product was washed with methanol, dissolved in 10 ml. of water, decolorized with carbon and precipitated with 50 ml. of methanol; 0.41 g. of amorphous white sulfate which was apparently identical with III was obtained.

Isolation of Streptolin A.—One gram of IV was dissolved in 500 ml. of 50% methanol at 55° and allowed to cool slowly to room temperature. The crystals which separated were similarly recrystallized five times. The results of this procedure and the analytical data for the product are given in Table I. The more insoluble helianthate V which had constant properties for the last three crystallizations appears to be a homogeneous product and is designated streptolin A, m.p. 206° (dec.). This procedure was repeated with similar results on a larger batch of material.

Streptolin A helianthate V yielded an amorphous sulfate VI which assayed 32,000 units per milligram, $[\alpha]^{25}D$ −20° (*c* 1.0).

The elemental analyses of the amorphous sulfates do not agree with that expected from the corresponding crystalline products. This is presumed to be due to formation of mixed salts (*i.e.*, tritetrabasic), and possible contamination with triethylamine.

Evaporation of the 2nd crystallization liquors gave a helianthate with higher biological activity (product 2-L, Table I). This yielded a sulfate VII which assayed 54,000 units per milligram, $[\alpha]^{25}D$ −22° (*c* 2). This has been shown by paper chromatography to be still a mixture of streptolins A and B.

The infrared spectra of streptolin A sulfate (VI) and the mixed sulfate VII, were determined in a nujol mull. A Baird recording infrared spectrophotometer with a sodium chloride prism was used. The spectrum for streptolin A is given in Fig. 1. The spectrum for the mixture was within experimental error of that from streptolin A.

Chromatographic Data.—The general procedure and equipment described by Karnovsky and Johnson[12] was used for developing and analyzing downflow chromatograms. One-half inch strips of Eaton-Dikeman No. 613 paper were soaked in a solution containing 0.95 *M* sodium sulfate and 0.05 *M* sodium acid sulfate and air dried. Strips were cut such that twenty inches was allowed for development of the chromatogram. After application of the antibiotic sample, the strips were placed in the developing chamber such that the point of sample application was about three inches from the liquid level in the reservoir. Development was carried out at 25° for 30 to 50 hr. The solvent front went beyond the end of the strip.

At the end of the development period, the tapes were air-dried, split lengthwise and analyzed by plating on agar inoculated with *B. subtilis*. The agar medium was the same as the streptomycin assay medium of Loo, *et al.*, except that when diffusion of the streptolin antibiotics was desired, 1% sodium chloride was added. The plates were incubated at 25°.

Quantitative estimations of the chromatogram zones were made by cutting the strips into one-fourth inch squares and assaying these against squares containing known amounts of streptolin A on the streptolin assay agar. The analyses of the various preparations are given in Figs. 2 and 3.

(12) M. L. Karnovsky and M. J. Johnson, *Anal. Chem.*, **21**, 1125 (1949).

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[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY, THE OHIO STATE UNIVERSITY]

Hydrolysis of Proteins by Ion Exchange Resin Catalysis[1]

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The hydrolysis of casein by ion exchange resin catalysis proceeds very near to the point of completion after refluxing the protein with 0.05 *N* hydrochloric acid and Dowex 50 for a period of 48 hours. Low recoveries of certain amino acids are believed to be due to the presence of unhydrolyzed peptide residues. The rate of protein hydrolysis by ion exchange resin catalysis is affected by the nature of the resin and the nature of the protein. Among the proteins investigated, this type of hydrolysis indicates a peptide bond selectivity for certain amino acids.

Introduction

It has been shown that rates of protein hydrolysis by dilute acid depend not only on temperature and acidity, but also on the nature of the anion of the acid used.[2] It was demonstrated that certain high molecular weight sulfonic acids, at concentration levels less than 0.2 *M*, are more effective catalysts than the common mineral acids. The mechanism of catalysis was described as an increased basicity of the amide and peptide bonds as a result of a combination of these bonds with the large anion of the catalyst.

The practical difficulty of catalyst removal is overcome by using an insoluble sulfonic acid polymer such as an ion exchange resin. In the case of a cation exchange resin, the large nucleophilic anion should be effective in labilizing the protein peptide bond. Underwood and Deatherage[3] reported that coffee bean proteins can be hydrolyzed by refluxing with water and acid regenerated Dowex 50, a polystyrenesulfonic acid ion exchange resin. The present work is concerned with the extent of hydrolysis of casein by Dowex 50, an investigation of catalytic activity of certain other ion exchange resins, and the effect of the nature of the protein.

Experimental Procedure

Hydrolysis Procedure.—The casein was isolated from skim milk according to Cohn and Hendry.[4] Bovine serum albumin, egg albumin, gelatin and lactalbumin were commercial samples. Casein: moisture, 11.9%; ash, 0.75%; bovine serum albumin: moisture, 4.32%; ash, 0.53%.

Before use, the cation exchange resins were regenerated in the hydrogen cycle by repeated suspension and decantation with 4 *N* hydrochloric acid. The resin was then rinsed with water until the filtrate was chloride free, drained free of excess water, and stored in the moist form. The anion exchange resins were regenerated in the same manner with 5% sodium hydroxide.

The hydrolysis of casein was carried out by refluxing at atmospheric pressure 3 g. of casein with 300 ml. of 0.05 *N* hydrochloric acid and 15 g. of 200–400 mesh approximately 12% cross linked Dowex 50. Since the rate of resin-catalyzed hydrolysis depends upon the hydrogen ion concentration, hydrogen ions were added within the limits of stability of the resin. Provisions must be made for stirring in order to prevent bumping and to permit good contact be-

(1) Presented before the Biological Division of the American Chemical Society at Atlantic City, N. J., September 18, 1952.

(2) J. S. Steinhardt and C. H. Fugitt, *J. Research Natl. Bur. Standards*, **29**, 315 (1942).

(3) G. E. Underwood and F. E. Deatherage, *Science*, **115**, 95 (1952); *Food Research*, **17**, 425 (1952).

(4) E. J. Cohn and J. L. Hendry in Blatt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943.

tween the resin and the protein substrate. Sealed tube reactions at 100° in which there was no provision for shaking were found to proceed at a much slower rate. The refluxing was continued for 48 hours which is about 20 hours beyond the point at which the biuret test is negative. At the end of the reflux period, the hydrolytic products are held on the resin catalyst by linkage through their free amino groups. Inasmuch as the degree of hydrolysis was being investigated, it was necessary to displace the products of hydrolysis from the resin under conditions which would not favor any additional hydrolysis. Aqueous ammonia was found to be an effective eluant at low concentration levels. The resin was filtered off into a Pyrex column 9 mm. in diameter and then eluted with 0.15 *N* aqueous ammonia until the effluent showed a negative ninhydrin reaction. The total volume of eluate was about 500 ml. and the ammonia breakthrough point occurred at about 400 ml. The initial liquid portion of the hydrolyzate and the resin eluate were combined and the total volume was adjusted to 500 ml. by reduced pressure distillation.

Partial hydrolyzates were prepared by refluxing and stirring 0.5 g. of protein with 50 ml. of 0.05 *N* hydrochloric acid and 2.5 g. of Dowex 50. At the end of the hydrolysis period, the *p*H of the liquid portion of the hydrolyzate was adjusted to the isoelectric point of the original protein and any insoluble material was removed by centrifugation. In the case of bovine serum albumin, such slightly altered protein is present up to about 30 hours of hydrolysis time. The resin was eluted in the same manner as previously described.

Oxidation of Bovine Serum Albumin.—Three grams of bovine serum albumin was dissolved in 300 ml. of 0.05 *N* hydrochloric acid. Three milliliters of 30% hydrogen peroxide and 3 drops of 3% ammonium molybdate solution was added and the mixture was refluxed for one hour. The *p*H of the solution was adjusted to 5.3 with sodium hydroxide and the resulting precipitate was removed by centrifugation and dried over calcium chloride in a vacuum desiccator. The product was a dark brown granular material. Cystine and cysteine were oxidized to cysteic acid and part of the tyrosine was oxidized.

Amino Acid Estimations.—These were made by a preliminary chromatographic separation of the hydrolyzates on Dowex 50 in the sodium cycle according to Moore and Stein.[5] One-milliliter fractions were collected with the aid of a fraction collector constructed according to Varner and Bulen.[6] These fractions which represented one amino acid (ninhydrin spot test) were combined and made up to a known volume, and aliquots of these solutions were analyzed by the colorimetric ninhydrin method.[7] Serine and threonine were determined together.

Ammonia was determined by steam distillation at *p*H 10.1 on a separate resin hydrolyzate in which the hydrolytic products were displaced from the resin with 3 *N* hydrochloric acid.

Hydrolysis in dilute acid solution is accompanied by partial conversion of glutamic acid into its internal anhydride, pyrrolidonecarboxylic acid.[8] The presence of pyrrolidonecarboxylic acid was tentatively confirmed by using the paper chromatographic isolation technique of Consden.[9] A water-saturated phenol paper chromatogram of the Dowex 50 casein hydrolyzate was cut into sections and each section was eluted with water. The residues were taken up in a few microliters of 9% hydrochloric acid and heated for 2 hours at 100° in sealed 1-mm. capillary tubes in order to convert the pyrrolidone form back to glutamic acid. The glutamic acid fractions were identified by paper chromatography. The same procedure was repeated for the second dimension of a two-dimensional paper chromatogram in which the second solvent was *n*-butanol, glacial acetic acid, water (4:1:5).[10] Sections containing glutamic acid after treatment with hydrochloric acid were: phenol, R_f 0.65–0.75; butanol–acetic acid, R_f 0.35–0.45. These values were in agreement with those for known samples of pyrrolidonecarboxylic acid. The fact that pyrrolidonecarboxylic acid is not adsorbed by Dowex 50 during the column separation of the hydrolyzate served as the basis for the quantitative estimation of this substance. Pyrrolidonecarboxylic acid appears in fractions 23–27 ml. on a 0.9 × 100-cm. column of 12% cross-linked Dowex 50 while aspartic acid begins at about fraction 50 ml. The pyrrolidonecarboxylic acid fractions were combined, made 9% with respect to hydrochloric acid, and heated at 100° for a period of two hours. The glutamic acid was then determined by the colorimetric ninhydrin method.

(5) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).
(6) J. E. Varner and W. A. Bulen, *J. Chem. Ed.*, **29**, 625 (1952).
(7) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).
(8) H. Wilson and R. K. Cannan, *ibid.*, **119**, 309 (1937).
(9) R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, **41**, 590 (1947).
(10) S. M. Partridge, *ibid.*, **42**, 238 (1948).

Results

Hydrolysis of Casein.—As shown in Table I, the recoveries of amino acids by 48-hour Dowex 50 hydrolysis of casein are significantly lower than the 20-hour 6 *N* hydrochloric acid control in the case of glutamic acid, valine and isoleucine. In order to determine whether these low recoveries were due to incomplete hydrolysis, an attempt was made to isolate unhydrolyzed peptide residues using paper chromatographic techniques. The resin hydrolyzate and control were developed on Whatman No. 1 filter paper in two dimensions using water-saturated phenol, and butanol–acetic acid. In the case of the resin hydrolyzate, there were no well defined spots which could not be accounted for in terms of known amino acids. The two-dimensional chromatogram was divided into sections and each section was eluted with water and hydrolyzed with 6 *N* hydrochloric acid in the same manner as described for the isolation of pyrrolidonecarboxylic acid. Sections of the original chromatogram in the vicinity of proline yielded traces of valine, leucine and glutamic acid after treatment with 6 *N* hydrochloric acid. Small amounts of glutamic acid were also found after hydrolysis of areas in the vicinity of aspartic acid. The remainder of the areas of the chromatogram did not show any additional amino acids after hydrolysis. This evidence suggests that the low recoveries of certain amino acids were due to incomplete hydrolysis. Low recoveries of the basic amino acids may also have been due to incomplete elution from the resin catalyst. It has been reported[5] that it is not possible to obtain complete recovery of basic amino acids from cation exchange resins when alkaline eluants such as ammonium hy-

TABLE I

RECOVERIES OF AMINO ACIDS OF CASEIN BY ION EXCHANGE RESIN HYDROLYSIS AND BY 6 *N* HYDROCHLORIC ACID HYDROLYSIS

Amino acid	Millimoles/g. protein 6 *N* HCl	Dowex 50, 48 hours
Aspartic acid	0.45	0.48
Serine, threonine	0.89	.95
Glutamic acid	1.40	.88
Pyrrolidonecarboxylic acid	..	.34
Proline	0.56	.56
Glycine, alanine	.61	.62
Valine	.57	.41
Methionine	.20	.19
Isoleucine	.40	.32
Leucine	.70	.68
Tyrosine, phenylalanine	.60	.58
Tryptophan	..	..
Histidine	.18	.16
Lysine	.54	.52
Arginine	.26	.26
Ammonia	1.24	1.09

droxide are used. The higher recoveries of serine and threonine by resin hydrolysis indicate less destruction of these acid labile amino acids.

In Table II are shown the recoveries of the neutral and acidic amino acids obtained from a 6-hour partial Dowex 50 hydrolyzate of casein. Under these conditions, there was no accumulation of any peptide of a given species such that it could be clearly detected by paper chromatographic separation. While aspartic acid is liberated preferentially, valine and isoleucine are released at much slower rates than the other amino acids. This tends to support the evidence in favor of incomplete hydrolysis, since isoleucine and valine were found to be the amino acids lowest in yield.

TABLE II

AMINO ACID RECOVERIES FROM A SIX-HOUR DOWEX 50 PARTIAL HYDROLYZATE OF CASEIN

Amino acid	Millimoles/g. protein Complete hydrolysis[a]	Partial, 6-hour	Amino acid liberated, %
Aspartic acid	0.482	0.206	43
Serine, threonine	0.893	.119	13
Glutamic acid	1.398	.083	5.9
Glycine, alanine	0.614	.120	19
Valine	.574	.040	7.0
Methionine	.202	.034	17
Isoleucine	.396	.026	6.5
Leucine	.703	.114	16
Tyrosine, phenylalanine	.602	.093	15

[a] Hydrolysis by refluxing with 6 *N* hydrochloric acid for 20 hours.

Nature of the Resin.—Additional cation exchange resins which were investigated included Amberlite IRC-50, a carboxylic type resin, and Dowex 30, Amberlite IR-105 and Zeo Rex which are phenolsulfonic acid type resins. The hydrolyses with casein were carried out under the same conditions as previously described, but without the addition of hydrogen ions. A variation in catalytic activity was observed among the different resins. The phenolsulfonic type resins catalyzed the hydrolysis of casein at a greater rate than Dowex 50, while the carboxylic type resin was found to be less effective. These differences in rate appear to be due to variations in the *p*H of the hydrolyzates resulting from the number of hydrogen ions furnished by the resins of different mesh size and capacity. Differences in inherent catalytic activity, if they exist, are obscured by this variation in *p*H. The phenolsulfonic type resins were found inferior to Dowex 50 with respect to heat stability.

Among the anion exchange resins which were tried were Amberlite IR-4B and XE-67, weak base resins and Dowex 2 which is a stronger base resin. The mixtures were refluxed for 150 hours with and without the addition of hydroxide ions. In each case the biuret test was strongly positive at the end of the reflux period; when no hydroxide ions were added, the casein was only partially in solution.

Nature of the Protein.—It was found that the rate of resin-catalyzed hydrolysis depends upon, among other things, the nature of the protein. Gelatin is hydrolyzed quite rapidly by Dowex 50; a negative biuret test is obtained after refluxing the protein with Dowex 50 and 0.05 *N* hydrochloric acid for 10–12 hours. As contrasted to gelatin and casein, proteins such as lactalbumin, egg albumin and bovine serum albumin are much more resistant to hydrolysis. In the case of egg albumin, hydrolysis periods much in excess of 50 hours are required. In Table III are shown the amino acid estimations of a 6-hour partial Dowex 50 hydrolyzate of bovine serum albumin. With the exception of aspartic acid which is liberated preferentially, most of the amino acids have been liberated to the extent of 7–9%. The relatively slower rates of release of isoleucine and valine are much more pronounced at 14 and 30 hours of hydrolysis. Low recoveries of glutamic acid may be due in part to the secondary formation of pyrrolidonecarboxylic acid. At 6 hours, only small amounts of free cystine could be detected by paper chromatography. A comparison of the data in Table III with the same type of data for casein in Table II indicates that hydrolysis of casein proceeds at about twice the rate of bovine serum albumin during the first 6 hours.

TABLE III

AMINO ACID RECOVERIES FROM A SIX-HOUR PARTIAL DOWEX 50 HYDROLYZATE OF BOVINE SERUM ALBUMIN

Amino acid	Millimoles/g. protein Complete hydrolysis[a]	Partial, 6-hour	Amino acid liberated, %
Aspartic acid	0.820	0.221	27
Serine, threonine	0.893	.104	12
Glutamic acid	1.122	.034	3.0
Glycine, alanine	0.943	.077	8.2
Valine	0.506	.037	7.3
Leucine, isoleucine	1.135	.056	4.9
Tyrosine, phenylalanine	0.678	.056	8.3

[a] S. Moore and W. H. Stein, *J. Biol. Chem.*, 178, 79 (1949).

The cystine peptide linkage appears to be very resistant to resin-catalyzed hydrolysis and the resistance of proteins to hydrolysis of this type seems to parallel the cystine content. It was suggested that an important factor involved may be the aggregation of protein molecules during denaturation due to the formation of disulfide cross linkages. If this is the case, cleavage of the disulfide bonds by chemical agents should accelerate hydrolysis rate. This factor was not found to be significant in the case of hydrogen peroxide oxidized bovine serum albumin. There was no significant increase in the rate of hydrolysis, nor was there any preferential increase in the rate of hydrolysis of cysteic acid peptide bonds. An increase in rate was observed when peroxide was added with the resin catalyst during hydrolysis, but this may have been due to breakdown of the resin and a subsequent lowering of the *p*H.

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