CRYSTALLINE TRYPSIN

I. Isolation and Tests of Purity

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(Accepted for publication, June 22, 1932)

It has been known since the time of Corvissart and Kühne that pancreatic juice possesses the property of digesting proteins. Kühne assumed that this property was due to the presence in the juice of an unorganized ferment or enzyme which he called trypsin. Subsequent work by Fisher and Abderhalden, Cohnheim, Bayliss, Vernon, Schaffer and Terroine, Abderhalden, Willstätter, and Waldschmidt-Leitz and their collaborators has added greatly to our knowledge of the enzymatic properties of pancreatic juice (1). The kinetics of these reactions have also been partially worked out. According to Waldschmidt-Leitz the activated pancreatic juice contains at least five proteolytic enzymes—trypsinogen, trypsin-kinase, carboxy-peptidase and amino-peptidase, which hydrolyze polypeptides, and erepsin. There is no positive evidence[1] as yet that any of these enzymes has ever been obtained in pure form or separated completely from each other and it is probable that the separation can be carried still further, as already indicated by Abderhalden (3). The enzyme described in this paper differs essentially from any previously described. In the meantime the existence of these separate enzymes has been assumed purely from the behavior of certain solutions, as Linderström-Lang has pointed out (4).

In contrast to the marked advances in knowledge of the properties

[1] In one sense this will always be the case since it is not possible to furnish positive proof of the purity of a substance but only negative proof consisting of the fact that no evidence of mixtures can be found under conditions which would be expected to show such evidence. For a discussion of the difficulty of proving the existence of, or defining a "pure substance" see Lunn and Senior (2).

of the enzymes of the pancreatic juices, as shown by their catalytic effect in various reactions, little or no knowledge has been gained as to the chemical nature of these enzymes. The early workers, Kühne, Mays, Hammarsten, and Michaelis, considered them to be associated with the nucleic acid fraction; but Levene (5) showed that they were not nucleic acids themselves since hydrolysis of the nucleic acids did not destroy the activity. Willstätter and his collaborators (6) concluded that the enzymes belong to an unknown class of chemical compounds associated with proteins or other substances of high molecular weight and a somewhat similar point of view is expressed by Fodor (7). This conclusion, however, rests only on the purely negative evidence that no pure substance, *i.e.* one having constant properties, has been obtained which showed enzymatic activity. The known chemical properties of enzymes, on the other hand, such as the temperature coefficient of inactivation, effect of acid and alkali on stability, and the reactivation sometimes observed, as well as the ease with which they are adsorbed on colloidal particles, are those of proteins. In addition, crystalline proteins of constant composition and activity have been isolated by Sumner (8) in the case of urease, by Northrop (9) in the case of pepsin, and by Caldwell, Booher, and Sherman (10) in the case of amylase. The existing positive evidence, therefore, indicates the protein nature of enzymes and the conditions and methods used in the present work were those known to be favorable for the isolation of proteins; *i.e.*, concentrated solutions in concentrated neutral salt and low temperatures. These methods have led to the isolation of a crystalline protein having constant physical and chemical properties, including constant proteolytic activity. This enzyme attacks only proteins, and pepsin-peptone, so far as we have determined. Since the protease of pancreas has always been called trypsin the present enzyme will be referred to as trypsin. It differs markedly from the trypsin-kinase of Waldschmidt-Leitz in that it does not carry the hydrolysis of proteins nearly so far as does trypsin-kinase.

Methods of Determining Activity

The activity of the various preparations was determined by the change in viscosity of gelatin and casein, the increase in formol titra-

tion of gelatin and casein, and the formation of non-protein nitrogen in casein solutions, etc. The following definitions and abbreviations are used in the paper.

$[\text{T. U.}]^{4g\,V}_{\text{mg. N}}$—per cent change in viscosity per minute per mg. trypsin nitrogen contained in 5.2 ml. 2.5 per cent gelatin, pH 4.0.

$[\text{T. U.}]^{\left\{\begin{smallmatrix}\text{Cas. } F\\ \text{Gel. } F\end{smallmatrix}\right.}_{\text{mg. N}}$—milliequivalents carboxyl groups liberated per minute, per mg. trypsin nitrogen contained in 6.0 ml. 4 per cent $\left\{\begin{matrix}\text{casein}\\ \text{gelatin, pH 7.6.}\end{matrix}\right.$

$[\text{T. U.}]^{\text{Cas. } S}_{\text{mg. N}}$—milliequivalents nitrogen soluble in 5 per cent trichloracetic acid formed per minute per mg. trypsin nitrogen contained in 6.0 ml. 4 per cent casein, pH 7.6

$[\text{T. U.}]^{\text{Ren.}}_{\text{mg. N}}$—per cent increase in viscosity per minute per mg. trypsin nitrogen contained in 5.2 ml. of standard milk solution.

$[\text{T. U.}]^{\text{Clot.}}_{\text{mg. N}}$—ml. magnesium sulfate plasma clotted by 1 mg. trypsin nitrogen in 18 hours, 6°C.

$[\alpha]^{D}_{\text{mg. P. N.}}$—degrees optical rotation in a 1 dm. tube of a solution in $\frac{1}{4}$ saturated ammonium sulfate, M/10 pH 4.0 acetate, containing 1 mg. protein nitrogen per ml.; sodium D line, at 20°C.

The methods used are described in the experimental part of the paper (11).

The determinations were confined in all cases except the rennet action, to the initial slope of the curves in which region the specific activity obtained is independent of the concentration of enzyme used.

Preliminary Method of Fractionation

The first attempts at purification of the enzyme were similar to the experiments of Michaelis and Davidsohn (12) and consisted in a study of the precipitate obtained from crude trypsin extracts at about pH 3.0. The crude material used was Fairchild's trypsin which is prepared from beef pancreas.[2] If an aqueous extract of the dry powder is titrated to about pH 3.0 with acid a precipitate forms, as Michaelis found. Most of the activity is found in this precipitate. Upon repeated solution and reprecipitation much of the activity is lost and the specific activity of the precipitate becomes less and less. A number of other methods of fractionation were tried but it was found that

[2] The writers are indebted to Mr. Benjamin Fairchild of Fairchild Bros. and Foster for this information.

the precipitate obtained with strong ammonium sulfate was the only one which could be dissolved and reprecipitated indefinitely without loss of activity either as a whole or in regard to the precipitate. Systematic fractionation of an extract of the commercial preparation in $\frac{1}{4}$ saturated ammonium sulfate was then undertaken with various concentrations of ammonium sulfate. As the ammonium sulfate concentration is increased a series of precipitates is obtained which become more and more active. The most active precipitate appeared at about 0.6 saturated ammonium sulfate and further fractionation of this precipitate did not change its activity appreciably. The precipitate was a protein and gave some indications of crystallizing so that a large number of experiments were done in an attempt to crystallize it. It was found eventually that crystals could be obtained by adding saturated ammonium sulfate very cautiously to a 5 per cent solution of the precipitate (1 part filter cake dissolved in five times its weight of solution) in $\frac{1}{4}$ saturated ammonium sulfate made up in M/10 acetate buffer, pH 4.0, temperature 25–30°C. The first precipitate which appears under these conditions is usually amorphous. If this is filtered off and the filtrate allowed to stand at 25–30°C., small crystals of cubic form begin to appear in the solution and increase rapidly in amount. Good crystals are obtained only with slow crystallization. Otherwise the crystals are not well formed and usually appear spherical. The process is favored by stirring. The crystallization may be hastened by continued addition of ammonium sulfate (with stirring) and eventually practically all the protein may be obtained in crystalline form. This is the method of preparation already reported (13). Material obtained in this way is about ten times as active as the original commercial product on a total dry weight basis and about three times as active per milligram soluble protein nitrogen.

Repeated crystallization does not change the specific activity to any extent but it was noted that the first small amount of precipitate formed was always slightly less active than the succeeding crops of crystals. The results of such an experiment are shown in Table I.

It was very difficult to decide whether this result was due to actual fractionation or to loss in activity during the experiment. In order to test the purity of the preparation in another way a series of solubility experiments was done in $\frac{3}{4}$ saturated magnesium sulfate. The crys-

talline precipitate was stirred for 10 to 15 minutes with a mixture made up of 75 ml. saturated magnesium sulfate and 25 ml. M/10 pH 4.0 acetate. The suspension was then filtered and the precipitate again

TABLE I

100 gm. poorly crystalline cake dissolved in 600 ml. ¼ saturated ammonium sulfate in M/10 acetate buffer, pH 4.0. Saturated ammonium sulfate added with stirring to faint turbidity. More saturated ammonium sulfate run in very slowly. Crystalline precipitate formed and filtered off from time to time. Saturated ammonium sulfate added to filtrate until no further crystals are obtained. The precipitates were then combined, dissolved in 6 volumes ¼ saturated ammonium sulfate, pH 4.0 and the crystallization of Fraction b repeated four times. Samples of each fraction were analyzed for protein nitrogen, optical activity, and proteolytic activity by gelatin viscosity method.

Crystallization No.	Weight of cake	Fraction	Precipitate	$[T.U.]^{4gV}_{mg.N}$	$[\alpha]^{D}_{mg.N}$
	gm.		*gm.*		
1	100	1 a	11	40	
		1 b	17	55	0.33
		1 c	15	57	
		1 d	11	57	
		Mother liquor		42	
2	39	2 a	1.3	42	
(1a + 1b +		2 b	26.5	47	0.33
1c + 1d)		Mother liquor		36	
3	26	3 a	2.5	43	
		3 b	11.5	47	0.37
		Mother liquor		50	
4	11	4 a	1		
		4 b	7.5	45	0.39
		Mother liquor		40	
5	7	5 a	0.5	42	
		5 b	3.6	45	0.36
		Mother liquor			

stirred with the same solvent. This was continued until the precipitate had nearly all gone into solution and the filtrates and residue then analyzed for nitrogen and activity. The results of the experiments are shown in Table II.

The solubility decreases rapidly with successive extractions while the specific activity of the protein in solution also decreases to some extent. The final residue, however, has about the same activity as the original material. These results show definitely that the crystalline material prepared by this method is not a pure protein since the solubility depends on the quantity of precipitate. It is similar to the type of result found by Sörensen (22) with other proteins and indicates that the substance is probably a solid solution. There was some loss in activity during this experiment but hardly sufficient to account for the observed results.

TABLE II

Solubility in Magnesium Sulfate, pH 4.0 Acetate =
{75 Ml. Saturated Magnesium Sulfate
{25 Ml. M/10 pH 4.0 Acetate

20 gm. crystalline filter cake stirred for 10–15 minutes with 75 ml. solvent. Filtered and filtrate analyzed for total nitrogen and activity. Extraction repeated eight times.

Extract No.	1	3	5	7	9	Residue from 9th extract
N/ml.	1.92	0.94	0.80	0.49	0.29	
$[T. U.]^{4gV}_{mg. P. N.}$	60	36	54	40	47	60

Effect of Heating in Acid Solution

It was noted when a solution of the material was boiled in dilute hydrochloric acid and then cooled that a form of protein appeared which precipitated on the addition of magnesium sulfate although there was not very much loss in activity. This remarkable stability in dilute acid is a characteristic property of trypsin and has been noted by Mellanby and Wooley (14). It was thought at first that the precipitate formed on adding a solution, which had been heated and then cooled, to salt solutions was probably denatured trypsin protein and an experiment was made in order to determine whether the loss in activity was proportional to the quantity of this denatured protein formed. A solution of the material in N/10 hydrochloric acid was heated to 95°C. and the total activity of the solution

determined as well as the quantity of denatured protein present. The results of an experiment of this kind are shown in Table III.

The table shows that the total protein nitrogen remains constant while the activity per milliliter of solution decreases. The specific activity per milligram total protein nitrogen therefore decreases with time. However, if instead of total protein nitrogen the concentration of protein which does not precipitate with magnesium sulfate is considered, the experiment shows that the specific activity, referred to this soluble protein, increases nearly 100 per cent in the first few minutes, remains nearly constant for a while, and then decreases. The experiment shows conclusively that the material is not a pure substance but contains a protein which is denatured by heat and which has little or no activity, and another protein of high activity which either is not denatured by heat or which reverts to the native condition on cooling.[3] This behavior is so unusual that a detailed study of

TABLE III

Loss in Activity and Formation of Denatured Protein in Dilute Acid Solution at 95°C.

2 gm. of crystalline filter cake dissolved in 50 ml. of M/10 hydrochloric acid and kept at 95°C. 1 ml. samples taken and added to 4 ml. M/10 pH 4.0 acetate buffer and this solution analyzed for protein nitrogen and total activity. Another set of 1 ml. samples taken, cooled, and added to 4 ml. cold M/10 magnesium sulfate in 0.5 normal sulfuric acid. The suspension centrifuged and supernatant liquid analyzed for protein nitrogen and activity.

Time	Analysis from samples in acetate			Analysis of filtrate from $MgSO_4$ samples		
	Protein N	$[T.U.]^{4gV}_{ml.}$	$[T.U.]^{4gV}_{mg.P.N.}$	Protein N	$[T.U.]^{4gV}_{ml.}$	$[T.U.]^{4gV}_{mg.P.N.}$
hrs.	*mg.*			*mg.*		
0	1.6	100	62	1.6	100	62
0.1	1.57	80	51	0.58	63	110
0.2	1.5	64	43	0.56	54	97
0.4	1.7	43	25	0.52	27	52
0.8	1.7	18.5	11	0.45	7.4	16

[3] This result is very similar to that obtained recently by Waldschmidt-Leitz and Steigerwaldt (15) in following the digestion of urease with trypsin. It proves only that the material is not a pure protein but does not prove that no active protein is present.

the reaction was made and is reported in another paper (16). It turns out that the active protein is denatured when heated but reverts to the native condition very rapidly on cooling. At the same time the activity returns.

The experiment just described, however, also indicates a very efficient method of further fractionation. A study of the fractionation was therefore undertaken again. The raw material was either an aqueous extract of Fairchild's or other commercial trypsin, or pancreatic juice obtained from frozen pancreas. The frozen mass was sliced, spread on racks and allowed to thaw, and the liquid which drained out collected.

Final Method of Isolation

The method of fractionation finally worked out consists essentially of a preliminary precipitation of juice obtained in this way with strong acid which removes most of the inert proteins, as has previously been reported by Fodor and by Schönfeld-Reiner (17). The filtrate from this precipitate is then fractionated with ammonium sulfate and dissolved in dilute acid, heated to 80°C., and again fractionated with ammonium sulfate. By this method a protein was obtained which had about twice the specific activity of the original crystalline material but which crystallized under the same conditions and in a similar crystalline form (*cf.* Fig. 1). Up to the present it has not been found possible to increase the specific activity any further. Whenever any of the protein is destroyed or removed there is a corresponding loss in activity. The details of the preparation are shown in Table IV. In this experiment the heating and fractionation were repeated four times. Ordinarily the fraction obtained after the first heating (No. 5 in the table) is used as the final material. This fraction contains nearly one-half of the original proteolytic activity. Most of the loss occurs when the solution is heated. This is due to the fact that there are other proteins present in the solution, since, as is described in the paper on heat inactivation (16), the purified material may be heated and cooled again indefinitely without any loss in activity. The specific activity is about six times that of the original crude extract on the basis of protein nitrogen and considerably more than six times as active on the basis of total dry weight.

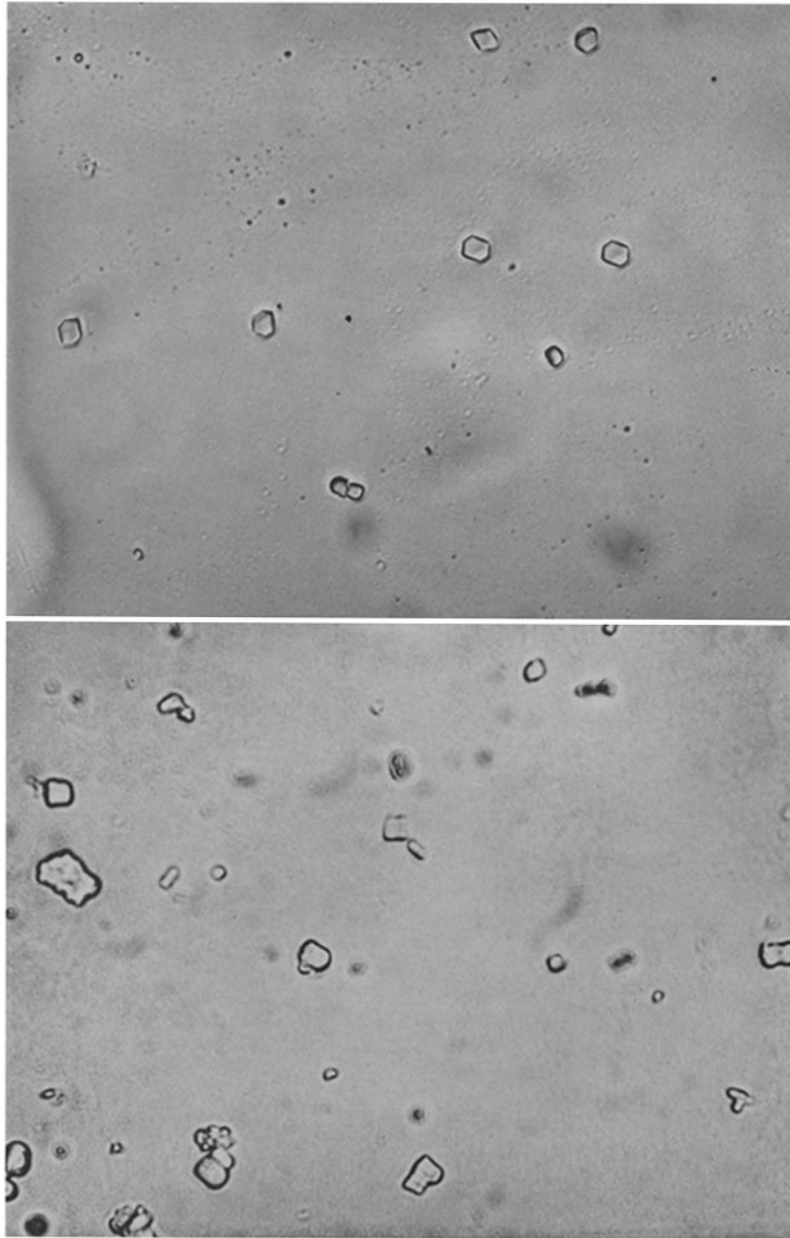

FIG. 1, *a* and *b*. (*a*) Trypsin crystals immediately after crystallization. (*b*) Trypsin crystals after standing in contact with the mother liquor for 1 or 2 months.

TABLE IV

Preparation of Crystalline Trypsin

	Fraction No.	Weight	$[T. U.]^{4gV}_{mg. P.N.}$	Total $[T. U.]^{4gV}$
		gm.		
150 kg. frozen pancreas cut in slices and allowed to thaw overnight at about 5°C. and the expressed fluid collected	1	12,000	18	12×10^5
Diluted with 1 volume water and 22 ml. concentrated hydrochloric acid added per liter (final concentration HCl 0.25 molar). Solution filtered in cold room through fluted paper (S. and S. No. 1450 1/2). Filtrate brought to 0.4 saturated ammonium sulfate by the addition of solid ammonium sulfate (250 gm. per liter) and refiltered through fluted paper. Filtrate brought to 0.7 saturated ammonium sulfate (250 gm. ammonium sulfate per liter) and filtered with suction. Precipitate	3	520	37	9×10^5
500 gm. Precipitate 3 dissolved in 12 liters N/20 hydrochloric acid, heated* rapidly to 90°C. and cooled to 20°C., brought to 0.4 saturated ammonium sulfate, and filtered. Filtrate brought to 0.7 saturated ammonium sulfate and filtered with suction. Precipitate	5	120	95	5×10^5
This completes the usual method of preparation. The succeeding fractionations are reported to show that no further change in activity occurs				
Precipitate 5 dissolved in 25 volumes M/20 pH 4 acetate buffer, heated rapidly to 90°C., cooled to 20°C., brought to 0.4 saturated ammonium sulfate, and filtered. Brought to 0.7 saturated ammonium sulfate and filtered with suction. Precipitate	7	70	100	4×10^5
No. 7 dissolved in 3 volumes $\frac{1}{4}$ saturated ammonium sulfate pH 4 brought to 0.4 saturated ammonium sulfate, filtered. Filtrate brought to 0.7 saturated ammonium sulfate, filtered with suction. Precipitate	8	50	110	3×10^5

TABLE IV—*Concluded*

	Fraction No.	Weight	$[\text{T. U.}]^{4gV}_{\text{mg. P.N.}}$	Total $[\text{T. U.}]^{4gV}$
		gm.		
No. 8 dissolved in 25 volumes N/20 hydrochloric acid, brought to 0.4 saturated ammonium sulfate, and filtered. Filtrate brought to 0.7 ammonium sulfate, filtered with suction. Precipitate	9	35	105	2×10^5
No. 9 dissolved in 3 volumes ¼ saturated ammonium sulfate, brought to 0.4 saturated ammonium sulfate by the addition of saturated ammonium sulfate, clear solution. Saturated ammonium sulfate added slowly until slight turbidity. Solution filtered, filtrate inoculated with crystals, and saturated ammonium sulfate added very slowly. Crystalline precipitate forms. Precipitate	10	20	110	1×10^5

* The heating and cooling of large quantities of solution may be most conveniently done by running the solution through a glass coil immersed in boiling water and then through a coil in cold water. The rate of flow is regulated so that the solution leaves the hot coil at a temperature of 80–85°C. The coil used in these experiments was of 5 mm. (inside diameter) thin walled tubing, about 2 m. long.

It is possible to prepare crystalline material having the maximum activity by fractionation with ammonium sulfate alone and without heating. The process is laborious and the yield poor so that, for practical purposes, the method is not satisfactory. The material obtained in this way, however, is identical with that obtained after heating. There is no reason to suppose, therefore, that the properties of the enzyme are changed by the heating.

The various fractions were tested for tryptic activity by gelatin and casein viscosity methods at pH 4.0, by the formol titration of gelatin and casein, and by the production of soluble nitrogen from casein. They were also tested for their blood-clotting power (18), for ability to clot milk, and for amylase, lipase, and erepsin. The optical activity at pH 4.0 and the total increase in formol titration with casein and gelatin in the presence of excess enzyme were also determined. The results of these determinations are shown in Table V.

The proteolytic activity, as measured by any of the methods and

TABLE V

Analysis of Fractions

Fraction No.	1	3	5	7	8	9	10	Glycerin extract of acetone dried pig pancreas	Crystalline pepsin
$[T. U.]^{4gV}_{mg. P. N.}$	18	37	100	100	110	105	110	20	13
$[T. U.]^{Gel. F}_{mg. P. N.}$	0.05	0.11	0.32	0.24	0.34	0.33	0.29		0.001
$[T. U.]^{Cas. V}_{mg. P. N.}$	160	330					870		
$[T. U.]^{Cas. F}_{mg. P. N.}$	0.053	0.084	0.19 0.13	0.15	0.16 0.12	0.23	0.18	0.04	0.20
$[T. U.]^{Cas. S}_{mg. P. N.}$	0.61	1.1	2.4 1.9	1.7	2.1	2.2	2.4	0.50	0.44
$[T. U.]^{Clot.}_{mg. P. N.}$		810		1500			1500		
$[\alpha]^{D}_{mg. N}$ (1 dm. tube) 25°C.		−0.458		−0.28	−0.29		−0.26		
Amylase—mg. P. N. for positive test, *mg*	<0.001	>2		>2	>2	>2	>2		

$[T. U.]^{Ren.}_{mg. P. N.}$		1000	4000	400	300	160	100	95		280,000
Lipase—$\frac{ml. N/10}{1 mg. N}$		0.6	0.4							
Erepsin (glycyl-glycine)		+	±		–	–	–	–		
Maximum increase in formol titration—ml. N/50 NaOH per 5 ml. protein solution	casein	>32	>15		9.1	9.0	8.9	9.0		17.0
	gelatin	>25	>10		6.9	7.0	7.0	7.0		7.0

also the blood-clotting property increases rapidly up to Fraction 5 and then remains constant. The percentage increase in activity between Fraction 1 and Fraction 5 is slightly more as determined by the gelatin viscosity method than by the other methods, which may be due either to the presence of more than one enzyme, one of which is removed during the course of purification, or to the fact that the other methods of measuring the activity measure secondary reactions due to peptone-splitting enzymes while the viscosity method measures only the change in the protein. The results as a whole indicate that the final preparation has constant activity as measured by any of the preceding methods and that no change in this activity occurs during the repeated fractionations between Fraction 5 and Fraction 10. The optical activity per milligram of nitrogen also reaches a constant value. The amylase activity is expressed as the number of milligrams protein nitrogen required to give a positive test under the conditions used by Willstätter. Fraction 1 has powerful amylolytic activity but no positive test for amylase can be obtained in succeeding fractions. The lipase activity, which is expressed as ml. N/10 alkali per milligram nitrogen under the conditions described in the experimental part (11) disappears in Fraction 5 as does the ability to digest dipeptides. The rennet action decreases rapidly as fractionation proceeds but even the last fractions have a weak effect on the clotting of milk which seems to remain constant. There is no doubt that there is originally present another enzyme with a much more powerful milk-clotting power than the final trypsin and it is possible that the small amount of rennet action noted with the final fractions is due to a trace of this enzyme carried through the preparation.

The specific activity of an activated glycerin extract of dried pancreas prepared according to Willstätter's method (19) is included for comparison. It has about the same specific activity on the basis of protein nitrogen as does the pancreatic extract used as the starting point of this fractionation. The specific activity of crystalline pepsin (20) is also shown in Table V. Pepsin is less active by most of the methods but very much more active in the clotting of milk. The relative activity of the two enzymes expressed in this way cannot be considered an absolute figure since the value obtained would vary with the conditions and methods used for the determination of the activity.

Hydrolysis of Peptides.—Waldschmidt-Leitz (24) has separated two enzymes from pancreatic extract, carboxy-polypeptidase and amino-polypeptidase capable of splitting some peptides, while his trypsin-kinase does not attack these compounds. According to Abderhalden (25), however, trypsin-kinase solution on standing in glycerin recovers its power to hydrolyze leucyl-glycine and other dipeptides. It was of interest, therefore, to determine the activity of the trypsin fraction obtained in the present experiments with peptides. The writers are indebted to Professor Waldschmidt-Leitz for kindly supplying them with some *d-l*-leucyl-*l*-tyrosine, leucyl diglycine, and chlor-

TABLE VI

Hydrolysis of Dipeptides with Various Trypsin Fractions

20 ml. M/25 peptide solution pH 7.5 in M/10 pH 7.5 phosphate buffer, about 5 mg. trypsin protein nitrogen added, 24 hours at 35°C. 5.0 ml. titrated with N/50 sodium hydroxide (formol titration).

Peptide	*d-l*-Leucyl-*l*-tyrosine	Leucyl diglycine	Chlora-cetyl *l*-tyrosine	Chlora-cetyl leucine	Glycyl-alanine	Glycyl-glycine	Glycyl aspartic acid
Hydrolysis after 24 hrs. with various trypsin preparations							
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Trypsin preparation No.							
1	25	30	100	70	14	12	7.5
3			0				
Crystalline	0	0	0	0	0	0	0

acetyl-*l*-tyrosine. 1/25th molar solutions of the preparations were prepared in M/10 phosphate buffer and titrated to pH 7.6. 1 ml. of various trypsin fractions containing 5 mg. of protein nitrogen was then added to 20 ml. of the peptide solution and hydrolysis allowed to proceed for 24 hours at 37°C. 5 ml. samples were then titrated with N/50 alkali in the presence of formalin, as usual. The method of titration is accurate to about 0.1 ml. N/50 alkali, which corresponds to a hydrolysis of about 1 per cent. The results of the experiment, expressed as per cent of the total hydrolysis are shown in Table VI. The original extract is quite active, especially with chloracetyl-*l*-tyrosine and therefore contains considerable carboxyl-polypeptidase.

The activity with respect to chloracetyl-*l*-tyrosine is lost at the first step in the fractionation; *i.e.*, treatment with strong acid. None of the succeeding fractions showed any activity. The crystalline material possessed no measurable activity with any of the peptides and in this respect agrees with results obtained with Waldschmidt-Leitz's trypsin-kinase.

The trypsin solutions used were in 75 per cent glycerin and had been stored in the ice chest for 4 or 5 months so that there is no recovery of activity under these conditions, as reported by Abderhalden for trypsin-kinase. In order to confirm this result a solution of trypsin was made up in 75 per cent glycerin, titrated to pH 7.5, and kept at 37°C. for 10 days. These conditions are described by Abderhalden (25) as favorable for the recovery of the activity. The solution of crystalline trypsin, however, remained inactive toward the peptides under these conditions so that in this respect it differs from the trypsin-kinase. The addition of glycine to the digestion mixture did not result in any activity (25).

Extent of Hydrolysis

The most striking difference between the various fractions is the extent to which the hydrolysis of casein and gelatin is carried when measured by the formol titration. The crude material causes a maximum increase in formol titration of more than 32 ml. N/50 sodium hydroxide per 5 ml. 5 per cent casein, and of more than 25 ml. per 5 ml. 5 per cent gelatin. The purified fractions only increase the formol titration of casein by 9 ml. M/50 and of gelatin by 7.0 ml. (*cf.* Paper II of this series).

Analysis

The results of ultimate analysis of the protein crystals are shown in Table VII. They are similar to those usually obtained for albumin. The protein contains no phosphorus.

Specific Activity of Different Preparations

About twenty different lots of crystalline trypsin have been prepared during the course of this work from different raw materials. The specific activity of most of these preparations together with that

TABLE VII

Elementary Analysis Crystalline Trypsin

Crystalline trypsin dissolved in N/10,000 hydrochloric acid and dialyzed against N/10,000 hydrochloric acid until free of sulfate. Poured into 10 volumes acetone at 0°, filtered. Dried to constant weight *in vacuo* at 60°C.

Element	Per cent	
	1	2
C	50.0	49.9
H	7.2	7.1
N	14.8	15.0
Cl	2.86	2.9
S	1.10	1.10
P	0.00	0.00
Ash	1.2	1.1

TABLE VIII

Activity of Various Preparations

Preparation	Raw material	Specific activity per mg. protein nitrogen by gelatin viscosity. $[T. U.]^{4gV}_{mg. P.N.}$	
		Raw material	Crystalline trypsin
1931			
June 17	Fairchild (Beef pancreas)	28	106
Nov. 4	Röhm and Haas, "Raw Degomma S" (pig)	7	106
" 13	Frozen beef pancreas juice	13	108, 104
" 17	" " " "	12	110, 94
" 27	" " " "	7	55
Dec. 7	" " " "	16	106, 110
1932			
Feb. 11	" " " "	19	85
May 18	Same material as Feb. 11 after 3 mos. in ice box		120
Mar. 14	Frozen beef pancreas juice		102
Apr. 15	" " " "		50
May 10	Fresh beef pancreas extracted 2 days at 20°C. with 1 volume water + 1 per cent cresol	8.7	62
" 13	Fairchild	25	108

of the crude material (Fraction 1) is shown in Table VIII. With the exception of four preparations, the specific activity was in each case 110 per milligram nitrogen, within the experimental error. The preparations which gave low results behaved abnormally in that heating in acid caused little or no increase in activity. It could easily be shown by repeated fractionation with ammonium sulfate that these low specific activities were not constant values but could be slowly increased. The loss involved in the fractionation was so large as to render the process impractical as a method of preparation. It is evident, however, that occasionally beef pancreatic juice contains a protein which cannot be conveniently fractionated out by the procedure usually followed. There is an indication that the more completely the pancreas is extracted the more of this troublesome protein is contained in the extract. No active material could be isolated from *fresh* (inactive) beef pancreas, so that in the other preparations spontaneous activation had undoubtedly occurred.

Tests of Purity

The fractionation experiment just described shows that the crystalline protein retains its constant chemical and physical properties, including its proteolytic activity, through a long series of successive fractionations and heatings. In these experiments, however, the principal fraction was analyzed. A much more sensitive test for the purity of a material consists in the comparison of the properties of the first small amount of precipitate obtained in a fractionation experiment with the properties of the final fraction left in the mother liquor after the bulk of the material has been precipitated. Theoretically, in either a mixture or a solid solution the maximum difference in composition would occur between these two fractions (21). A special series of experiments was therefore carried out in which a solution of the crystalline material was precipitated with ammonium sulfate and the specific proteolytic activity and optical activity of the first precipitate formed compared with that of the last small amount of material left in solution in the mother liquor. The results of this experiment are shown in Table IX.[4] The experiment shows that the

[4] The crystals as obtained directly always contain some nitrogen which is soluble in 2.5 per cent trichloracetic acid. This may be removed by repeated

TABLE IX

Fractional Precipitation with Ammonium Sulfate

80 gm. crystalline trypsin filter cake dissolved in 500 ml. ¼ saturated ammonium sulfate and precipitated by bringing ammonium sulfate concentration to 0.6 saturation. Repeated three times and precipitate washed with 0.7 saturated ammonium sulfate yield 30.0 gm. filter cake.

		Precipitate	$[T.U.]^{4g\,V}_{mg.\,P.N.}$	$[\alpha]^{D}_{mg.\,P.N.}$ 25°C.
		gm.		
7.5 gm. filter cake dissolved in 100 ml. ¼ saturated ammonium sulfate. Saturated ammonium sulfate added to slight precipitation. Filtered	Precipitate 1a	0.5	87	−0.235
Filtrate, saturated ammonium sulfate added until nearly all precipitated, filtered	Precipitate 1b	6.0	110	−0.258
	Mother Liquor 1c	1.0	119	−0.350
Precipitate 1b dissolved in 50 ml. ¼ saturated ammonium sulfate. Saturated ammonium sulfate added to slight precipitation, filtered	Precipitate 2a	0.1	103	−0.246
Filtrate, saturated ammonium sulfate added to heavy precipitate, filtered	Precipitate 2b	4.0	109	−0.257
	Mother Liquor 2c	1.5	124	−0.267
Precipitate 2b dissolved in 40 ml. ¼ saturated ammonium sulfate. Saturated ammonium sulfate added to slight precipitate, filtered	Precipitate 3a	0.3	95	−0.272
Filtrate, saturated ammonium sulfate added to heavy precipitate, filtered	Precipitate 3b	3.3	104	−0.267
	Mother Liquor 3c	0.3	106	−0.290

first small amount of precipitate obtained was slightly less active, but succeeding fractionations show no significant differences in either the

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extraction in saturated magnesium sulfate or ½ saturated ammonium sulfate. On standing this soluble nitrogen again appears so that it is necessary to use freshly fractionated material for any experiment in which the purity is to be tested.

optical activity or proteolytic activity; *i.e.*, no evidence even under these extreme conditions was found to indicate that the material was either a mixture or a solid solution. They show quite conclusively that it is not a mixture or an adsorption complex of a protein with a non-protein molecule since in either case the composition of the first fraction would be quite different from that of the small amount of material remaining in the mother liquor. If the material were an adsorption complex the first small amount of protein precipitate would be expected to carry down relatively more of the adsorbed compound since this is a general property of adsorption systems. If it were a mixture the hypothetical active non-protein molecule would certainly have a different solubility in ammonium sulfate from that of the protein. In either case the composition of the extreme fractions obtained would be quite different even though the difference in solubility were slight. If the material, however, is a solid solution it is quite possible that no indication of this fact would be obtained in this type of experiment. However, since the bulk of the material is undoubtedly a protein it is unlikely that a solid solution would be formed except with another protein. The existence of a solid solution is rendered more unlikely by the fact that the optical activity as well as the proteolytic activity is constant since the optical activity is a very specific property even of similar proteins.

Solubility Experiments

Another sensitive test for the purity of a protein consists in the determination of its solubility in the presence of varying amounts of solid phase. According to the phase rule, the solubility of a pure substance at constant temperature and pressure is constant and independent of the quantity of solid phase present, while the solubility of a mixture will increase at first with the quantity of solid present and will not be independent of this quantity until all the components present in the original solid are present in the solid phase. In the case of an ideal solid solution the solubility will increase gradually and indefinitely with increase in the quantity of solid present. The force of this method has already been illustrated in the case of the first crystalline protein obtained, since such experiments showed at once that the substance was not pure. A still more striking example is furnished

by Sörensen's experiments (22) with serum globulin and casein in which it was shown that these proteins although considered as chemical individuals for many years were not pure substances but were probably solid solutions of several similar proteins.

Unfortunately, owing to the unstable nature of the protein, it is impossible to carry out accurate solubility determinations with trypsin since even at 5°C. there is always some loss in activity during the course of the experiment. Another difficulty is that the solubility of the protein in ammonium sulfate is very sensitive to slight changes in the concentration of ammonium sulfate so that a difference of about 10 per cent in the ammonium sulfate concentration makes a difference of over 100 per cent in the solubility. This change in solubility with ammonium sulfate concentration is shown in Table X.

TABLE X

Solubility Crystalline Trypsin in Different Concentrations Ammonium Sulfate in N/10 pH 4.0 Acetate Buffer at 5°C.

M/10 pH 4.0 acetate buffer, *ml*	3	4	6
3.00 molar ammonium sulfate, *ml*	10	10	10
Ammonium sulfate concentration mols/liter	2.30	2.14	1.87
Protein nitrogen per ml., *mg*	0.30	0.74	>5.0

A good deal of time was spent in the attempt to obtain accurate solubility values but it was impossible to reach equilibrium conditions without some loss in activity. The experiments were made under the same general conditions as those already described for pepsin. The result of one such experiment is shown in Fig. 2 and represents the solubility of crystalline trypsin in a solvent prepared by mixing 4 ml. of N/10 pH 4.0 acetate buffer with 10 ml. 3 molar ammonium sulfate at 5°C. The points represented by open circles were obtained by dissolving the protein in the acetate buffer and precipitating the solution by adding ammonium sulfate; the points indicated by dots were obtained by stirring an excess of solid with the solvent. The fact that the values obtained in this way are about the same shows that the value is an equilibrium one. There is some indication that the solubility increases slightly with increasing quantity of precipitate but it is doubtful whether this increase is outside the experimental error.

The solid line in the figure represents the theoretical solubility curve for a substance having a solubility of 0.75 mg. protein nitrogen per ml., and a specific proteolytic activity, as measured by the change in viscosity of pH 4.0 gelatin of 100 T. U. per milligram protein nitrogen. The fact that the points indicating the activity per milliliter fall slightly below those indicating the milligrams protein nitrogen per milliliter shows that the specific activity, as determined in this particular experiment, was slightly less than 100. Determinations made with the same material somewhat later and with a new gelatin solution gave a value for the specific activity of 100 so that this low figure was probably due to a slight difference in the gelatin preparation.

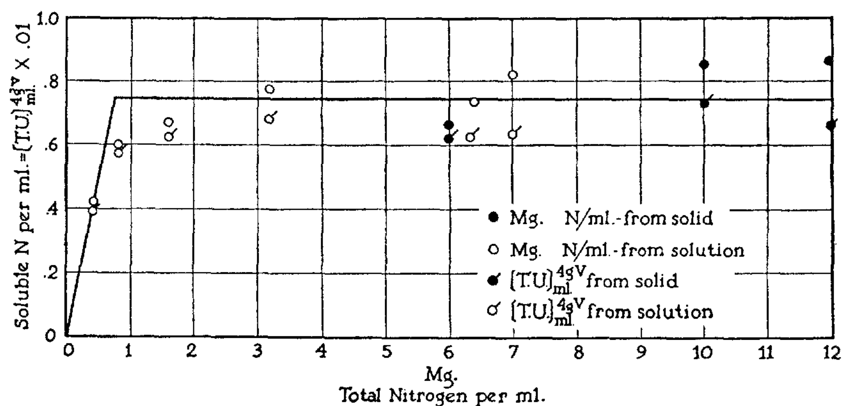

FIG. 2. Solubility of crystalline trypsin in {4 ml. N/10 pH 4.0 acetate buffer; 10 ml. 3 M. ammonium sulfate} at 5°C. with increasing quantities of solid trypsin.

A probable cause of diverging results in these experiments is the difference in solubility between the crystalline and amorphous forms of the precipitate. In the case of pepsin (9) this complicating effect could be clearly detected and experimentally verified by microscopic examination of the precipitate. In the case of trypsin, however, the crystals are so small as to render it difficult under the conditions of a solubility experiment to determine microscopically whether the precipitate is crystalline or amorphous. The determinations made with a total of 6 mg. total nitrogen per ml. were obtained with a precipitate which was undoubtedly crystalline and since they fall in

the same concentration range as the others, it is probable that the precipitate in all these experiments was crystalline since a marked difference in solubility between the crystalline and amorphous forms is generally found with proteins. As a whole the fractionation experiments show quite conclusively that the crystalline material is not a mixture nor an adsorption complex but they do not rule out the possibility that the material is a solid solution.

Relation of the Proteolytic Activity to the Protein

Heat Inactivation.—The experiments described show that under the conditions studied so far the material behaves like a pure substance, or

TABLE XI

Decrease in Activity and Protein Concentration on Heating Trypsin Solutions in N/10 Hydrochloric Acid

2 gm. crystalline filter cake dissolved in 40 ml. N/10 hydrochloric acid and solution heated to 95°C. by immersion in boiling water. 1 ml. samples taken, cooled to 0°C. for 2 minutes, and added to 4 ml. 0.7 saturated magnesium sulfate in N/1 sulfuric acid. Precipitate formed. Centrifuged and protein nitrogen and activity determined on supernatant liquid.

Time at 95°C., *hrs*	0	0.10	0.20	0.30	0.40
$[\text{T. U.}]^{4gV}_{\text{ml.}}$	118.0	32.8	22.4	7.0	0.94
Protein nitrogen per ml., *mg*	1.50	0.315	0.245	0.245	0.105
$[\text{T. U.}]^{4gV}_{\text{mg. P. N.}}$	80	104	92	29	9.0

possibly a solid solution. They indicate, therefore, that the proteolytic power of the preparation is a property of the protein molecule. This conclusion may be tested in a number of other ways. If the proteolytic activity is a property of the protein molecule then any chemical change in the protein should be reflected by a change in activity. When a solution of the protein is heated for a short time, the protein is denatured; *i.e.*, it is changed to a form which is precipitated by low concentrations of salt. At the same time the activity is lost. When the solution is allowed to cool, however, the protein is no longer precipitated by salt but reverts to its original native condition; at the same time its original activity is regained. This reaction has been studied in detail and

reported in another paper (16). If a solution of the protein is heated for a longer time, however, it does not return to its original soluble condition on cooling nor does the activity return. There is evidently a second non-reversible reaction which changes the denatured protein into another form. If the rate of formation of this permanently denatured protein be compared with the loss in activity of the solution, it is found that the decrease in activity is proportional to the decrease in native protein. The results of such an experiment are shown in Table XI. This experiment is carried out in the same way as that already reported with the first crystalline material obtained (Table III). In that case the specific activity increased rapidly at first, showing that the original material undoubtedly contained an inactive protein. In the present experiment there is also a slight increase in the specific activity at first. The specific activity then remains practically constant until only 20 per cent of the original total activity is left; it then decreases rapidly. Evidently on long heating there is formed in the solution a compound which does not precipitate with salt solution but does precipitate with trichloracetic acid. Such compounds are always found in the acid hydrolysis of proteins and it is not surprising that they are found under these conditions. The material used in this experiment originally had a specific activity of slightly more than 100. On standing the activity decreased somewhat, evidently with formation of a protein which is rapidly and permanently denatured by heat, so that the first few minutes heating results in a slight increase in the specific activity to its original value. This behavior has been noted consistently and indicates that on standing at room temperature the active protein becomes transformed into an inactive one which has lost the remarkable property of reverting to the native condition immediately on cooling after being heated. This experiment shows that when the protein is denatured by prolonged heating there is a corresponding loss in activity and therefore agrees with the result expected if the activity were a property of the protein molecule.

Pepsin Digestion.—It is known that pepsin digestion destroys the tryptic activity of a solution (Long and Johnson; Long and Hull (23)). If the proteolytic activity were a property of the native protein molecule it would be expected that the loss in activity during pepsin digestion would be proportional to the loss of native protein. If, on the

other hand, the proteolytic activity were due to some molecular species accompanying the protein, it would be expected that the protein concentration would decrease more rapidly than the activity since, so far as is known, pepsin acts only on proteins. This result would also be expected if the material were a mixture of an inactive with an active protein, since the rate of hydrolysis of different proteins with pepsin is highly specific. This would result in a change in the specific activity when calculated on the basis of total protein present. The same result would be obtained if any portion of the protein molecule

TABLE XII

Decrease in Activity and Protein Concentration in Trypsin Solutions Digested by Pepsin

1 gm. crystalline trypsin cake dissolved in 30 ml. M/100 hydrochloric acid at 35.5°C. 0.5 ml. crystalline pepsin solution containing 0.28 mg. protein nitrogen added. Protein nitrogen determined with 2.5 and 18 per cent trichloracetic acid. Activity determined by gelatin viscosity method. The activity due to pepsin was negligible.

Time at 35°C., *hrs.*	0	1	2	4	8	24	48
$[T. U.]^{4gV}_{ml.}$	125	99	74	56	40	20	10.3
Protein nitrogen per ml., *mg.*							
18 per cent CCl_3COOH	1.49	1.24	1.1	0.96	0.79	0.51	0.40
2.5 " " CCl_3COOH	1.21	0.93	0.62	0.51	0.40	0.23	0.20
$[T. U.]^{4gV}_{mg. P. N.}$							
18 per cent CCL_3COOH	84	80	67	58	51	39	26
2.5 " " CCL_3COOH	103	107	119	110	100	87	52

retained any appreciable activity. In this case the specific activity of the total protein left in solution would increase since the activity due to the fragment of the protein molecule would be added to that due to the unchanged protein itself. The result of an experiment in which crystalline trypsin was digested with a very small quantity of crystalline pepsin is shown in Table XII. The total protein present in solution was determined by precipitation with 2.5 per cent and also with 18 per cent trichloracetic acid. No quantitative method exists by which the native protein alone can be determined but the nitrogen precipitated by 2.5 per cent trichloracetic acid is probably not far

from the correct amount. 18 per cent trichloracetic acid precipitates considerably more nitrogen than 2.5 per cent and if any of the higher split products of the protein retained any activity, it might be expected that the specific activity calculated from nitrogen precipitated with 18 per cent trichloracetic acid would be more constant than that obtained when 2.5 per cent trichloracetic acid is used. The results of the experiment, however, show that the specific activity calculated on the basis of the nitrogen precipitated with 2.5 per cent trichloracetic acid remains constant until more than 70 per cent of the total activity is lost, while the specific activity calculated on the basis of 18 per cent trichloracetic acid decreases rapidly. Apparently, therefore, as soon as the protein is split so that it no longer precipitates with 2.5 per cent trichloracetic acid all its activity is lost and even the fragments still large enough to precipitate with 18 per cent trichloracetic acid possess no activity. The results indicate that the proteolytic activity is a property of the entire molecule as is the case with the (reversible) oxygen combining power of hemoglobin. It was found that this experiment was a very sensitive test for the purity of the preparation used, as is the effect of heating in acid. Preparations which have become partially inactivated show at first a rapid increase in the specific activity under these conditions due to the fact that the inactivated, denatured protein is more rapidly hydrolyzed than the active, native protein. With such preparations, therefore the initial specific activity is low but increases rapidly until it reaches a figure of about 100 and then remains constant.

Alkali Inactivation

When a solution of trypsin is allowed to stand in slightly alkaline solution the protein decomposes rapidly (16) so that these conditions furnish another method of comparing changes in the protein and changes in the activity. The results of an experiment under these conditions are shown in Table XIII. They are similar to those obtained with pepsin digestion in that the decrease in activity is very nearly proportional to the decrease in protein nitrogen as determined by 2.5 per cent trichloracetic acid, and is greater than the loss in protein nitrogen as determined by 18 per cent trichloracetic acid. They indicate again that the proteolytic property is lost so soon as any change occurs in the original native protein molecule.

The hydrolysis of the protein under these conditions may be ascribed to several possible mechanisms. It may be simply a case of the usual alkali hydrolysis of proteins, or it may be considered that the protein digests itself, and in either case it might be supposed that the formation of denatured protein was an intermediate step in the reaction so that the experiment as a whole might be considered simply as a variation of the result already obtained (16) to the effect that the activity is lost when the protein is denatured.

TABLE XIII

Decrease in Activity and Protein Nitrogen Concentration of Trypsin Solutions in Sodium Bicarbonate at 35.5°C.

0.50 gm. crystalline trypsin filter cake dissolved in 15 ml. N/100 sodium bicarbonate at 35.5°C. Protein nitrogen determined by precipitation with 2.5 or 18 per cent trichloracetic acid. Activity determined by viscosity pH 4.0 gelatin.

Time at 35.5°C., *hrs*		0	0.5	1.0
Protein nitrogen per ml. solution	18 per cent CCl_3COOH), *mg*	1.0	0.66	0.54
	2.5 " " CCl_3COOH), *mg*	0.85	0.46	0.40
$[T. U.]^{4gV}_{ml.}$		116	52	34
$[T. U.]^{4gV}_{mg. P. N.}$	18 per cent CCl_3COOH	116	79	62
	2.5 " " CCl_3COOH	(136)[1]	113	84

[1] This figure is based on one experimental value and is probably an experimental error.

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