[CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE]

Cobalt Ion Activation of Renal Acylase I

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RECEIVED APRIL 5, 1956

Acylase I, which was fully active after dialysis or in the presence of 1×10^{-3} *M* Versene and which contained no demonstrable Co^{++}, was accelerated in activity by addition of Co^{++} toward certain acetyl-L-amino acids and depressed in activity toward others. All acetyl-D-amino acids hydrolyzable by acylase I were cleaved at a greatly accelerated rate by this enzyme in the presence of Co^{++}. Maximal effective concentrations of Co^{++} were found to vary directly with the substrate concentration. Attempts to replace Co^{++} with other divalent ions were unsuccessful, for they either had no effect on the rate or else inhibited the enzyme completely. When Co^{++} was added to acylase I and the mixture dialyzed, centrifuged and lyophilized, the residual protein was found to contain cobalt to an amount which varied directly up to a maximum value with the concentration of the cobalt originally used. This preparation, referred to as acylase IA, hydrolyzed all substrates at a rate in excess of that of acylase I, including those whose hydrolysis was ordinarily depressed by the addition of Co^{++} to acylase I. Versene at 1×10^{-3} *M* had no effect on the activity of acylase IA.

Earlier studies revealed that the influence of added Co^{++} on the hydrolysis of a number of N-chloroacetyl-L-amino acids by renal acylase I varied with the nature of the substrate.[1] Thus, at 1×10^{-2} *M* Co^{++} concentration, the hydrolytic rate of chloroacetyl-L-threonine was increased by 33%, that of chloroacetyl-L-aspartic acid by 610%, whilst that of chloroacetyl-L-methionine was decreased by 62%.[2] There appeared in general to be some sort of relation between the capacity to be activated or inhibited by Co^{++} and the susceptibility of the substrate to the action of the enzyme, inasmuch as the less susceptible substrates were activated, and the more susceptible substrates were inhibited, in the presence of added Co^{++}. It seemed of interest to reëxamine these observations with closer attention to such details as variations of metal, enzyme and substrate concentrations, and to employ for this purpose a number of optically enantiomorphic N-acetyl amino acids as substrates.

Effect of Acylase I Concentration on Hydrolytic Rates.—When substrates of widely varying susceptibilities are simultaneously studied, it is usual to vary the enzyme levels accordingly. In the presence of a constant amount of Co^{++} the hydrolysis of a rapidly cleaved substrate with a relatively small amount of enzyme will be conducted in the presence of a higher cobalt:protein ratio than will be that of a more slowly cleaved substrate with a necessarily larger amount of enzyme. It was therefore desirable to study the effect of Co^{++} on the hydrolysis of a single substrate at different protein concentrations. Before undertaking this study the effect of enzyme concentration on activity was investigated, and it was observed that a linear relation was maintained for a 25-fold variation of enzyme protein level for the hydrolysis of acetyl-L-alanine. A similar relation was obtained for the hydrolysis of acetyl-L-isoleucine over a 100-fold variation in acylase I levels in the presence (1×10^{-3} *M*) and absence of added cobalt ions. Results on a study of the degree of activation by Co^{++} on acetyl-L-isoleucine at three quite different enzyme levels are given in Table I. It is evident that there is no change in the degree of activation with the same concentration of Co^{++} comparable to the very great changes in the enzyme levels employed. Thus, in the presence of a constant amount of substrate and of Co^{++}, under conditions in which the hydrolytic rate is relatively independent of the enzyme concentration, the degree of activation is apparently constant.

TABLE I

EFFECT OF Co^{++} ON THE HYDROLYTIC RATE OF ACETYL-L-ISOLEUCINE AT THREE DIFFERENT ACYLASE I LEVELS[a]

Mg. protein N present	Hydrolytic rate in the presence of Co^{++} at concn. 0	1×10^{-4} *M*	1×10^{-3} *M*	1×10^{-2} *M*	4×10^{-2} *M*
0.01	960	2150	2920	2960	1750
0.1	1100	1940	2590	3230	2190
1.0	1080	2340	2280	2100	1620

[a] Rates expressed as μmoles of substrate hydrolyzed per hour per mg. N in the reaction mixture at *p*H 7.0 at 37°; substrate at 0.017 *M* concentration; hydrolysis figures taken within 30% of complete cleavage so as to approximate a zero order reaction.

Specificity of Co^{++} as an Activator for Acylase I.—Acetyl-L-alanine at 0.017 *M* was used as the substrate for acylase I at *p*H 7.0 (veronal acetate buffer) in the presence of various acetates of Mn^{++}, Mg^{++}, Ca^{++}, Zn^{++}, Ni^{++} and Cu^{++} in concentrations ranging from 1×10^{-4} to 5×10^{-2} *M*. Copper and zinc salts in all concentrations inhibited the enzyme completely, whilst the other metals had no effect at the lower concentrations and a mild to severe inhibitory effect at the higher concentrations. As far as these ions are concerned, Co^{++} appears to function uniquely as an activator for certain substrates of acylase I.

Interaction Time of Cobalt and Acylase I.—In view of the slow activation of many enzymes by added metals, the effect of preincubating the enzyme with Co^{++} was studied for 2 minutes to 30 minutes of time in the presence of 1×10^{-3} *M* Co^{++} with acetyl-L-alanine at 0.017 *M* concentration as the substrate. In the absence of added Co^{++} the rate was 5800 μmoles of substrate hydrolyzed per hour per mg. N. Preincubated with Co^{++} for 2, 5, 10, 15 and 30 minutes at 37° and then mixed with the substrate, the hydrolytic rates were, respectively, 8750, 8940, 9030, 9480 and 9760

(1) K. R. Rao, S. M. Birnbaum, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **198**, 507 (1952).

(2) Renal acylase II, whose specific substrate is N-acyl-L-aspartic acid, is not affected by the presence of added Co^{++} or Mn^{++}. Even in the presence of Co^{++}, the hydrolytic rate of chloroacetyl-L-aspartic acid with acylase I falls considerably short of that with acylase II.[1] The relatively low hydrolytic action of acylase I on chloroacetyl-L-aspartic acid in the absence of added Co^{++} is not likely due to traces of adherent acylase II in the former preparation.

μmoles of substrate hydrolyzed per hour per mg. N. It would appear that under these conditions the enzyme was rapidly activated shortly after mixing with the Co^{++}, and thereafter was further activated at a very slow rate. When acylase I was preincubated with Co^{++} at 4×10^{-2} *M* concentration, the activation of the hydrolysis of acetyl-L-alanine was identical for preincubation periods of 2 and of 30 minutes. For nearly all of the work reported in this communication, a preincubation period of 2 minutes at 37° was employed.

Time Course of the Reaction.—Figure 1 shows the time course of the hydrolysis of acetyl-L-alanine at 0.017 *M* concentration by acylase I in the absence and in the presence of different amounts of added Co^{++}. The shapes of the curves are all very similar and suggest that the order of the reaction with respect to the substrate is not appreciably changed by the added metal ions. The initial hydrolytic rate is increased by addition of Co^{++} up to the highest concentration (0.04 *M*) employed when inhibition becomes evident. Included for comparison is the hydrolytic course of acetyl-L-capryline which more nearly approximates zero-order kinetics than does that of acetyl-L-alanine; this suggests that the Michaelis–Menten constant may be larger for the latter than for the former substrate. The possibility that the same enzyme may follow zero or first-order kinetics depending upon the substrate was shown by Smith and Spackman in the case of leucine aminopeptidase.[3]

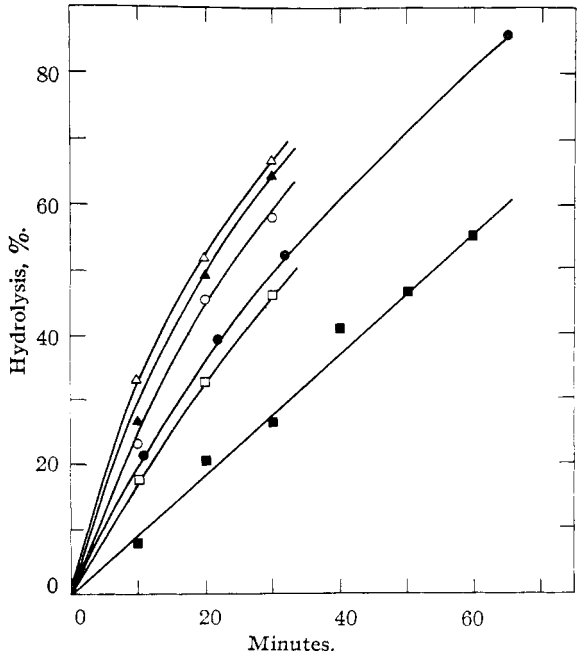

Fig. 1.—Relation between hydrolysis and time of incubation at 37° and *p*H 7.0. Substrate–acetyl-L-alanine at 0, ●, 1×10^{-4} *M* ○, 1×10^{-3} *M* △, 1×10^{-2} *M* ▲ and 4×10^{-2} *M* □ concentrations of Co^{++} added. Substrate–acetyl-L-capryline ■ with no added Co^{++}. Concentration of substrate 0.017 *M*, temperature 37°, 0.01 mg. protein N in each digest.

(3) E. L. Smith and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955).

Effect of Variation in Co^{++} Concentration.—The effect of varying amounts of added Co^{++} was studied at a single concentration (0.017 *M*) of a number of substrates (Table II). The D-isomers of the acetyl derivatives of isoleucine, aspartic acid, glutamic acid, arginine and histidine have been omitted from the table inasmuch as neither in the absence, nor in the presence of added Co^{++} at different levels, were they measurably hydrolyzed by acylase I.

Variation in Substrate Concentration at Several Concentrations of Co^{++}.—Results with acetyl-L- and D-alanine at two different concentrations differing by a factor of ten in the presence of various concentrations of Co^{++} are described in Fig. 2. In the case of acetyl-L-alanine at 0.017 *M* concentration, the per cent. increases in rate in the pres-

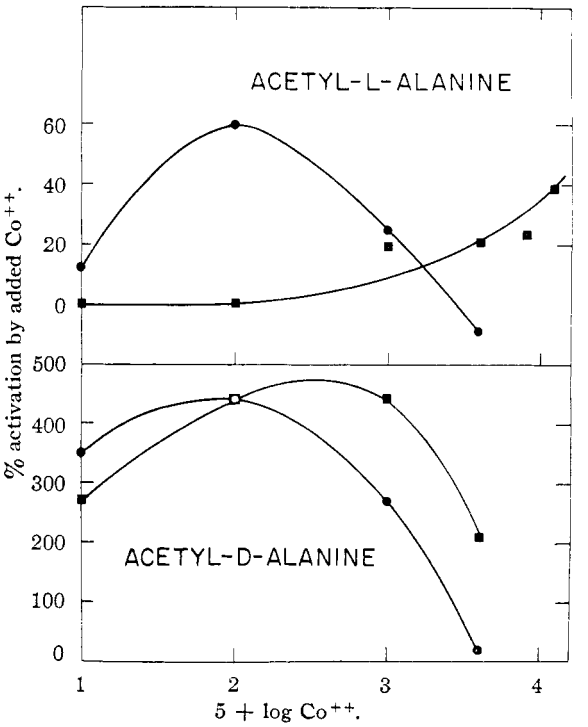

Fig. 2.—Relation between activation by cobalt and substrate concentration. Substrate concentration 0.017 *M* ●; 0.167 *M* ■, *p*H 7.0, temperature 37°. Acetyl L-alanine, 0.01 mg. protein N in each digest; acetyl D-alanine, 1 mg. protein N in each digest.

ence of 1×10^{-4} and 1×10^{-3} *M* Co^{++} are, respectively, 12 and 60; at the higher substrate concentration, namely, 0.167 *M*, there is no effect by added Co^{++} at the above concentrations, and only in the presence of more Co^{++} does an activating effect appear. In the case of acetyl-D-alanine whose hydrolytic rate is so markedly accelerated by Co^{++}, the maximum effective metal ion concentration is shifted to higher levels at the higher substrate concentration but to no such magnitude as that shown by the L-isomer (Fig. 2). Unfortunately, it was not possible to increase the Co^{++} concentration at *p*H 7.0 beyond that shown in the figure, and therefore the maximal activation of acetyl-L-alanine at the higher concentration could not be determined.

TABLE II

EFFECT OF ADDED Co^{++} ON THE HYDROLYTIC RATE OF ACYLASE I[a]

Substrate	Rate without added Co	% increase or decrease in rate on addn. of Co^{++} at concn. 1×10^{-4} M	1×10^{-3} M	1×10^{-2} M	4×10^{-2} M
Acetyl-L-alanine	5800	+12	+60	+25	−9
Acetyl-D-alanine	2.0	+350	+440	+270	+20
Acetyl-L-methionine	25800	−7	−19	−26	−62
Acetyl-D-methionine	10.9	+28	+80	+20	−21
Acetyl-L-isoleucine	970	+124	+240	−280	+82
Acetyl-L-aspartic acid	3.7	+200	+280	+290	+128
Acetyl-L-glutamic acid	3690	+30	+70	+27	−6
Acetyl-L-arginine	1110	−5	−36	−66	−78
Acetyl-L-histidine	740	+11	+31	−44	−75

[a] Rates defined in Table I; Substrate concentrations 0.017 *M*.

Similar results were observed with acetyl-L-isoleucine as substrate at concentrations varying over a sixteen-fold range (Table III). At the lower substrate concentrations, the maximal effective Co^{++} concentration appeared to be in the neighborhood of 1×10^{-2} *M*, whereas at the higher substrate concentrations this maximal concentration was seemingly shifted to a range about 1×10^{-2} to 4×10^{-2} *M*.

TABLE III

DEGREE OF ACTIVATION AND Co^{++} CONCENTRATION AT SEVERAL SUBSTRATE LEVELS FOR ACETYL-L-ISOLEUCINE[a]

Substrate concn., *M*	Rate without added Co^{++}	% activation by cobalt at concn. 1×10^{-4} M	1×10^{-3} M	1×10^{-2} M	4×10^{-2} M
0.021	1040	+83	+170	+210	+130
.083	1360	+80	+178	+365	+280
.167	1320	+77	+175	+367	+360
.333	1250	+85	+146	+450	+430

[a] Rate defined in Table I; 0.01 mg. protein N in each digest.

Inasmuch as for many substrates at 0.017 *M* a Co^{++} concentration of the order of 1×10^{-3} *M* gives rise to maximal activation, the effect of various substrate concentrations was examined at this Co^{++} level (Table IV). Marked differences are observed in the behavior of the various substrates, the hydrolysis of acetyl-L-methionine being inhibited by cobalt at all substrate concentrations studied.

The Cobalt–Acylase Interaction. Acylase IA.— Acylase I is active in the absence of added Co^{++}, and it has been found that in the presence of 0.001 *M* Versene the activity toward acetyl-L-alanine is not affected. A sample of acylase I was found to contain 0.13% of a tan-colored ash. Cobalt could not be detected in this ash either by the chemical procedure of Kitson[4] or by a spectroscopic method sensitive to at least one part in a million.[5] The greater part of the ash was composed of calcium, magnesium, sodium, phosphate and zinc, with traces of aluminum, chromium, manganese, iron, silicon, strontium and barium.

When acylase I was dialyzed against several changes of distilled water, no change was found in its specific activity toward a number of substrates, although only about 65% of the protein was recovered after centrifugation and lyophilization. Similarly, it was found that when acylase I was mixed with cobaltous acetate at 4×10^{-2} *M* in Veronal acetate buffer at *p*H 7.0 for 10 minutes and then dialyzed, no change in the activated condition of the enzyme could be observed, although again only 65% of the protein was recovered after lyophilization. From this observation, a cobalt-activated enzyme containing cobalt in a non-dialyzable form, and referred to hereinafter as acylase IA, was prepared. A number of such acylase IA preparations were made by mixing acylase I at 36 mg. per ml. with cobaltous acetate at various concentrations. The mixtures were dialyzed against several changes of distilled water and then lyophilized. Regardless of how much cobalt was employed in the mixture, the recovery of close to 65% was the same. Table V describes the results on the various preparations, together with the activity of these preparations toward a number of substrates.

Determinations on two preparations of acylase IA which had been derived from a mixture of acylase I and 4×10^{-2} *M* Co^{++} revealed 0.5 and 0.6% ash. The greater part of the ash was now cobalt, and there was a diminution in the amount of calcium, magnesium, manganese and strontium; traces of nickel now appeared as a result of the contamination of the cobalt by this metal. The nitrogen and cobalt values given in Table V are based on the whole protein. The total N of all the preparations was very much the same, but the proportion of cobalt increased up to a limiting value of 0.26% as the amount of cobaltous acetate present in the original mixture was increased. Paralleling this increase in bound Co^{++} is an increase in the hydrolytic rate of all of the substrates tested up to a limit between 0.1 and 0.2% of bound Co^{++} to protein or between the concentrations of 1×10^{-3} and 1×10^{-2} *M* Co^{++} with which the original acylase I had been mixed. This range is the same as that found for the maximal activation of acylase I in the presence of added Co^{++} at these substrate concentrations (Table II, Fig. 2) and suggests that in the presence of an excess of added Co^{++} it is only that which is bound to the protein which is effective.

In the presence of 1×10^{-4} *M* cobaltous acetate and at *p*H 7.0, 36 mg. of acylase I combined with cobalt to 0.016% or in molar concentration to $1 \times$

(4) R. E. Kitson, *Anal. Chem.*, **22**, 664 (1950).

(5) Preliminary spectroscopic analyses were performed by Mr. B. F. Scribner of the National Bureau of Standards, to whom the authors' thanks are due.

TABLE IV

DEGREE OF ACTIVATION OF HYDROLYTIC RATE BY ACYLASE I BY 1×10^{-3} M Co^{++} WITH VARIOUS CONCENTRATIONS OF SUBSTRATE[a]

Substrate	Enzyme concn. as mg. N	% activation at substrate concn. 0.008 M	0.017 M	0.033 M	0.067 M	0.167 M	0.333 M	0.667 M
Acetyl-L-alanine	0.01	+84	+68	+24	+19	0		
Acetyl-L-butyrine	.01	+50	+15	+4	+6			
Acetyl-L-norvaline	.01	−42	−46	−39	−45			
Acetyl-L-norleucine	.01	−32	−33	−36	−38			
Acetyl-L-heptyline	.01	−18	−16	−15	−20			
Acetyl-L-capryline	.01		−27					
Acetyl-D-alanine	1.0	+260	+410	+630	+660	+440	+503	+600
Acetyl-L-methionine	0.01	−5	−16	−31	−40		−11	−19
Acetyl-D-methionine	1.0	+35	+51	+78	+119		+277	+300
Acetyl-L-aspartic acid	1.0	+154	+276	+311	+510		+396	+291
Acetyl-L-glutamic acid	0.01	+50	+81	+67	+45			

[a] Rate defined in Table I.

TABLE V

COBALT-ACTIVATED ACYLASE I PREPARATIONS—ACYLASE IA

Cobaltous acetate used in prepn., M	Anal. of active enzyme Nitrogen,[a] %	Cobalt, %	Rates of hydrolysis of N-acetyl derivatives of[c] L-Alanine	D-Alanine	L-Methionine	D-Methionine	L-Ornithine (α)
0	15.4	0[b]	6100	2.1	24200	10.4	15.0
1×10^{-4}	15.8	0.016	8440	5.2	26500	51.0	33.4
1×10^{-3}	15.6	.096	10300	9.0	27400	80.7	36.6
1×10^{-2}	15.6	.194	12100	9.9	26900	85.8	36.2
4×10^{-2}	15.5	.262	11400	10.0	28700	83.9	36.4
1×10^{-1}	15.6	.264	11400	10.6	30100	84.9	35.9

[a] Not corrected for ash. [b] Not detected by chemical or spectroscopic procedures employed. [c] Rates defined in Table I; substrate concentration 0.017 M.

10^{-4}. In the presence of 1×10^{-3} M cobaltous acetate, 6×10^{-4} M Co^{++} was bound, at 1×10^{-2} M cobaltous acetate about 1.2×10^{-3} M Co^{++} was bound and at 4×10^{-2} and 1×10^{-1} M cobaltous acetate about 1.6×10^{-3} M Co^{++} was bound. Beyond the first concentration studied, there was a definite excess of cobaltous acetate present over and beyond that bound by the acylase I protein. Presumably the excess was removed during the dialysis procedure. When amounts of acylase I of 12 mg. to 36 mg. were mixed with 4×10^{-2} M cobaltous acetate and the mixtures subsequently dialyzed, centrifuged and lyophilized, the preparations of acylase IA obtained all effected the hydrolysis of acetyl-D-alanine (0.017 M concentration) at rates which varied between 9.2 and 10.2 μmoles per hour per mg. N (*cf.* Table V).

Of quite striking interest in Table V is the fact that the hydrolysis of acetyl-L-methionine, which was inhibited when Co^{++} was added to digests with acylase I, was now accelerated at all concentrations of Co^{++} studied provided that the metal was combined with protein in that form characteristic of acylase IA.

The susceptibility of a number of substrates at 0.017 M concentration to acylase I and to acylase IA (prepared by previous mixing with 4×10^{-2} M cobaltous acetate) is recorded in Table VI. Included for comparison are results obtained using acylase I in the presence of added 1×10^{-3} M Co^{++}; omitted from this particular series are studies on acetyl-D-amino acids whose hydrolytic rates are so low that differences in terms of per cent. change when Co^{++} is added might be so far in error as to be meaningless.

Acylase IA is an activated form of acylase I from which it differs both in reactivity and to some extent in stereospecificity. The marked effect of acylase IA on the D-substrates is noteworthy. That this effect may extend to a β-D-configuration is evident from the data in Table VI on acetyl-L-isoleucine and acetyl-L-alloisoleucine. The amino acid components of these two substrates both possess an α-L-configuration, but the β-configuration of L-isoleucine is L- and that of L-alloisoleucine is D.[6] It has been shown that acylase I is an L-directed enzyme, favoring the hydrolysis of acylated diastereomeric amino acids, with an L-configuration not only at the α- but also at the β-carbon.[7] Thus (Table VI), acetyl-L-isoleucine is hydrolyzed twice as fast by acylase I as is acetyl-L-alloisoleucine, which is what would be expected of a strongly L-directed enzyme. But, with acylase IA, the rates of hydrolysis of the two substrates are not so greatly different, for this enzyme hydrolyzes acetyl-L-isoleucine three times as rapidly as does acylase I and acetyl-L-alloisoleucine four times as rapidly as does acylase I. Thus, acylase IA is not as strongly L-directed as is acylase I, or conversely, acylase IA is a more strongly D-directed enzyme than is acylase I.

Acylase IA, like acylase I, is not affected in its activity toward acetyl-L-alanine in the presence of 1×10^{-3} Versene, although the former contains cobalt. A comparison of the changes in activity in columns 4 and 5 in Table VI is of interest and

(6) J. Trommel, *Proc. Koninkl. Ned. Akad. Wetenschap.*, **B56**, 272 (1953); **B57**, 364 (1954); S. Stälberg-Stenhagen and E. Stenhagen, *Arkiv. Kemi. Mineral. Geol.*, **24B**, 1 (1947); M. Winitz, S. M. Birnbaum and J. P. Greenstein, THIS JOURNAL, **77**, 3106 (1955).

(7) M. Winitz, L. Bloch-Frankenthal, N. Izumiya, S. M. Birnbaum, C. G. Baker and J. P. Greenstein, THIS JOURNAL, **78**, 2423 (1956).

TABLE VI

SUSCEPTIBILITY OF SUBSTRATES TO ENZYMATIC ACTION OF ACYLASE I AND ACYLASE IA

Acetyl derivative[a] of the amino acids	L-form Rate[a] with Acylase I	L-form Rate[a] with Acylase IA	L-form Change in activity of acylase IA over acylase I, %	L-form Change in activity of acylase I by added 1×10^{-3} M Co^{++}%	D-form Rate[a] with Acylase I	D-form Rate[a] with Acylase IA	D-form Change in activity of acylase IA over acylase I, %	D-form Change in activity of acylase I by added 1×10^{-3} M Co^{++}%
Alanine	6330	11000	+ 73	+ 68	2.6	9.8	+270	+440
Butyrine	12700	15300	+ 21	+ 15	0.5	1.5	+200	...
Norvaline	21200	23300	+ 10	− 46	0.9	2.9	+220	...
Norleucine	18800	19600	+ 4	− 33	1.5	5.7	+280	...
Heptyline	10500	12300	+ 17	− 16	0.5	2.5	+400	...
Capryline	2730	3210	+ 18	− 27	0	0.5	...	...
Methionine	25500	32800	+ 28	− 19	11.6	75.2	+550	+ 80
Aspartic acid	4.0	38.3	+860	+276	0	0	...	...
Glutamic acid	3600	4290	+ 20	+ 81	...	...	...	...
Isoleucine	1010	3090	+200	+240	0	0	...	...
Alloisoleucine	570	2240	+290	+210	0	0	...	...
Valine	3300	12400	+280	+ 25	0	0	...	...
Leucine	10800	16700	+ 55	+ 22	0	0	...	...
Histidine	740	1140	+ 54	+ 31	0	0	...	...
Arginine	1110	1160	+ 5	− 36	0	0	...	...
Ornithine (α)	15	36.4	+143	+ 67	...	...	...	...
α-Chloroacetyl-DL-lysine	23.7	75.3	+218	+ 53	...	...	...	...
α,ϵ-Dichloracetyl-DL-lysine	104	420	+304	+ 21	...	...	...	...
α,δ-Dichloracetyl-DL-ornithine	500	675	+ 35	+ 24	...	...	...	...

[a] Rates defined in Table I; substrate concentrations 0.017 M. Acetylglycine studied under the present conditions possessed a rate with acylase I of 2940, with acylase IA of 4740, the per cent. change in activity of acylase IA over acylase I being +61. The change in activity of acylase I toward this substrate by added 1×10^{-3} M Co^{++} was +57%.

may be based on what appears to be a difference in many cases between bound cobalt (column 4) and free cobalt ions (column 5). In the presence of free Co^{++}, a high order of activation is observed for acetyl-L-aspartic acid, acetyl-L-isoleucine and acetyl-L-alloisoleucine, but in the presence of bound cobalt, as high if not a higher order of activation is observed not only for these substrates but also for acetyl-L-valine, α-acetyl-L-ornithine, α-chloroacetyl-DL-lysine and α,ϵ-dichloroacetyl-DL-lysine.

The considerable degree of activation of the hydrolysis of certain of the acetyl-L-amino acids suggested a modification in the resolution procedure developed in this Laboratory.[8] This procedure depends in part upon the asymmetric action of acylase I upon N-acyl-DL-amino acids, and if Co^{++} should be added to the reaction mixture, it would be expected that the reaction would be accelerated and less enzyme need be used. Inasmuch as cobalt would tend to accelerate the hydrolysis of certain of the N-acyl-D-isomers, it would be necessary to avoid adding Co^{++} when such isomers are present. Judged on the data in Table VI, the resolution of isoleucine could be conducted safely from this standpoint, and a brief description of this resolution as modified by adding Co^{++} to the digestion mixture is given below.

Effect of Added Co^{++} on Acylase IA.—When Co^{++} is added at the higher levels to acylase I, acylase IA is formed by an apparently irreversible combination with the metal. The mixture may therefore be considered to be acylase IA in the presence of excess Co^{++}. It is probable that it is this excess of Co^{++} which is responsible for the diminished rate of hydrolysis of such compounds as acetyl-L-methionine and acetyl-L-norvaline, and the observed rate may be the resultant of the activating influence of acylase IA and the stronger inhibitory effect of free Co^{++}. In the case of other compounds, the excess of Co^{++} may produce an accelerating effect over and beyond that of acylase IA (acetyl-D-alanine and acetyl-L-glutamic acid), an inhibitory effect insufficient to overcome the accelerating influence of acylase IA (acetyl-D-methionine, acetyl-L-valine, etc.), and no appreciable effect at all (acetyl-L-alanine, acetyl-L-butyrine, etc.).

It should be possible in part at least to reproduce the same activation or inhibition phenomena by adding Co^{++} to acylase IA as those found by adding Co^{++} to acylase I. Under the same conditions described in Table VI except that Co^{++} to 1×10^{-3} M concentration was added, the hydrolytic rates by acylase IA of the following acetylated amino acids were diminished by an extent shown in the accompanying parentheses in terms of per cent.: L-butyrine, (−6), L-norvaline (−14), L-norleucine (−27), L-heptyline (−13), L-capryline (−6), L-methionine (−27) and L-arginine (−14). Similarly, the hydrolytic rates of the following acetylated amino acid were accelerated by the extent shown: glycine (+30) L-alanine (+13), L-aspartic acid (+39), L-glutamic acid (+43), L-isoleucine (+80), L-alloisoleucine (+62), L-valine (+48), L-histidine (+9), D-alanine (+11) and D-methionine (+20). It would appear that al-

(8) S. M. Birnbaum, L. Levintow, R. B. Kingsley and J. P. Greenstein, *J. Biol. Chem.*, **194**, 455 (1952); J. P. Greenstein, S. M. Birnbaum and M. C. Otey, *ibid.*, **204**, 307 (1953).

though the inhibitory phenomena displayed by Co^{++} added to acylase IA were those which might in general have been expected, the accelerating phenomena were not all that were expected. Thus, the added impetus to the hydrolytic rates by addition of Co^{++} to acylase IA, although expected for acetyl-D-alanine and acetyl-L-glutamic acid, was surprising in the case of the other substrates whose rates should have been either unchanged or diminished. The fact that they were increased suggests that further sites on the acylase IA molecule were capable of binding cobalt and that either full saturation of the acylase IA with cobalt had not been originally achieved when acylase I was mixed with an excess of Co^{++} or else that during the dialysis procedure some bound cobalt had been stripped off its binding site in acylase IA.

Michaelis–Menten Constants.—The usual constants[9,10] have been obtained through the use of the Lineweaver–Burk relation[11]: $S/v = S/V + K_s/V$, where S is the substrate concentration, v the rate of hydrolysis and V the rate at the saturation level of the enzyme. The results are shown in Table VII. It is noted that the K_s values for acylase IA are uniformly smaller for each substrate than with acylase I. For the homologous series from acetylglycine to acetyl-L-capryline, the K_s values diminish progressively with both kinds of enzyme. The values of the maximum rates for each compound in this series are very nearly the same, although the values of K_s are in most cases considerably different.

TABLE VII

MICHAELIS–MENTEN CONSTANTS

Substrate	Acylase I Max. rate V	Acylase I $K_s \times 10^3$, mole/l.	Acylase IA Max. rate V	Acylase IA $K_s \times 10^3$, mole/l.
Acetyl-glycine	6480	40.8	6410	9.4
Acetyl-L-alanine	15500	25.6	15600	7.1
Acetyl-L-butyrine	20700	8.3	19900	5.0
Acetyl-L-norvaline	26900	3.0	25500	1.0
Acetyl-L-norleucine	22300	2.0	19000	v.s.[a]
Acetyl-L-heptyline	14700	1.3	16200	v.s.[a]
Acetyl-L-capryline	2690	v.s.[a]	3300	v.s.[a]
Acetyl-L-methionine	30700	3.7	32800	1.8
Acetyl-L-leucine	19500	12.8	25700	5.6
Acetyl-L-valine	10200	30.6	12500	13.2
Acetyl-L-isoleucine	1820	17.8	3770	15.1
Acetyl-L-alloisoleucine			3970	13.9
Acetyl-L-aspartic acid	44.5	409	142	178
Acetyl-L-glutamic acid	17900	63.1	17800	28.8
Acetyl-L-arginine			2120	27.6
Acetyl-D-alanine	6.1	34.4		
Acetyl-D-methionine	62.0	77.8		

[a] Very small.

Experimental

The acetyl-L- and D-amino acids were prepared from optically active amino acids prepared in this Laboratory by resolution.[8] The acetyl derivatives of alanine,[8] leucine,[8] methionine,[8] alloisoleucine,[8] arginine[12] and α-ornithine[13] have been described, as have the chloroacetylated derivatives of racemic ornithine[14] and lysine.[15] Acetyl-L-glutamic acid and acetyl-L- and D-histidine were prepared by the method of Bergmann and Zervas.[16] The synthesis of the latter compounds was slightly modified in that the mixture of L- or D-histidine with glacial acetic acid and an equivalent amount of acetic anhydride was rapidly brought to the boiling point, held there for 30 seconds and immediately chilled; $[\alpha]^{25}D$ for the L-isomer was +46.2° (c 1, H_2O). The L-isomer prepared by the Schotten–Baumann procedure using LiOH as base yielded $[\alpha]^{25}D$ +46.8° (c 1, H_2O). The acetyl-D-histidine was found to be slightly racemized by the synthesis using acetic anhydride in glacial acetic acid solution and was purified by first treating the compound with acylase I to hydrolyze the acetyl-L-histidine contaminant followed by treatment with *Crotalus adamanteus* venom L-amino acid oxidase to remove the L-histidine formed,[17] $[\alpha]^{25}D$ −46.0° (c 1, H_2O). Bergmann and Zervas reported an $[\alpha]D$ value of +44.7° for acetyl-L-histidine.[16] Acetylglycine was purchased from the Eastman Kodak Company and was recrystallized from water, m.p. 207°. The remainder of the compounds employed in the present study were acetylated by the usual Schotten–Baumann procedure using acetic anhydride and NaOH. Crystallization media included water, ethanol and ethyl acetate–petroleum ether. Their physical constants are listed in Table VIII.

Salts.—All metal salts (Fisher Certified Reagents) were employed as the acetates.

Enzymic Procedure.—The digests were prepared as follows. One ml. of veronal acetate buffer at pH 7 (or 0.7 ml. of buffer plus 0.3 ml. of a solution in the buffer of metal ion or Versene at ten times the desired final concentration) was incubated at 37° with 1 ml. of acylase I solution in buffer for a given period of time. For most of the experiments reported a period of two minutes was used. One ml. of substrate solution at 37° which had been adjusted to pH 7 with dilute NaOH was then added. After a measured time interval, 3 ml. of saturated picric acid solution was added and the amount of free amino acid determined by COOH–N in the Van Slyke apparatus. In the case of acetylaspartic acid, the reaction was stopped by the addition of 3 ml. of 0.5 M citrate buffer at pH 4 inasmuch as picric acid has a tendency to hydrolyze this substrate. Blank determinations were performed in all cases. The pH was carefully checked on a reaction mixture prepared in the same manner, and any solution which varied from 7.0 by more than ±0.1 was rejected. The concentration of the enzyme solution was 1.0 mg. N in the case of all of the D-substrates, for acetyl-L-aspartic acid and for all of the derivatives of ornithine and lysine, whereas for all of the remaining L-substrates 0.01 mg. N was employed. A quoted rate of zero indicates that there was no measurable evolution of ninhydrin-CO_2 after 3 hr. of incubation at 37° with an enzyme level of 1 mg. N. Initial rates within 30% of the complete hydrolysis of the substrates were expressed in terms of μmoles of substrate hydrolyzed per hour per mg. N.

Preparation of Enzymes.—Acylase I was prepared as described.[8] Acylase IA was prepared as follows. Acylase I was dissolved in veronal acetate buffer at pH 7.0, and sufficient cobaltous acetate solution in veronal buffer was added to attain the desired concentration, and buffer finally added to a definite volume. For most of the preparations of this modified acylase, the final concentration of cobalt was 4×10^{-2} M and that of the protein was 12–36 mg. per ml. The solution was allowed to stand at 37° for 10 minutes and then dialyzed at 5° against distilled water frequently changed. The contents of the dialysis sack were centrifuged at 4000 r.p.m. for 30 minutes and the light orange-brown colored supernatant solution quickly frozen and lyophilized. The recoveries were about 65%.

Resolution of Isoleucine in the Presence of Co^{++}.—An epimeric mixture of 1972 g. of acetyl-L-isoleucine and acetyl-D-alloisoleucine was dissolved in 30 l. of water and the solution adjusted to pH 7.5 with ammonium hydroxide. An amount of 9.5 g. of cobaltous acetate tetrahydrate was dis-

(9) L. Michaelis and M. L. Menten, *Biochem. Z.*, **49**, 333 (1913).

(10) G. E. Briggs and J. B. S. Haldane, *Biochem. J.*, **19**, 338 (1925).

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TABLE VIII

PHYSICAL CONSTANTS AND ANALYSES

N-Acetyl derivative of	M.p.,[a] °C.	$[\alpha]_D$,[b] deg.	Calcd. for	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
L-Butyrine	131	−40.0(c)	$C_6H_{11}O_3N$	49.7	7.6	9.7	49.6	7.8	9.7
D-Butyrine	131	+40.0(c)					49.6	7.9	9.6
L-Norvaline	100	−35.0(c)	$C_7H_{13}O_3N$	52.8	8.1	8.8	52.8	8.2	8.9
D-Norvaline	100	+35.0(c)					52.5	8.6	8.8
L-Norleucine	112	−20.0(c)	$C_8H_{15}O_3N$	55.5	8.7	8.1	55.6	9.0	8.2
D-Norleucine	112	+20.0(c)					55.3	8.9	8.1
L-Heptyline	108	− 8.0(d)	$C_9H_{17}O_3N$	57.7	9.1	7.5	57.9	9.3	7.6
D-Heptyline	108	+ 8.0(d)					57.9	9.2	7.6
L-Capryline	105	+ 7.5(e)	$C_{10}H_{19}O_3N$	59.7	9.5	7.0	60.1	9.7	6.9
D-Capryline	105	− 7.5(e)					60.1	9.7	7.0
L-Valine	168	+ 7.5(e)	$C_7H_{13}O_3N$	52.8	8.1	8.8	52.5	8.4	8.7
D-Valine	168	− 7.5(e)					52.8	8.5	8.8
L-Aspartic acid	142	+57.0(e)	$C_6H_9O_5N$	41.1	5.1	8.0	41.1	5.2	8.1
D-Aspartic acid	142	−57.0(e)					41.3	5.5	8.0

[a] All melting points corrected. [b] All solutions at 1–2% at 25°. [c] In water. [d] In 50% acetic acid. [e] In glacial acetic acid.

solved in this solution and the total volume brought to 38 l. Two hundred mg. of acylase I powder was dissolved in the solution which was then incubated at 38°. The initial rate of hydrolysis under these conditions was 6600 μmoles of substrate hydrolyzed per hour per mg. N. Less than one-third the amount of enzyme was employed than customary in the absence of added cobalt. The reaction was complete in 24 hr., but the solution was allowed to stand for another 24 hr. In working up each of the optical antipodes[18] no difficulty due to the presence of Co^{++} was encountered, and the isomers were obtained in a state of high optical and chemical purity, in yields of 50–60% of the theoretical.

(18) J. P. Greenstein, L. Levintow, C. G. Baker and J. White, *J. Biol. Chem.*, **188**, 647 (1951); J. P. Greenstein, S. M. Birnbaum and L. Levintow, *Biochem. Preparations*, **3**, 84 (1953).

Product of Hydrolysis of Acetyl-D-alanine by Acylase I in the Presence of Co^{++}.—The action of acylase I on acetyl-D-methionine led to a product which was isolated in good yield and identified as D-methionine.[8] The experiment was repeated with acylase I using acetyl-D-alanine as substrate and in the presence of 4×10^{-2} Co^{++}. At the end of the reaction D-alanine was isolated in 62% of the theoretical yield, $[\alpha]^{25}D$ −30.4 (*c* 0.6, glacial acetic acid). The rotation of L-alanine under these conditions has been reported as +29.4°.[8]

Acknowledgments.—We wish to express our appreciation to Mr. R. J. Koegel and Miss Rita McCallum of the analytical unit of this Laboratory for the determination of the elemental analyses.

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[CONTRIBUTION FROM THE DEPTS. OF ANATOMY, MICROBIOLOGY AND IMMUNOLOGY, AND BIOCHEMISTRY, MARQUETTE UNIVERSITY SCHOOL OF MEDICINE]

A Deoxyribonuclease of *Micrococcus pyogenes*[1]

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RECEIVED APRIL 11, 1956

Micrococcus pyogenes var. *aureus* was found to release large quantities of a calcium-activated deoxyribonuclease (DNase) into the culture medium when grown with vigorous aeration. This enzyme was unusually stable and could be purified by boiling, followed by precipitation from the culture fluid with $(NH_4)_2SO_4$ and a series of washings, first with saturated NH_4Cl containing 2.5% trichloroacetic acid, and then with 83% ethanol. The *p*H optimum was 8.6. After completion of the action of the *M. pyogenes* DNase on deoxyribonucleic acid, studies with the aid of ion-exchange chromatography indicated that the number of bonds in the substrate which were attacked was greater than that for pancreatic DNase and less than that for snake venom phosphodiesterase. Mononucleotides liberated by the action of the bacterial enzyme were not dephosphorylated by snake venom 5′-nucleotidase, indicating that *M. pyogenes* DNase, unlike pancreatic DNase and various phosphodiesterases, could split the 5′-phosphodiester bond in deoxyribonucleic acid.

In the course of a survey of deoxyribonucleases (DNases) from various sources, it was found that an enzyme present in the supernatant fluid of centrifuged cultures of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) had several unusual properties. It required calcium, instead of magnesium, as cationic activator. Prior to separation from the original culture medium, it could be boiled with little or no loss of activity. The enzyme passed through a dialyzing membrane in easily detectable quantities, providing further evidence that it was a rather small molecule.

The unusual stability of the enzyme gave promise that it might be obtainable sufficiently free of interfering enzymes, so that a study of the mechanism of action on deoxyribonucleic acid (DNA) could be undertaken. After prolonged digestion of the substrate with various preparations of the enzyme, mononucleotides and other small molecules were obtained in rather large yield, without the production of inorganic phosphate in detectable quantity. The patterns produced by chromatog-

(1) A preliminary report was presented at the meeting of the American Chemical Society in Minneapolis, September, 1955.