VIII (13.5 g.) into XI; 5.7 g. (30%) yield, m.p. 307–308° dec.

Anal. Calcd. for $C_{10}H_8Cl_2NAsO_3$: N, 4.17; As, 22.29. Found: N, 4.15; As, 21.97.

5-Amino-8-chloro-2-hydroxy-6-methylquinoline (XIII).—XII (19.7 g.) was dissolved in absolute ethanol and reduced catalytically under conditions similar to those used for reducing I and VII. Crude XIII was liberated from its hydrochloride with ammonia–water prior to purifying by crystallization from hot ethanol–water solution (50:50 by volume); 10 g. (58.5%) of yellow crystals, m.p. 195–197°.

Anal. Calcd. for $C_{10}H_9ClN_2O$: N, 13.43; Cl, 17.00. Found: N, 13.26; Cl, 16.99.

5-Acetamido-8-chloro-2-hydroxy-6-methylquinoline (XV).—XIII (1.5 g.) was changed into XV under conditions similar to those used for converting II and VIII into III and IX, respectively. The crude XV was purified by crystallization from hot ethanol–water solution; 1.2 g. (67%) yield, m.p. 305–307°.

Anal. Calcd. for $C_{12}H_{11}ClN_2O_2$: N, 11.18; Cl, 14.14. Found: N, 11.15; Cl, 14.07.

5-Benzamido-8-chloro-2-hydroxy-6-methylquinoline (XIV).—The method that de Arce, Greene and Capps[3] used to convert 5-amino-8-bromo-2-hydroxy-6-methylquinoline into its 5-benzamido derivative was resorted to for changing XIII (1.5 g.) into XIV; 2 g. (92%) yield, m.p. 260–262°.

Anal. Calcd. for $C_{17}H_{13}ClN_2O_2$: N, 8.96; Cl, 11.34. Found: N, 8.79; Cl, 11.32.

8-Chloro-2-hydroxy-6-methyl-5-quinolinearsonic Acid (XVI).—XIII (4.5 g.) was diazotized and converted into XVI under conditions similar to those used for converting II into V and VIII into XI; 1.9 g. (27.8%) yield, m.p. 310–312° dec.

Anal. Calcd. for $C_{10}H_9ClNAsO_4$: N, 4.41; As, 23.59. Found: N, 4.21; As, 23.77.

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Separation of the Glycyl from the Phenylalanyl Chain of Oxidized Insulin by Countercurrent Distribution[1]

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The initial step in the determination of the amino acid sequence of insulin by Sanger and his colleagues[2–4] was a cleavage of the molecule with performic acid which produced two peptide chains, one with phenylalanine as the N-terminal amino acid, the other with glycine in the N-terminal position. The amino acid composition of these chains is such that a separation could be achieved by methods of precipitation utilizing changes in *p*H, or salt, or ethanol concentrations.[2] However, the yields of the two chains were relatively low with a combined recovery of 40–50% of theory. The experiments described here are an application of the technique of countercurrent distribution to the quantitative separation of the two chains to determine the maximum possible yield of the chains which can be obtained by the performic acid cleavage. A complete separation of the glycyl chain from the phenylalanyl chain was achieved by only a few transfers with the solvent system, 2-butanol/aqueous 0.077 *M* *p*-toluenesulfonic acid. Under the best conditions a combined yield of the two chains of approximately 95% has been obtained. The remaining material was mainly a fraction insoluble in the solvent system. The phenylalanyl chain was not separated from any traces of unoxidized insulin which might have been present.

Experimental

Insulin Preparations.—The insulin was beef insulin (Boots Pure Drug Co., Ltd., Nottingham, England). One experiment with essentially the same results was carried out with crystalline beef insulin (Eli Lilly Co.).

Oxidation with Performic Acid.—Oxidations were carried out at −5 to −10°, 25 and 50°. Conditions including those of Sanger,[2] the milder ones of Sanger and Thompson[4] and those with preformed performic acid[5] were used. Initially the reaction mixtures, following oxidation, were diluted with several volumes of water and lyophilized. In later experiments the following procedure led to much less insoluble material and more reproducible results. After oxidation, the reaction mixture was diluted with 10 volumes of water, cooled to 0° and concentrated to a thin sirup with a rotary evaporator[6,7] with 5 ml. ethanol added to prevent foaming. The solvent system was then added immediately. Concentration was completed within 30 minutes after oxidation.

Determination of Insoluble Material.—The insoluble material, which collected at the interphase when oxidized material was placed in the solvent system, was removed before distribution with a dropping pipet and placed in a small centrifuge tube. Then most of the remaining solvent was removed by pipet and dry 2-butanol was added to take up the residual aqueous phase. The insoluble material was centrifuged, washed three times with ether after removal of the butanol, dried and weighed.

Countercurrent Distribution.—Distributions were carried out at room temperature in glass apparatus with 10 or 40 ml. in each phase.[8] From 15 to 50 mg. of material was used in each distribution. The solvent system was prepared by equilibrating equal volumes of 2-butanol and 0.077 *M* *p*-toluenesulfonic acid (Eastman Kodak Co.). Determination of the distribution curves was as previously described[9] with a quantitative ninhydrin reaction[10] after hydrolysis of the peptides. Results are expressed in mg. of peptide determined by comparison with a standard curve of hydrolyzed insulin.

Recovery of Glycyl Chain for Chromatography.—The contents of the tubes containing the slow moving component were combined and taken nearly to dryness at low temperature in the rotary evaporator. *p*-Toluenesulfonic acid and traces of water were removed by repeated extraction with ether. The peptide remained as a dry film on the sides of the flask and was recovered by solution in water. After hydrolysis, two-dimensional paper chromatograms were run on this material and on the insoluble fraction by the ascending method[11] with *n*-butanol-acetic acid[12] and phenol–water[13] solvents.

Results and Discussion

The distribution curve of unoxidized insulin after 49 transfers showed a single peak agreeing

(1) Supported by a research grant, No. C-2290 from National Cancer Institute, U. S. Public Health Service, National Institutes of Health.

(2) F. Sanger, *Biochem. J.*, **44**, 126 (1949).

(3) F. Sanger and H. Tuppy, *ibid.*, **49**, 463, 481 (1951).

(4) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353, 366 (1953).

(5) J. M. Mueller, J. G. Pierce, H. Davoll and V. du Vigneaud, *J. Biol. Chem.*, **191**, 309 (1951).

(6) E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, **57**, 33 (1954).

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

(8) L. C. Craig and O. Post, *ibid.*, **21**, 500 (1949). Such apparatus are obtainable from H. O. Post Scientific Instruments Co., 6822 60th Road, Maspeth 78, N. Y.

(9) J. G. Pierce, *Biochem. J.*, **57**, 16 (1954).

(10) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(11) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

(12) R. J. Block, *Anal. Chem.*, **22**, 1327 (1950).

(13) R. J. Block, "Paper Chromatography," Academic Press, Inc., New York, N. Y., 1952, p. 53.

well with a theoretical distribution for a single substance.[14]

Figure 1 shows a distribution curve of oxidized insulin. In those oxidations where less of the slow moving component appeared, some increase of material distributing in tubes 11–20 (Fig. 1) occurred. In such cases, somewhat greater deviation of the curves from theory was found.

The slow moving component (tubes 1–8) was identified as the glycyl chain. Paper chromatograms of this component after hydrolysis showed all the amino acids of the glycyl chain.[2] The basic amino acids and threonine, which are absent from this chain, were missing. The missing amino acids were readily detected in control chromatograms of hydrolyzed oxidized insulin. Paper chromatograms of the material insoluble in the solvent system were identical with those run with an equivalent amount of an oxidized insulin control. The material probably is similar to Sanger's fractions X and M.[2]

The best conditions found for the maximum yield of the two chains with a minimum amount of insoluble material were oxidation at 50° for 15 minutes using proportions of formic acid and H_2O_2 of 9:1[2]; followed by concentration in the rotary evaporator. With these conditions duplicate distributions of material combined, in each case, from two separate oxidations gave nearly identical distribution curves. From the previously published data[2–4,15] one can calculate the ratio of the glycyl to the phenylalanyl chain that would prevail if the insulin molecule is made up solely of the two chains. This ratio, with the chains as cysteic acid peptides, is 0.72. The ratios between the chains in the two distributions just described were 0.67 and 0.68 (calculated from weight in tubes 1–11 and 33–45). The use of preformed performic acid at −5 to −10°[5,6] did not yield as much glycyl chain. The results of oxidations under several sets of conditions are summarized in Table I. It can be seen that where freeze drying was used considerable insoluble material was found when appreciable amounts of the glycyl chains were liberated. A number of these experiments, however, also gave rise to ratios close to that demanded by theory. Treatment with preformed performic acid at 50° was too strenuous; the glycyl chain peak was skewed on the left side indicating a new component, presumably arising from non-specific destruction.

TABLE I

RATIO OF GLYCYL CHAIN TO PHENYLALANYL CHAIN UNDER DIFFERENT CONDITIONS OF OXIDATION[a,b]

Time of preforming performic acid at room temp., min.	Temp. of oxidation, °C.	Time of oxidation min.	Insol. material, %	Ratio of chains: Theory, 0.72
		Concn. by rotary evaporator		
0[c]	50	15	3,3,3,6[d]	0.67, 0.69
25	−5 to −10	45	4,4,4,4[d]	0.45, 0.53
		Concn. by lyophilizing		
0[c]	50	15	18,26	0.73
0	50	15	16,16	.72
45	50	15	20,20	.82
35	50	15	22,21	.76
25	−5 to −10	45	20,–	.63
45	−5 to −10	60	31,28	.56
0[e]	0	60	9,6	Negligible glycyl chain

[a] The peak of the distribution curves representing the phenylalanyl chain would contain unreacted insulin if present. [b] The performic acid was formic acid: H_2O_2, 9:1; 4 ml. reagent per 100 mg. of insulin was used.[2] Results of oxidations at 25° (with lyophilizing) were similar to those shown. [c] The performic acid was prepared and immediately heated in the 50° bath for 3 minutes. The insulin was then added. [d] Values from four separate oxidations; material from two oxidations was combined and distributed. [e] This oxidation was carried out using the milder conditions of Sanger and Thompson[4]; at 25°, 35% insoluble material was obtained (with lyophilizing); a distribution was not carried out.

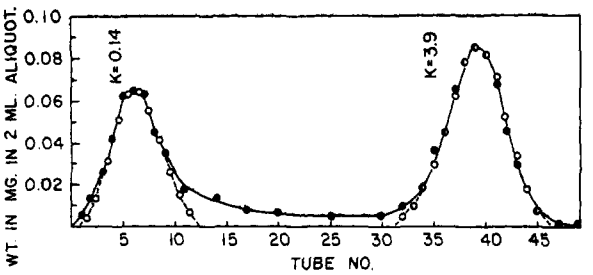

Fig. 1.—Distribution curve of Boots beef insulin following performic acid oxidation; 50 mg. oxidized at 50° for 15 min.; concentration in rotary evaporator; insoluble material approx. 5%; ●, weight in 2-ml. aliquot after phases broken[9]; O, theoretical.

The recovery of approx. 95% of the insulin as two major components in a ratio close to that demanded by theory from a molecule made up solely of glycyl and phenylalanyl chains offers strong additional evidence to that of Sanger and his colleagues[3,4] and of Harfenist[15] that such a representation is correct. The data are also of interest with regard to the relationship between partition ratios of large peptides or proteins, their amino acid composition and the acid used in the solvent system. In examining the homogeneity of insulin preparations, Harfenist and Craig employed a solvent system with 0.077 *M* dichloroacetic acid in which insulin has a partition ratio (K) of approx. 0.5.[16] In the present experiments, *p*-toluenesulfonic acid of equal molarity was used giving a (K) for insulin of approx. 6. Thus intact insulin and the phenylalanyl chain which contains the basic amino acids[2,3] both moved rapidly through the apparatus allowing their separation in a few transfers from the glycyl chain.[17]

The use of such solvent systems with variations

(14) The closely related second component which is found after several hundred transfers was not revealed by 49 transfers (ref. 16). These components appear to differ only by one amide group (ref. 15).

(15) E. J. Harfenist, THIS JOURNAL, **75**, 5528 (1953).

(16) E. J. Harfenist and L. C. Craig, *ibid.*, **74**, 3083 (1952).

(17) Other examples of the relationship are beef vasopressin which contains one arginine residue per molecule and has a (K) of 1 in a system with 0.1 *M* *p*-toluenesulfonic acid (R. A. Turner, J. G. Pierce and V. du Vigneaud, *J. Biol. Chem.*, **191**, 21 (1951)) and pituitary growth hormone with a very large number of basic groups and a (K) of approx. 10 in a system with only 0.005 *M* *p*-toluenesulfonic acid (ref. 9).

in the organic acid and its concentration should aid in the separation of relatively large cleavage products of other proteins. The need for such separations recently has been emphasized.[18,19]

Acknowledgments.—The author is indebted to Prof. A. C. Chibnall and Dr. O. K. Behrens for samples of insulin. The assistance of Burton D. Wilson and Daniel E. Ott is gratefully acknowledged.

(18) A. C. Chibnall, in "Les Proteines, Rapports et Discussions," R. Stoops, Ed., Institut International de Chimie Solvay, Brussels, 1953, p. 128.

(19) R. L. M. Synge, *ibid.*, p. 153.

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The Resolution of DL-*threo*-1-*p*-Methylsulfonylphenyl-2-amino-1,3-propanediol

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The preparation of D-*threo*-1-*p*-methylsulfonylphenyl-2-dichloroacetamido-1,3-propanediol by the oxidation of D-*threo*-1-*p*-methylmercaptophenyl-2-dichloroacetamido-1,3-propanediol has been reported by Cutler, Stenger and Suter.[1] Although the synthesis of the racemate of the former compound from 4-methylsulfonylacetophenone was described by Suter, Schalit and Cutler[2] in a subsequent publication, no resolution of the final product or of its intermediates has appeared in the literature. Because of the high order of antibacterial activity of the D-*threo*-*p*-methylsulfonyl compound, it is of interest to describe a method for resolving DL-*threo*-1-*p*-methylsulfonylphenyl-2-amino-1,3-propanediol developed in our laboratory during a series of studies of N-acylated compounds of this type.[3]

The recovery of racemic *threo*-1-*p*-methylsulfonylphenyl-2-amino-1,3-propanediol from the hydrolysis of the corresponding acetamide or dichloroacetamide presented a problem because of the extreme solubility of this compound in water. Suter, *et al.*,[2] described recovery of a 19.5% yield of base from the acid hydrolysis of the acetamide. The product was isolated by extracting a strongly alkalinized solution of the hydrolysate with butanol and evaporating the butanol extract. In our laboratory it was found that the recovery of the base could be substantially improved by passing the acid hydrolysate over an XE 98[4] basic resin column and evaporating the eluate containing the liberated base to dryness under reduced pressure. This method has been used also to recover the resolved bases from the optically active mandelate salts. In the latter case the salt was first treated with an equivalent amount of hydrochloric acid to liberate the mandelic acid and the resolving agent was removed by ether extraction. The aqueous residue containing the optically active hydrochloride salt was then passed over an XE 98 column and the D- or L-*threo* base isolated as above.

The resolution of DL-*threo*-*p*-methylsulfonylphenyl-2-amino-1,3-propanediol was achieved by fractional crystallization of the appropriate active mandelic acid salt from ethanol. When (+)mandelic acid was used, the mandelate salt of the levorotatory base was less soluble in ethanol and could be isolated in the optically pure state by repeated crystallization. The dichloroacetamide of this base proved to be identical with the product obtained by oxidizing D-*threo*-1-*p*-methylmercaptophenyl-2-dichloroacetamido-1,3-propanediol. The antibacterial activity was twice that of the racemate. On the other hand, the dichloroacetamide of the dextrorotatory base obtained when (−)-mandelic acid was used as the resolving agent had substantially no *in vitro* antibacterial activity.

Experimental

The Preparation of DL-*threo*-1-*p*-Methylsulfonylphenyl-2-amino-1,3-propanediol.—A 5-g. sample of DL-*threo*-1-*p*-methylsulfonylphenyl-2-dichloroacetamido-1,3-propanediol[5] was treated with 40 ml. of 1.0 *N* hydrochloric acid for 2.5 hours on the steam-bath. The chilled hydrolysate was diluted with 125 ml. of distilled water and put over a basic XE 98[4] column which had been washed previously to *p*H 9.4 with distilled water. The combined alkaline eluates were evaporated under reduced pressure and the residue dried by repeated evaporation with ethanol and benzene to a crystalline solid. The base was recrystallized from ethanol to give 2.23 g. of product (m.p. 121–122°).

Anal. Calcd. for $C_{10}H_{15}NO_4S$: C, 48.96; H, 6.16; N, 5.71. Found: C, 49.14; H, 6.21; N, 5.73.

The Resolution of DL-*threo*-1-*p*-Methylsulfonylphenyl-2-amino-1,3-propanediol.—A 2-g. sample of DL-*threo*-1-*p*-methylsulfonylphenyl-2-amino-1,3-propanediol was treated with 1.24 g. of (+)mandelic acid in 20 ml. of absolute ethanol. After standing overnight at room temperature, 2.7 g. of crystalline salt melting at 138–140° was obtained. The mandelate salt was then dissolved in 100 ml. of absolute ethanol and allowed to stand for 48 hours at room temperature while the resolved product slowly separated. It was found that separation could be materially hastened when a few crystals of D base (+)mandelate were added as seeds. The 630 mg. of crystalline product which was obtained in this manner melted at 145–147°. Recrystallization from 20 ml. of methanol raised the melting point to 151–152°. The salt was filtered after two hours. A final recrystallization yielded 430 mg. of salt with no change in melting point, $[\alpha]^{22}D$ +24.8° (*c* 1.37% in water).

Anal. Calcd. for $C_{18}H_{23}NO_7S$: C, 54.39; H, 5.83; N, 3.52. Found: C, 54.55; H, 5.68; N, 3.41.

The 430 mg. of resolved base mandelate salt was dissolved in 13 ml. of distilled water containing 0.8 ml. of 2 *N* hydrochloric acid. The aqueous solution containing the base hydrochloride was passed over a basic XE 98 column and the resolved base isolated from the eluates in the same manner as was the racemic base. The recrystallized product melted at 140–142°, $[\alpha]^{22}D$ +20° (*c* 2% in ethanol).

Anal. Calcd. for $C_{10}H_{15}NO_4S$: C, 48.96; H, 6.16; N, 5.71. Found: C, 49.10; H, 6.46; N, 5.78.

The above product was converted to the dichloroacetamide by heating with excess methyl dichloroacetate in ethanol solution for 1 hour on the steam-bath. The product was worked up in the usual manner.[6] The dichloroacetamide melted at 164–165°, $[\alpha]^{22}D$ +12.8° (*c* 2% in ethanol), $[\alpha]^{22}D$ −16.2° (*c* 11.3% in dimethylacetamide). The resolved product was twice as active in *in vitro* antibacterial

(1) R. A. Cutler, R. J. Stenger and C. M. Suter, THIS JOURNAL, **74**, 5475 (1952).

(2) C. M. Suter, S. Schalit and R. A. Cutler, *ibid.*, **75**, 4330 (1953).

(3) We are indebted to Dr. A. S. Schlingman, Mrs. Della Fox and Miss Mary Manning and co-workers for detailed antibacterial studies of these compounds. The antibacterial activity found independently by these workers is qualitatively and quantitatively in agreement with that reported by Cutler, *et al.*[1]

(4) Rohm and Haas Company.

(5) DL-*threo*-1-*p*-Methylsulfonylphenyl-2-dichloroacetamido-1,3-propanediol was prepared by the same method used by Suter, *et al.*[2]

(6) M. C. Rebstock, THIS JOURNAL, **72**, 4800 (1950).