thyroacetic acids, were the most potent compounds tested. In the test system employed, VII and XIII were as potent as L-T_3, one of the most active hypocholesteremic thyromimetic agents reported in the literature.

It can be seen that replacement of the 3′-iodine atom in VII with an isopropyl group has had no effect on hypocholesteremic activity (compare VII with XIII). This finding is consistent with previous observations noted with compounds containing an alanine side chain.[10] Although the number of compounds screened was quite small it appears that a structure–function relationship does exist. Maximum activity resides in those compounds with a two-carbon side chain and a 3′-iodine atom or isopropyl group (VII, VIII, and XIII). Increasing or decreasing the length of the side chain or replacing the 3′-iodine or isopropyl group with hydrogen decreases cholesterol-lowering activity. Formation of the methyl ether in most cases also somewhat lessens hypocholesteremic activity (compare VII with VIII).

A more comprehensive study of the thyromimetic activities of VII and XIII has been reported recently.[20]

TABLE III

PLASMA CHOLESTEROL VALUES

Compound no.	Activity[a]
I	0.030
II	< .001
III	.030
IV	.040
V	.025
VI	.006
VII	1.000
VIII	0.500
IX	< .001
X	< .001
XI	.060
XII	.030
XIII	1.000

[a] Activity is expressed in terms of L-T_3 having an arbitrary value of 1.

Acknowledgment.—We wish to express our gratitude to Mr. Roger O'Connor for technical assistance throughout this study. We also wish to thank Mrs. Doris Ralston and staff for elemental analyses.

(20) C. M. Greenberg, B. Blank, F. R. Pfeiffer, and J. F. Pauls, *Am. J. Physiol.*, in press.

Synthesis, Properties, and Enzymatic Reactions of Some Aminoacyladenines[1]

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A series of aminoacyladenines was prepared by a procedure involving the condensation of the carbobenzoxy derivatives of glycine, alanine, valine, leucine, isoleucine, and phenylalanine with adenine by the carbodiimide method with dimethyl sulfoxide as the solvent. Chemical, spectral, and chromatographic characteristics of the above compounds are described. Neither rat organ homogenates nor a variety of proteolytic enzymes hydrolyzed the aminoacyladenines, with the exception of leucine aminopeptidase, which showed activity against L-leucyladenine.

Interest in aminoacylamidopurines and specifically aminoacyladenines stems from the observation that ribonucleic acids (RNA) isolated by a variety of methods contain amino acids or peptides.[3-5] Aside from the binding of amino acids by ester linkage to the terminal ribose moiety of soluble RNA,[6] other binding sites have not been definitely established. It is conceivable that high molecular weight RNA may bind amino acids as acid anhydrides similar to the amino acid nucleotidates found in yeast[7] or as amides by combination of the carboxyl group of the amino acids with the amino nitrogen of adenine, guanine, and the cytosines.

This paper describes the synthesis and properties of a series of aminoacyladenines as a preliminary step in establishing whether or not such compounds occur in nucleic acids or nucleoproteins.

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(2) Stanford Research Institute, Menlo Park, California. This work was taken in part from a doctoral dissertation presented to the Graduate College of the University of Vermont, 1962.

(3) J. L. Potter and A. L. Dounce, *J. Am. Chem. Soc.*, **78**, 3078 (1956).

(4) V. Habermann, *Biochim. Biophys. Acta*, **32**, 297 (1959).

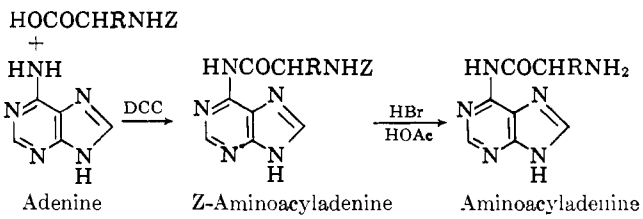
(5) U. Z. Littauer and H. Eisenberg, *ibid.*, **32**, 320 (1959).

(6) M. Hoagland, *ibid.*, **16**, 288 (1955).

(7) A. H. Cook and G. Harris, *Rev. Pure Appl. Chem.*, **10**, 61 (1960).

Results and Discussion

The aminoacyladenines were prepared by condensing a carbobenzoxyamino acid (Z-amino acid) and adenine in dimethyl sulfoxide solution with N,N′-dicyclohexylcarbodiimide (DCC) and decarbobenzoxylating the product with anhydrous HBr in glacial acetic acid.



Adenine Z-Aminoacyladenine Aminoacyladenine

The values for C, H, and N (Table I) of both carbobenzoxy- and aminoacyladenines support a structure having an amino acid:adenine ratio of 1.

The physical properties and behavior on paper chromatograms of both carbobenzoxy- and aminoacylade-

TABLE I

MELTING POINTS AND ANALYTICAL DATA OF AMINOACYLADENINES

Compound[a]	Formula	M.p., °C.[b]	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found
Z-Gly-Ad	$C_{15}H_{14}N_6O_3$	234–235	55.3	55.4	4.30	4.23	25.8	25.6
Z-L-Ala-Ad	$C_{16}H_{16}N_6O_3$	218–220	56.5	56.6	4.71	4.61	24.7	24.3
Z-DL-Ala-Ad	$C_{16}H_{16}N_6O_3$	231–233	56.5	55.9	4.71	4.70	24.7	24.9
Z-DL-Val-Ad	$C_{18}H_{20}N_6O_3$	183–185	58.8	59.2	5.44	5.45	22.8	22.1
Z-L-Leu-Ad	$C_{19}H_{22}N_6O_3$	149–150	59.7	59.8	5.70	6.05	22.0	22.2
Z-L-Ileu-Ad	$C_{19}H_{22}N_6O_3$	196–197	59.7	59.9	5.70	6.13	22.0	22.0
Z-DL-Ileu-Ad	$C_{19}H_{22}N_6O_3$	182–184	59.7	60.3	5.70	5.90	22.6	22.2
Z-L-Phe-Ad	$C_{22}H_{20}N_6O_3$	207–209	63.5	63.5	4.80	5.07	20.2	20.7
Z-DL-Phe-Ad	$C_{22}H_{20}N_6O_3$	181–183	63.5	63.5	4.80	4.90	20.2	20.2
Adenine[d]	$C_5H_5N_5$	360 dec.	44.4		3.70		51.8	51.6
Gly-Ad	$C_7H_8N_6O$	165–280 dec.	43.7	43.2	4.15	4.23	43.7	43.4
L-Ala-Ad	$C_8H_{10}N_6O$	152–154	46.6	43.8	4.86	5.25	40.7	39.1
DL-Val-Ad	$C_{10}H_{14}N_6O$	146–148	51.4	49.5	5.99	5.93	35.9	35.8
L-Leu-Ad	$C_{11}H_{16}N_6O$	138–140	53.3	52.6	6.45	6.53	33.8	34.3
L-Ileu-Ad	$C_{11}H_{16}N_6O$	145–147	53.3	53.4	6.45	6.32	33.8	33.8
L-Phe-Ad	$C_{14}H_{14}N_6O$	102–105	59.8	58.4	4.96	4.86	29.9	29.1

[a] The abbreviations used are those accepted by the *Journal of Biological Chemistry* for naming of peptides. [b] Capillary melting points are corrected. [c] Carbon, hydrogen, and nitrogen analyses were done by Weiler and Strauss, Oxford, England. [d] Adenine is included as a standard for comparison.

TABLE II

PHYSICAL AND CHROMATOGRAPHIC PROPERTIES OF AMINOACYLADENINES

Compound	λ_{max}, mμ[a]	ϵ_{max} × 10^{-3}	Optical rotation[b] $[\alpha]^t_D$, deg.	t°, C.	R_f in solvent[c] A	B	C
Z-Gly-Ad	280	13.0			0.79	0.40	0.73
Z-L-Ala-Ad	281	13.3	−68.0	28	0.86	0.56	0.81
Z-DL-Val-Ad	281	13.5			0.90	0.65	0.85
Z-L-Leu-Ad	282	13.0	−45.0	26	0.91	0.69	0.86
Z-L-Ileu-Ad	282	13.0	−28.5	24	0.92	0.66	0.86
Z-L-Phe-Ad	282	13.5	+21.5	21	0.90	0.60	0.83
Gly-Ad	280	12.3			0.27	0.016	0.62
L-Ala-Ad	282	12.3	−12.4	24	0.43	0.096	0.71
DL-Val-Ad	282	10.2			0.74	0.30	0.78
L-Leu-Ad	282	12.1	+4.2	27	0.76	0.36	0.81
L-Ileu-Ad	282	12.0	+14.6	23	0.77	0.35	0.78
L-Phe-Ad	282	13.1	+44.0	27	0.70	0.19	0.74

[a] Concentrations and solvent employed were 5–7 × 10^{-5} *M* in methanol. [b] Optical rotations in all cases were determined on 1% solutions in methanol. [c] Solvent A: ethanol–*M* ammonium acetate pH 7.5, 7:3. Solvent B: methanol. Solvent C: methanol–glacial acetic acid–water, 12:3:5. In all cases only one spot was observed. Spots were located visually with an ultraviolet lamp.

nines are shown in Table II. The ultraviolet absorption spectrum of carbobenzoxy-L-isoleucyladenine, (Z-L-Ileu-Ad) with an absorption maximum at 280 mμ (Fig. 1) is characteristic of all the carbobenzoxyaminoacyladenines. Removal of the carbobenzoxy group results in minor changes in absorption in regions below 260 and above 300 mμ (Fig. 2).

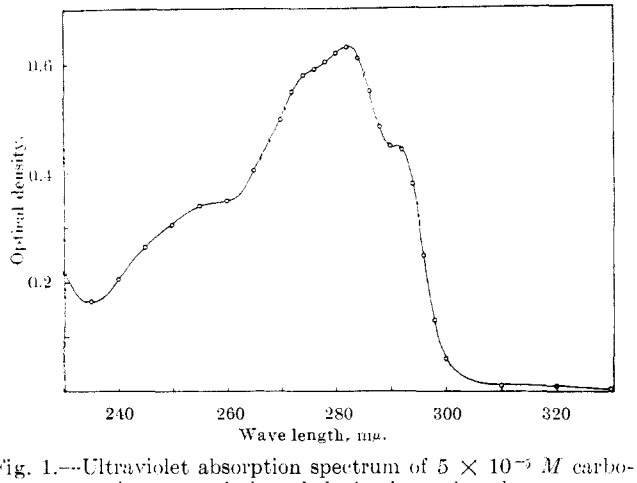


Fig. 1.—Ultraviolet absorption spectrum of 5 × 10^{-5} *M* carbobenzoxy-L-isoleucyladenine in methanol.

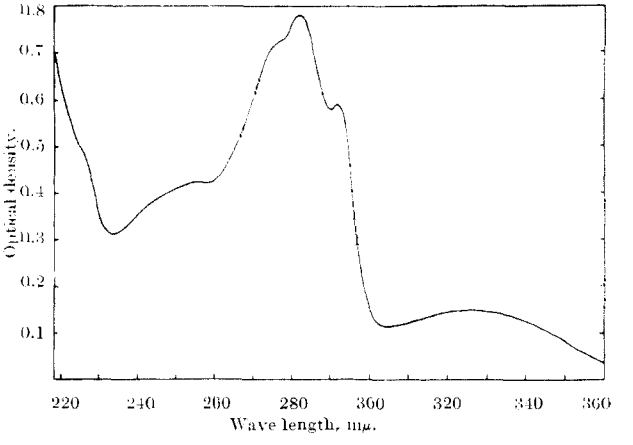


Fig. 2.—Ultraviolet absorption spectrum of 5.8 × 10^{-5} *M* L-isoleucyladenine in methanol.

Evidence for the structure of carbobenzoxy- and aminoacyladenines was obtained from deamination of these compounds. Wilson[8] and Schein[9] have shown that adenine yields 1 mole of nitrogen with nitrous acid at room temperature. Carbobenzoxyaminoacyladenines

(8) D. W. Wilson, *J. Biol. Chem.*, **56**, 183 (1923).
(9) A. H. Schein, *J. Med. Pharm. Chem.*, **5**, 302 (1962).

did not yield any nitrogen with nitrous acid. A mixture of 1 mole of adenine and 1 mole of phenylalanine yielded 2 moles of amino nitrogen, while 1 mole of Gly-Ad, L-Ala-Ad, and DL-Val-Ad each yielded 1.0 mole of amino nitrogen. Slightly higher yields of nitrogen were obtained from L-Leu-Ad (1.2 moles), L-Ileu-Ad (1.2 moles), and L-Phe-Ad (1.1 moles). The excess nitrogen from the latter three compounds may be derived from partial hydrolysis of the aminoacyladenines in the acetic acid–nitrous acid solution during deamination. It is evident that there are no free amino groups in carbobenzoxyaminoacyladenines and one free amino nitrogen in the aminoacyladenines. Of the various sites for substitution on the adenine molecule, these data support substitution on the 6-amino group. Deamination of adenine containing an amino acid substituent at any position but the 6-amino N would yield two moles of nitrogen per mole of compound.

Treatment of carbobenzoxyaminoacyladenines with either *N* HCl or *N* NaOH at 100° for 1 hr. resulted in complete hydrolysis of the amide bond between the carbobenzoxyaminoacyl moiety and adenine. Milder conditions, 0.5 *N* NaOH for 20 min. at 100°, sufficed to hydrolyze aminoacyladenines to yield 1 mole of free adenine per mole of aminoacyladenine. Calculation of the extent of hydrolysis was based on the absorbance ratio 260/275 mμ and is described in a subsequent section. The reaction was also followed by paper chromatography and, where possible, by measuring the increase in ninhydrin-reacting material during the heating period.

The ultraviolet absorption spectra of aminoacyladenine solutions in water and in methanol changed on standing at room temperature for short periods of time. Since the compounds were to be utilized for enzymatic studies it was necessary to determine their stability in aqueous solutions at various pH values. In acidic media the absorption maximum of Gly-Ad is at 275 mμ. After 90 hr. at pH 2 the peak at 275 mμ is replaced by a broad absorption band with a peak at 263 mμ (Fig. 3). At pH 12 (Fig. 4) the absorption peak of

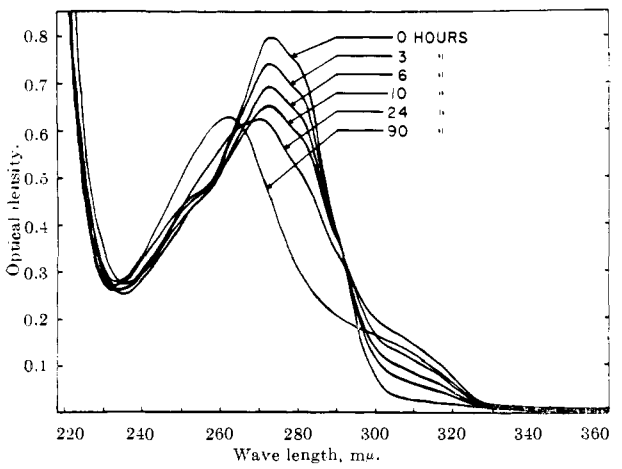


Fig. 3.—Changes in the ultraviolet absorption spectrum of 6.0 × 10^{-5} *M* glycyladenine solution as a function of time at pH 2.

Gly-Ad at 280 mμ is rapidly replaced by a peak at 269 mμ, representing hydrolysis to adenine. Confirmation was obtained by chromatography of aliquots at different time intervals; there was an increase in intensity of an

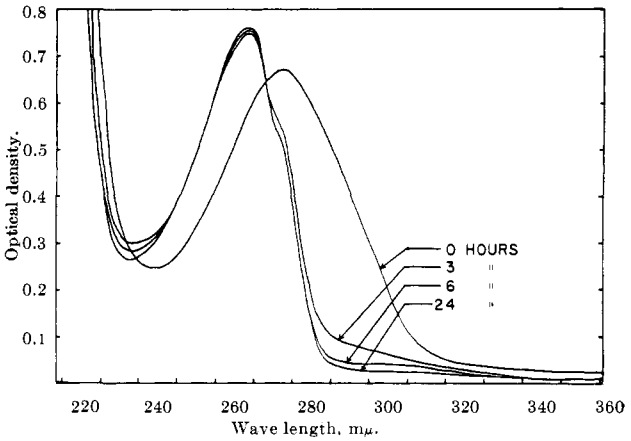


Fig. 4.—Changes in the ultraviolet absorption spectrum of a 6.0 × 10^{-5} *M* glycyladenine solution as a function of time at pH 12.

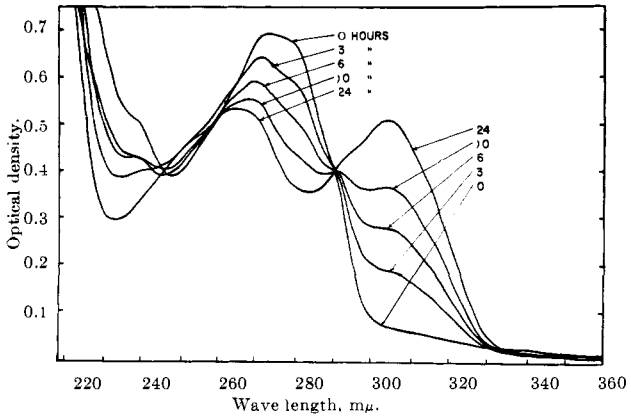


Fig. 5.—Changes in the ultraviolet absorption spectrum of a 6.0 × 10^{-5} *M* glycyladenine solution as a function of time at pH 7.

adenine spot, a decrease in intensity of the Gly-Ad spot, and an increase with time of a ninhydrin-reactive spot corresponding to glycine. At pH 7 (Fig. 5), major spectral changes in Gly-Ad occur in a region (310 mμ) not associated with simple hydrolysis, the significance of which has not been established.

Stability studies were also carried out at other pH values and with other aminoacyladenines. The results were not significantly different from those with Gly-Ad.

Enzymatic Studies.—In order to establish whether aminoacyladenines may or may not exist in tissues, the susceptibility of these compounds to hydrolysis by various enzymes and tissue preparations was studied. Commercial preparations of pepsin, trypsin, α-chymotrypsin, papain, ficin, bromelin, pancreatin, acylase, and leucine aminopeptidase were incubated with aminoacyladenines. Hydrolysis was measured by the change in absorbance ratio 260/275 mμ. As a standard for the calculation of hydrolysis, the absorbance of different proportions of pure aminoacyladenine and adenine in acid solution was measured at 260 and 275 mμ. The absorbance ratio 260/275 was determined for each proportion. This ratio is a constant for each pure compound; it varies linearly from 0.56 for aminoacyladenine to 1.71 for adenine and hence can be used to calculate the extent of hydrolysis of aminoacyladenines. Incubation mixtures of aminoacyladenines and enzyme were acidified after 1 hr. at 37° and the per cent of hy-

drolysis was determined. Suitable enzyme and substrate controls were carried through the procedure to correct experimental values for absorption by the enzyme and for spontaneous hydrolysis of the substrate.

No significant enzymatic activity was observed for the aminoacyladenines except for the pair, L-leucyladenine and leucine aminopeptidase. In this case approximately 25% hydrolysis occurred. Similar experiments were conducted using rat organ homogenates of liver, kidney, brain, heart, spleen, intestinal mucosa, muscle, and lung prepared in 0.25 *M* sucrose. No significant hydrolysis above that of the experimental variations in the substrate and enzyme controls was obtained with any of the homogenates.

Samples of aminoacyladenines were tested for growth inhibitory effects against *in vitro* cultures of cells of the KB line at the Cancer Chemotherapy National Service Center. None of the compounds showed sufficient antitumor activity to warrant further testing.

Experimental

6-(N-Carbobenzoxy)aminoacyladenines.—The N-carbobenzoxy derivatives of glycine, L-alanine, DL-valine, L-leucine, L-isoleucine, and L-phenylalanine were prepared essentially by the method of Bergmann and Zervas.[10] Adenine (Nutritional Biochemicals Co.), the respective carbobenzoxyamino acid, and N,N'-dicyclohexylcarbodiimide (Aldrich Chemicals Co.) in the molar proportion 1:1:1 were dissolved in hot (80°) dimethyl sulfoxide (0.4 *M* adenine) and allowed to cool at room temperature for 48 hr. Large needles of dicyclohexylurea formed and were removed by filtration. The filtrate was evaporated to dryness at 70° *in vacuo* and the residue was suspended in warm toluene and filtered. The insoluble residue was extracted successively in a Soxhlet extractor with toluene, ethyl acetate, and ether. The insoluble residue was finally washed with three portions of 0.5 *N* HCl to remove unreacted adenine, then with H_2O, and dried.

In the preparation of Z-Val-Ad, Z-Leu-Ad, Z-Ileu-Ad, and Z-Phe-Ad, removal of dimethyl sulfoxide yielded an oily suspension rather than a solid. The oily suspensions were miscible with hot toluene. Residual adenine was removed by filtration, and, after removal of the toluene from the filtrate *in vacuo*, a clear oil was obtained. Addition of ether to the oils of Z-DL-Val-Ad and Z-L-Phe-Ad resulted in the formation of a heavy phase which solidified on standing for 24 hr. at room temperature. With Z-L-Leu-Ad and Z-L-Ileu-Ad addition of ether did not cause the formation of a heavy phase, but when the clear ethereal solutions were shaken with 5% $NaHCO_3$ and allowed to stand at room temperature for 24 hr., the carbobenzoxyaminoacyladenines crystallized in plates at the ether–aqueous $NaHCO_3$ interphase. Recrystallization was effected from ethanol-ether. Yields varied from 9% for Z-DL-Val-Ad to 30% for Z-L-Phe-Ad, with yields of approximately 18% for Z-Gly-Ad, Z-L-Ala-Ad, Z-L-Leu-Ad, and Z-L-Ileu-Ad.

Aminoacyladenines.—Treatment of the carbobenzoxyaminoacyladenines with a 34% solution of anhydrous HBr in glacial acetic acid[11] resulted in removal of the carbobenzoxy groups. Ten grams of HBr solution per gram of carbobenzoxy compound was stirred magnetically for 1 hr. at room temperature. Subsequent dilution with anhydrous ether yielded a white solid which was separated by filtration and washed with ether. The hydrobromide salt (90–100% yield) was dried *in vacuo* over NaOH pellets for 24 hr.

Removal of HBr was effected by treatment of a chilled suspension of the salt in chloroform with an equimolar quantity of triethylamine. After being stirred for 10 min., the thick white precipitate which formed was collected on a filter and washed successively with cold chloroform and ether. The dry residue was washed with a minimal amount of ice-cold water and dried *in vacuo* over P_2O_5. Yields varied from 43–92% (35–47% overall) for the various aminoacyladenines.

Deamination with Nitrous Acid.—The calculated amount of aminoacyladenine required to yield 0.5 mmole of nitrogen was dissolved in 10 ml. of 50% acetic acid and deaminated for 30 min. in a mixture of 30% $NaNO_2$ and glacial acetic acid in a Van Slyke amino nitrogen apparatus.[12] The volume of nitrogen evolved was corrected for temperature, pressure, and a reagent blank. Controls of adenine alone, an amino acid, or a mixture of adenine and amino acid yielded in each case the expected molar quantities of nitrogen.

Stability Studies.—Freshly prepared 5×10^{-3} *M* aminoacyladenine in methanol was immediately diluted to 5×10^{-5} *M* with aqueous solutions of the following composition: 0.01 *N* HCl, pH 2.0; 0.01 *M* phosphate buffer, pH 7.0; and 0.01 *N* NaOH, pH 12.0. Spectral curves were recorded in a Beckman DK 2 recording spectrophotometer at successive time intervals against appropriate blank solutions.

Enzymes and Homogenates.—The activity of pepsin, trypsin (Nutritional Biochemicals Corp.), papain, ficin (Worthington Biochemical Corp.), α-chymotrypsin, pancreatin, and bromelin (Mann Research Lab.) was measured by the method of Anson using denatured hemoglobin (Nutritional Biochemical Corp.) in 0.1 *M* phosphate buffer, pH 7.4.[13,14] The plant proteolytic enzymes papain, ficin, and bromelin were activated by the addition of 5×10^{-3} *M* cysteine. The proteolytic activity of pepsin was determined at pH 2. One milliliter of a solution containing 10–100 μg. of enzyme in the appropriate buffer was added to 5 ml. of a 2% denatured hemoglobin solution similarly buffered. The mixture was incubated at 37° for 30 min. The reaction was terminated by the addition of 5% trichloroacetic acid. After filtration, the solution was made alkaline by the addition of 0.5 *N* NaOH and was treated with the Folin–Ciocalteau reagent.[15] The absorbance was measured at 660 mμ against a similarly-treated blank. The activities in terms of μmoles of tyrosine formed/mg. enzyme/ml./30 min. were calculated from values of standard tyrosine solutions treated in the same manner. In these units, the activities of each enzyme were: pepsin, 8.1; trypsin, 2.7; α-chymotrypsin, 1.8; papain, 1.2; ficin, 0.9; bromelin, 0.3; and pancreatin, 0.3.

Acylase (Pentex Inc.) activity was determined by measuring the decrease of absorbance of N-acetyl-DL-methionine at 239 mμ.[16] Leucine aminopeptidase (Worthington Biochemical Corp.) was assayed by measuring the decrease in absorbance of L-leucine amide at 238 mμ.[6] The activities of these two enzymes, respectively, were 1.4 and 8.1 mmoles of substrate hydrolyzed/mg. of enzyme/ml./30 min.

A suspension containing 100 mg. wet weight of each homogenate[18] was added to the aminoacyladenines in pH 7.4 phosphate buffer and incubated for 1 hr. at 37°. The reaction was halted by the addition of trichloroacetic acid, the solution was filtered, and the absorbance at 260 and 275 mμ was measured.

(10) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(11) D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954).

(12) D. D. Van Slyke, *J. Biol. Chem.*, **12**, 275 (1912).

(13) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

(14) R. M. Hoveloc, "Methods in Enzymology," Colowick and Kaplan, Vol. II, Ed., Academic Press, New York, N. Y., 1955, p. 3.

(15) O. Folin and V. Ciocalteau, *J. Biol. Chem.*, **73**, 627 (1927).

(16) M. A. Metz and R. J. Seldeatec, *Biochim. Biophys. Acta*, **27**, 168 (1958).

(17) F. Binkley and C. Toveos, *Arch. Biochem. Biophys.*, **86**, 201 (1960).

(18) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, in "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1957.