patterns a and b, came as a surprise since this particular preparation had not shown heterogeneity by the solubility method, electrophoresis or the ultracentrifuge.[2] The possibility of transformation in the system was therefore suspected. However, it was found that the insulin from either peak could be recovered easily in crystalline form in a high yield approximately that for the recrystallization of the original. Moreover, both fractions were highly active biologically. Analytically, the two fractions were indistinguishable. When partition ratios of the two crystalline fractions were determined in the DCA system, they were found to be different from each other and in agreement with the *K*'s calculated from the positions of the peaks in the pattern, 0.49 and 0.59. This result indicated a true separation.

The experiment was repeated at double the concentration level. Patterns c and d of Fig. 1 confirm the results of the first experiment. There is evidence for a degree of association of the two bands which results in a somewhat poorer separation than would be expected from the difference in partition ratios. A cut from the main band, tubes 410–460, was recovered in crystalline form for a redistribution experiment.

The patterns obtained on redistributing this material, e and f of Fig. 1, still showed the presence of a small amount of the B component, a result not surprising in view of the width of the cut and the evidence for association of the two bands in the first distribution. Of more importance is the closeness of approach of the main component to the theoretical curve after such a degree of fractionation. These experiments indicate that a preparation of insulin is perfectly capable of behaving almost ideally as a discrete single solute in a countercurrent distribution experiment.

It seemed of interest to determine whether other insulin preparations would show a similar behavior. Boots ox insulin gave the patterns a and b shown in Fig. 2. These data are in accord with the results of Fig. 1, but apparently much less of component B is present in the preparation. Other preparations received from the Boots Co. also showed the same smaller percentage of the B component. However, the left limb of the peak in Fig. 2b showed considerably more divergence from the theoretical than in the case of the Lilly preparation. A cut, tubes 320–350, was therefore taken and the protein recovered in crystalline form. This material had essentially as much activity per mg. as did components A and B. It probably represents a third active component which can tentatively be called C.

A cruder preparation of Boots ox insulin, crystallized only once and run at a 2.5-g. level, gave difficulty with emulsions. The first pattern at 389 transfers was obtained satisfactorily notwithstanding, but, on continuing, the insulin began to precipitate as fibrils and the run had to be abandoned. An attempt at the 0.5-g. level ended the same way. This is the only experience of fibril formation encountered thus far. The pattern at 389 transfers indicated very little more inhomogeneity than with the six times recrystallized preparation.

Comparison of crystalline ox, pork and sheep insulins did not reveal striking differences. The major components of all three showed the same partition ratio, and were otherwise indistinguishable. Only differences in the minor components were observed. The B component of the sheep insulin had a lower extinction at 277 mμ. The sheep pattern also showed a small peak where the C component would occur.

A few distributions were also made with insulins which had not been so highly purified as those for which patterns are given here. These showed much more material in regions other than those where the A, B and C components would occur, but the main peaks all had the same partition ratio. Patterns a and b of Fig. 3, are examples of the best amorphous preparation studied. Apparently the first crystallization has the greatest purifying effect and further crystallizations are of little help in eliminating the minor components.

Distribution of highly purified insulin preparations has not resulted in a component of higher

(7) G. T. Barry, Y. Saito and L. C. Craig, *J. Biol. Chem.*, **174**, 209, (1948).

activity than heretofore reported. This is because all the components of such preparations have nearly the same biological activity. There was no indication of loss of total activity during a run, but it has not been feasible to assay quantitatively all the fractions in order to settle this point.

Acknowledgment.—We are greatly indebted to Dr. E. D. Campbell of the Eli Lilly Co., Sir Jack Drummond of the Boots Pure Drug Co., and Dr. K. Linderstrøm-Lang for generous supplies of insulin. All biological assays reported here were made by the companies supplying the insulin. The technical assistance of Miss Dorris McNamara is acknowledged. Microanalyses were made by Mr. D. Rigakos.

NEW YORK 21, N. Y.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Molecular Weight of Insulin

BY ELIZABETH J. HARFENIST AND LYMAN C. CRAIG

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The molecular weight of insulin has been examined using the method of partial substitution by 1-fluoro-2,4-dinitrobenzene, separation of the reaction products and colorimetric analysis of the monosubstituted derivative for the dinitrophenyl group. A molecular weight of 6,500 has been found.

In a previous report[1] it was shown that it is possible to fractionate insulin preparations by countercurrent distribution and thereby obtain a preparation whose behavior approaches that of a single solute partitioning almost ideally. The next step in the characterization of insulin is therefore the determination of the molecular weight.

Molecular weights for insulin determined by several methods have already been published. The earlier work with diffusion and the ultracentrifuge[2] indicated values in the range 36,000 to 48,000, but more recently Gutfreund[3] observed that the sedimentation was less rapid at lower protein concentrations and *p*H values. As a result of osmotic pressure measurements he was able to assign a value of 12,000 for the molecular weight at infinite dilution. This value is in agreement with later studies.[4] The earlier higher values were interpreted as being due to association of monomer units in solution. Even the value of 12,000 has been questioned by Fredericq and Neurath[5] whose results indicated that in the proper environment insulin may be dissociated still further to a species with a molecular weight of about 6,000.

This state of affairs makes another completely independent approach to the question of molecular weight highly desirable. For this reason it appeared of great interest to learn whether the approach suggested by Battersby and Craig[6] for the molecular weight determination of the higher peptides could be applied to a substance of the complexity of insulin. The results of such studies up to the present time are reported in this paper.

(1) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

(2) (a) B. Sjögren and T. Svedberg, *ibid.*, **53**, 2657 (1931); (b) G. L. Miller and K. J. I. Andersson, *J. Biol. Chem.*, **144**, 459 (1942); (c) H. Gutfreund and A. G. Ogston, *Biochem. J.*, **40**, 432 (1946).

(3) (a) H. Gutfreund, *ibid.*, **42**, 156 (1948); (b) H. Gutfreund, *ibid.*, **42**, 544 (1948).

(4) (a) J. L. Oncley, E. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952); (b) F. Tietze and H. Neurath, *J. Biol. Chem.*, **194**, 1 (1952). NOTE ADDED IN PROOF: While this paper was in press, two papers appeared supporting the value of 12,000 as the molecular weight of insulin: H. Gutfreund, *Biochem. J.*, **50**, 564 (1952); P. Doty, M. Gellert and B. Rabinovitch, THIS JOURNAL, **74**, 2065 (1952).

(5) E. Fredericq and H. Neurath, *ibid.*, **72**, 2684 (1950).

(6) A. R. Battersby and L. C. Craig, *ibid.*, **73**, 1887 (1951).

Experimental

The sample of insulin chosen for the initial molecular weight studies was one which was well characterized by countercurrent distribution and known to contain 90% or better of the A component. The method used in the work reported here was partial substitution by the Sanger reagent, 1-fluoro-2,4-dinitrobenzene.[7]

Five hundred milligrams of insulin (Boots Lot 9011 G) was dissolved in 25 ml. of a 1% aqueous solution of sodium bicarbonate at a temperature of 24°. To this was added a solution of 250 mg. of 1-fluoro-2,4-dinitrobenzene (FDNB) in 50 ml. of ethanol. At the same time a control solution, of the same composition except that the insulin was omitted, was made up. The progress of the reaction was followed by measuring the ultraviolet absorption at 350 mμ in a Beckman quartz spectrophotometer, using $1/_{20}$-cm. cells. The reaction was quenched by the addition of 0.25 ml. of glacial acetic acid when the optical density had reached 0.65, a value which corresponds to the substitution of 1.5 moles of FDNB per insulin molecule if the molecular weight is 12,000. The reaction time was about 15 minutes. Unreacted FDNB was removed by extraction with ether, followed by evaporation to dryness of the ether–alcohol layer. The residue was taken up in water and ether and the aqueous layer from this was added to the aqueous layer from the first extraction. The combined aqueous extracts were then evaporated to dryness in the rotary evaporator.[8]

The entire yellow residue was distributed in the system used for the original characterization of the protein, 2-butanol/1% aqueous dichloroacetic acid (DCA). A 220 tube automatic countercurrent distribution apparatus[9] was used. After 216 transfers analyses by weight and by ultraviolet absorption at 277 mμ and at 350 mμ gave the pattern shown in Fig. 1a. Bands 1, 2 and 3, all colorless, were withdrawn and the apparatus was arranged for recycling the yellow bands. At 530 transfers the pattern shown in Fig. 1b was obtained.

Material was recovered from bands 2–6 of Fig. 1a. Band 1 was found to contain only inorganic salts from the reaction. Band 2 contained unchanged insulin which was isolated in crystalline form by the usual procedure. Since intact solute from band 3 was not needed for study, material for hydrolysis was recovered by the addition of hydrochloric acid, extraction of the DCA and 2-butanol with ether, and evaporation of the aqueous layer. A portion of the residue was hydrolyzed and studied by paper chromatography, but no spots other than those obtained from an hydrolysate of insulin were observed.

Products from bands 4, 5 and 6 were all isolated in the following manner. The solutions were concentrated *in*

(7) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(8) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

(9) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *ibid.*, **23**, 1236 (1951).