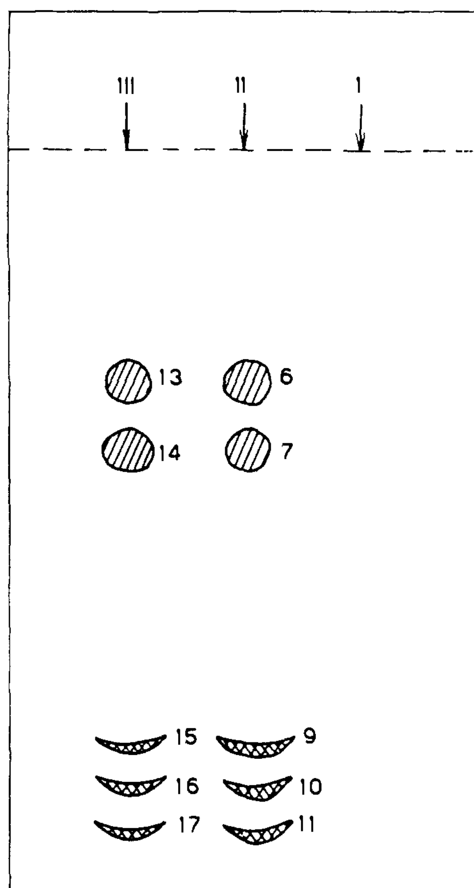

Fig. 3.—Descending chromatogram in alkaline solvent, after the orthophosphate has been cut off, developed with ninhydrin: columns I, II and III and spots as in Fig. 2.

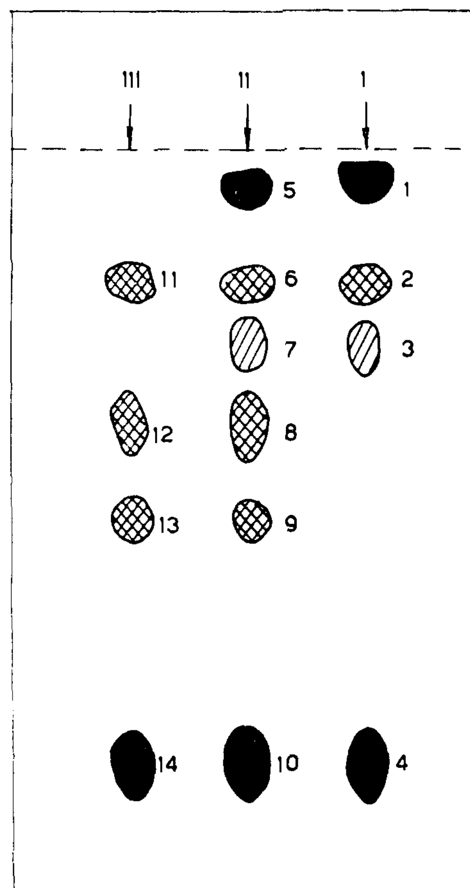

Fig. 4.—Descending chromatogram in *p*-toluenesulfonic acid solvent: columns I, II, III as in Fig. 2. Spot 1, ATP + ADP; spots 2, 6, 11, inorganic pyrophosphate; spots 3, 7, AMP; spots 4, 10, 14, inorg. orthophosphate; spot 5, ADP; spots 8, 9, 12, 13, peptides.

matographically using the Eggleston and Hems method.[2]

Chromatography.—First an ascending chromatogram was made, using 90 cc. of isopropyl alcohol + 60 cc. of 90% formic acid as solvent. Figure 1 is a typical run for a reaction mixture composed of leucine phosphate and ADP. The rapidly wandering orthophosphate (Spot 3 Fig. 1) is cut off from the rest of the chromatogram. Subsequently the paper was turned upside down and the chromatogram spread out in the descending direction with an alkaline solvent—240 cc. of propanol + 120 cc. of ammonia + 40 cc. of 0.002 *M* ethylenediamine tetraacetate.

Figures 2 and 3 represent two descending chromatograms spread out by the alkaline reagent. The chromatographic runs were usually carried out with two controls for comparison of rates, designated on Figs. 2, 3 and 4 as: (I), a solution containing a mixture of ATP (adenosine triphosphate), ADP (adenosine diphosphate), AMP (adenylic acid) and inorganic ortho- and pyrophosphates; and (III), a solution of free leucine phosphate. Figure 2 was developed by ninhydrin for free amino acid and polypeptides obtained by the self polymerization of leucine phosphate. It will be observed from Fig. 3 that polypeptides of various degrees of polymerization are formed both in the control and in the reaction mixture (III), Spots 13, 14, 15, 16, 17 and (II) 6, 7, 9, 10, 11, and inorganic pyrophosphates appears as a result of self-phosphorylation (Fig. 2, (III) spot 12 and part of (II) spot 5). However, Fig. 2 discloses that in addition to the phosphate salts of the peptides, adenosine diphosphate (Fig. 2, (II) spot 5) and traces of adenosine triphosphate have been obtained.

As the spot for adenosine diphosphate coincided with that for inorganic pyrophosphoric acid, the two substances were separated by the upper phase of the following mixture of solvents: 20 g. of *p*-toluenesulfonic acid + 60 ml. of *t*-amyl alcohol + 300 ml. of water.[3] This solvent, though not suitable for the separation of ATP from ADP, discriminates clearly between the inorganic pyrophosphate and ADP. The chromatograph represented by Fig. 4 demonstrates that phosphorylation has taken place, and that adenosine diphosphate, (II) spot 5, has been formed by the interaction of leucine phosphate with adenylic acid.

Spot 5, containing ADP formed by the reaction described, was washed out from another undeveloped chromatogram and its phosphorus content determined after 7 minutes of hydrolysis. The analytical results confirmed the fact that ADP was formed.

Acknowledgment.—It is a pleasure to thank Dr. Avidor for numerous helpful suggestions with regard to the chromatographic analysis.

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Conditions for Rapid Hydrolysis of Some Proteins by Dowex 50 Catalysis[1]

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Application of ion-exchange resins as catalysts for the essentially complete hydrolysis of proteins

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is often limited by the necessary long periods of hydrolysis.[2,3] Conditions of hydrolysis were studied with the purpose of obtaining hydrolysis of certain proteins within the relatively short period of 24 hours. For comparison hydrolyses were also carried out using 6 *N* hydrochloric acid.

Experimental

Commercial samples of bovine serum albumin, insulin and edestin were used. Casein was isolated from skim milk.[4] A sample of insulin[5] was oxidized with performic acid according to Sanger.[6]

The preparation of 200–400 mesh, 12% cross-linked Dowex 50 for use as a catalyst has been described.[3] A nitrated derivative of Dowex 50 was prepared by refluxing 12 g. of Dowex 50 with 20 ml. of concd. HNO_3 and 30 ml. of concd. H_2SO_4 for a period of 3 hours. The mixture was poured into a beaker of ice, washed free of acid with water and air-dried.

In carrying out the hydrolyses, the ratio of air-dried Dowex 50 to protein, R/S, was 5/1 or 20/1. The ratio of liquid phase to protein was 100/1. A typical hydrolysate contained 150 mg. of protein, 3 g. of Dowex 50 and 15 ml. of 0.05 *N* HCl. The mixtures were sealed in Pyrex tubes and rotated at 10 r.p.m. in a constant temperature (±0.2°) oven for the periods indicated. The elution of the hydrolytic products from resin catalyst, the separation of the amino acids on 100-cm. columns of Dowex 50, and the amino acid estimations by the ninhydrin reaction have been described.[3] The separation of the basic amino acids was carried out on a 15-cm. column of Dowex 50 according to Moore and Stein.[7]

Hydrolysis with 6 *N* HCl was also carried out in sealed tubes. The HCl was removed in a vacuum desiccator over solid KOH. The residue was dissolved in 100 ml. of water, the *p*H adjusted to 2.8, and a 2-ml. sample applied to the column for amino acid separation.

Edestin.—In Table I are shown the recoveries of the neutral and acidic amino acids of edestin under various conditions of hydrolysis. R/S, temperature and nature of the resin are the variables. Hydrolysis with 6 *N* HCl at 105° for 24 hours is used as the standard of comparison although it is realized that hydrolysis for some proteins is not quite complete even under these conditions.[8,9] At 100° for 90 hours with R/S = 5/1, as shown in column 1, most of the amino acids investigated, with the exception of glutamic acid, valine, isoleucine and leucine, have been completely liberated as compared with 6 *N* HCl hydrolysis in column 5. Increasing the temperature to 105° and R/S to 20/1 but decreasing the hydrolysis time to 24 hours, column 2, has no positive effect upon the resistant peptides involving the amino acids given above. The yields of valine, tyrosine and phenylalanine are significantly lower. The decreased *p*H of the mixture apparently reduces the formation of pyrrolidonecarboxylic acid giving a greater yield of free glutamic acid. The use of the nitrated form of Dowex 50 under the same conditions shows no improvement in amino acid yields. When the temperature is increased to 110° for 24 hours with R/S = 20/1, column 5, the amino acid yields agree quite well with those for 6 *N* HCl hydrolysis. It appears that temperature is a critical factor in obtaining complete hydrolysis of edestin in a 24-hour period. Although there is no significant decrease in serine and threonine yields at this temperature, more sensitive methods are necessary to measure any change in the degree of destruction of these amino acids.

Bovine Serum Albumin.—As compared to edestin, bovine serum albumin is somewhat easier to hydrolyze. The amino acid analyses are shown in Table II. Under the limits of experimental error of the method of estimation, maximum yields of most of the neutral and acidic amino acids can be obtained at 105° for 24 hours when R/S = 20/1. The standards of comparison for complete hydrolysis are the literature values reported by Moore and Stein.[10] Increasing the temperature to 110°, column 3, shows a significant increase in the yield of free glutamic acid. Pyrrolidonecarboxylic acid estimations were not carried out on the hydrolysates in columns 2 and 3.

TABLE I

RECOVERIES OF THE NEUTRAL AND ACIDIC AMINO ACIDS OF EDESTIN UNDER VARIOUS CONDITIONS OF HYDROLYSIS

	1	2	3	4	5
Catalyst	Dowex 50 0.05 *N* HCl	Dowex 50 0.05 *N* HCl	Dowex-NO_2 0.05 *N* HCl	Dowex 50 0.05 *N* HCl	6 *N* HCl
Hydrolysis time, hr.	90	24	24	24	24
Resin/substrate, R/S	5/1	20/1	20/1	20/1	
Temp., °C.	100	105	105	110	105
Amino acids	Mmoles/g. protein				
Aspartic acid	0.90	0.88	0.81	0.92	0.92
Threonine	.28	.27	.23	.28	.28
Serine	.52	.48	.48	.50	.51
Glutamic acid	.55	.80	.88	.92	1.26
	1.12			1.25	
Pyrrolidonecarboxylic acid	.57	..	..	.33	
Glycine	.52	.54	.48	.55	0.53
Alanine	.48	.46	.45	.49	.49
Valine	.35	.23	.21	.41	.41
Isoleucine	.26	.24	.28	.33	.33
Leucine	.50	.50	.50	.58	.58
Tyrosine plus phenylalanine	.61	.46	..	.54	.54

TABLE II

RECOVERIES OF THE NEUTRAL AND ACIDIC AMINO ACIDS OF BOVINE SERUM ALBUMIN UNDER VARIOUS CONDITIONS OF HYDROLYSIS

	1	2	3	4
Catalyst	Dowex 50 0.05 *N* HCl	Dowex 50 0.05 *N* HCl	Dowex 50 0.05 *N* HCl	Lit. values
Hydrolysis time, hr.	100	24	24	
Resin/substrate, R/S	5/1	20/1	20/1	
Temp., °C.	100	105	110	
Amino acids	Mmoles/g. protein			
Aspartic acid	0.68	0.81	0.83	0.82
Threonine	.94	.51	.49	.49
Serine		.44	.43	.40
Glutamic acid	.49	.72	.85	1.12
	0.72			
Pyrrolidonecarboxylic acid	.23	..	..	
Glycine	.94	.24	.28	0.24
Alanine		.72	.71	.70
Valine	.43	.47	.48	.51
Isoleucine	1.05	.20	.21	.20
Leucine		.89	.94	.94
Tyrosine plus phenylalanine	0.69	.69	.65	.68

Insulin.—Attempts to hydrolyze zinc insulin were not successful. In each case, heating insulin with the resin and 0.05 *N* HCl and subsequent cooling resulted in the formation of an opaque, thixotropic gel which contained the resin dispersed throughout. Apparently conditions were favorable for the aggregation of the insulin molecules into fibrils and spherites which make the substrate resistant to hydrolysis. The phenomenon of insulin aggregation has been reported by Waugh.[11]

The condition of molecular aggregation may be eliminated by first oxidizing the disulfide bonds of insulin with perfor-

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mic acid. One hundred and fifty mg. of oxidized insulin was hydrolyzed with 3 g. of Dowex 50 and 15 ml. of 0.05 *N* HCl for 24 hours at 105°. Estimation of free amino acids indicated that, among the neutral and acidic amino acids, only aspartic acid and glycine were completely liberated. Insulin, even in its oxidized form, represents a protein which is difficult to hydrolyze and would require temperatures of the order of 110° under the conditions employed.

Casein and Tryptophan.—An attempt was made to hydrolyze casein and at the same time to protect tryptophan which is destroyed under ordinary conditions of resin hydrolysis. Fifteen ml. of 0.296 *N* H_2SO_3 was substituted for 0.05 *N* HCl to provide reducing conditions according to Pederson and Baker.[12] To the H_2SO_3 were added 150 mg. of casein and 3 g. of Dowex 50; the mixture was sealed in a Pyrex tube and heated at 105° with rotation for 20 hours. The products of hydrolysis were separated on a 15-cm. column of Dowex 50 and the 1-ml. eluate fractions were examined for tryptophan by the method of Graham, *et al.*[13] No tryptophan is found under these conditions.

In order to obtain essentially complete hydrolysis of edestin, oxidized insulin and bovine serum albumin in a 24-hour period by ion-exchange resin catalysis, it is necessary to use a large excess of Dowex 50 and temperatures of the order of 110°. The peptides of glutamic acid, leucine, isoleucine and valine have relatively high stabilities and, as a result, they persist through the final stages of hydrolysis. The use of H_2SO_3 in conjunction with Dowex 50 does not protect tryptophan from destruction during the hydrolysis of casein.

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Acetylation of Some Aminopyrimidines

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In the synthesis of various purines it is usual to combine 4,5-diaminopyrimidines, which may also have other substituents such as amino, hydroxyl, mercapto, etc., in the 2- and/or 6-position, with suitable acid derivatives. Invariably it is assumed that the acid derivative acylates the 5-amino position preferentially if not exclusively, and this 5-acylamino compound is subsequently cyclized to the purine. No report has been found describing simultaneous acylation of amino groups in the 2-, 4- or 6-positions of the pyrimidine ring. Since no systematic work has been reported on the acylation of amino groups in positions other than the 5-position, it seemed to be of interest to study the acetylation of several simple pyrimidines bearing one or more amino groups along with certain other substituents in the 2-, 4- and 6-positions of the pyrimidine ring.

Although no systematic work has been reported on the acylation of assorted aminopyrimidines occasional isolated instances have been found in which, incidental to other work, the preparation of some monoacetylaminopyrimidines has been described. Thus, for example, Wheeler[1] reported 4-acetylaminopyrimidine. Wheeler and Johnson[2] have made 2-acetylamino-4-hydroxypyrimidine which has also been made and included in this paper for comparison purposes. Although all the cases where an individual aminopyrimidine has been acetylated for protective or preparative purposes cannot be included here, one very recent example will be mentioned. Baker and co-workers[3] have acetylated 2-methylmercapto-4-amino-6-dimethylaminopyrimidine to give the 4-acetylamino product. This paper, important in adducing evidence for the structure of the purine antibiotic puromycin, is significant in the current work in that these authors[3] have seemingly established the position of acetylation as on the 4-amino group.

The substituted pyrimidines used in the acetylation experiments were 2-aminopyrimidine (I), 2-amino-4-hydroxypyrimidine (II), 2-amino-4-piperidino-6-methylpyrimidine (III), 2-amino-4,6-dihydroxypyrimidine (IV), 4-amino-2,6-dihydroxypyrimidine (V), 2,4-diamino-6-hydroxypyrimidine (VI) and 2,4,6-triaminopyrimidine (VII). A standard acetylation procedure was used for all the compounds, and this is described in the Experimental part. The results and analytical data are summarized in Table I.

In general it can be said that where the number of amino groups present was equal to or greater than the number of hydroxy groups in a particular pyrimidine molecule acetylation of all the amino groups resulted under the conditions used, and the products formed were stable to various purification procedures. When more hydroxyl groups than amino were present on a molecule as in compounds IV and V, acetylation was not easy or complete.

Compound IV, malonylguanidine, gave less than 50% yield of an insoluble substance different in certain properties from IV. This product was extremely difficult to purify since it was nearly insoluble in the variety of solvents tried. It gave analyses which did not check with the theoretical for either the starting compound or a monoacetyl derivative, but which were much closer to the values for the former. Malonylguanidine can be purified either by solution in alkali and reprecipitation with acid or by the reverse process. The acetylation product dissolved less readily in either acid or alkali than the parent compound, and then could not be reprecipitated by neutralization.

Compound V gave no acetylation product that could be isolated, and after several reprecipitations from dilute alkali with dilute acid all of the V was recovered unchanged.

The simpler monoacetylaminopyrimidines, obtained from compounds I, II and III, could be purified easily by recrystallization from common organic solvents. The products from VI and VII on the other hand were much more insoluble and more difficult to purify. The acetyl derivative of VI was soluble in cold alkali and could be reprecipitated unchanged by addition of acid. It was best purified, however, by dissolving it in a little cold concentrated hydrochloric acid from which it separated unchanged on simple dilution with water. The product so obtained analyzed well for the diacetylamino

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