that Kiliani reports that "Gitalin," made according to Kraft's process, is a mixture containing some digitoxin, this cold-water extract requires further investigation, especially as very little is known of the chloroform-insoluble portion.

3. The saponing substance of digitalis leaves, digitsaponin, is a totally inactive and non-hemolytic susbtance when purified. This disposes of one possible source of error in pharmacological tests on the leaf.

4. Hatcher's chloroform-soluble fraction of the infusion is a mixture of "Gitalin" and digitoxin. If the accepted theory that the cause of the deterioration of digitalis preparations is the water-soluble, chloroform-soluble substance or substances which we have designated as "Gitalin" is true, then such a fraction should also be unstable. Work is being continued on this point.

The writer is indebted to Dr. Henry Kraemer for his many suggestions, and for his assistance in obtaining the crude drug used in this investigation, which was obtained from plants grown by him at the Frederick Stearns & Co. Medicinal plant garden at the University of Michigan during 1918 and 1919.

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A FURTHER STUDY OF THE PROCESS OF PURIFYING PANCREATIC AMYLASE.

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In a recent paper from this laboratory[1] the proteolytic and amylolytic activities of some of the fractions obtained in the purification of pancreatic amylase were compared. The results showed that the attempts to purify pancreatic amylase by fractionation of pancreatin result in greatly concentrating both amylolytic and proteolytic activities in certain fractions, but also revealed large losses of both types of enzyme activity and indicated to us that the purification process should be studied in some detail in order that more light might be thrown upon the place and nature of the losses. Especially was it sought to determine in so far as practicable whether the loss of amylolytic activity which occurs, for example, when the partially purified amylase is dissolved and reprecipitated, is due to incompleteness of precipitation, to deterioration (inactivation) of the enzyme under such treatment, or conceivably to a change of amylase into protease or a shifting of amylolytic to proteolytic activity. Accordingly, the process of purification previously developed in this laboratory[2] was carried out with determinations of total solids and enzyme activity at

[1] Sherman and Neun, THIS JOURNAL, 41, 1855–62 (1919).

[2] Sherman and Schlesinger, *ibid.*, 33, 1195–1204 (1911).

each step in the process where such determinations were feasible. The colloidal nature of the materials dealt with and the very great instability of the active enzyme[1] necessarily render conclusive results exceptionally difficult of attainment.

Effect of Alcohol and Ether upon the Activity of the Pancreatic Amylase.

Since the solutions to be withdrawn for testing at different stages in the purification process all contain alcohol and many of them contain ether, experiments were first made to determine what effect, if any, would be exerted upon the activity of the enzyme by alcohol or alcohol and ether in such amounts as might be introduced with the enzyme in the various solutions and mother liquors to be tested. For this purpose equal portions of the same amylase preparation were treated for the determination of "amylolytic power"[2] with and without the addition of alcohol or alcohol and ether. The substrate was made up in the usual way[2] and the alcohol or alcohol and ether added to it before it was placed in the 40° water-bath. Some of the ether was doubtless evaporated while the substrate was coming to the temperature of the bath and during the digestion, but this is equally true of the later experiments which these tests were intended to control. The results are shown in Table I.

TABLE I.—EFFECT OF ALCOHOL AND ETHER ON THE ACTIVITY OF PANCREATIC AMYLASE.

Per cent. alcohol by volume.	Per cent. ether by volume.	"Amylolytic power" shown by pancreatin.
0	0	259
0	0	273
0.1	0	280
2.0	0	280
2.0	0	274
5.0	0	258
2.0	2.0	270
2.0	6.0	258

Greater concentrations of alcohol and ether were used here than in the later experiments and since the observed variations in the enzyme activity are no greater than the usual experimental variations, it is concluded that these reagents, in the concentration used, are without effect on the activity of the enzyme.

Extraction of the Pancreatin.

In every case the extraction was made with 50% alcohol by grinding 20 g. of pancreatin with the alcohol in a mortar. In the first 2 preparations the pancreatin was ground with the bulk of the 200 cc. of alcohol, only a few cc. being retained to rinse the mortar, and the maceration filtered through a fluted filter, which filtration required nearly 2 hours. In the third prep-

[1] Sherman and Schlesinger, THIS JOURNAL, 37, 1309 (1915).

[2] Sherman, Kendall and Clark, *ibid.*, 32, 1073–86 (1910).

aration the alcohol was used in portions of 75 cc., 75 cc., and 50 cc., and drawn off each time on a Büchner funnel with suction. In the fourth preparation two 100 cc. portions were used and each filtered with suction.

The result of these variations in the method of extraction was not very marked. Somewhat more of the solution was recovered in the later cases, but the more efficient extraction of solids did not result in more efficient extraction of the amylase. However, the use of suction reduces the time required for filtration very greatly. The factors are too variable to afford a constant time of filtration, but in the third experiment only 20 minutes was required.

Precipitation with Alcohol and Ether.

A mixture was made containing one part of absolute alcohol and 4 parts of anhydrous ether. In Preparations 1, 2 and 3 the filtered, 50% alcohol extract was added to 7 times its volume of this mixture and the whole was rotated somewhat and allowed to stand for 30 minutes in the ice-box in order to induce coagulation of the precipitate. At the end of this time a yellow viscous precipitate had stuck to the bottom of the jar. The liquid (Mother Liquor 1) which was still slightly turbid was decanted.

In Preparation 4 the ratio of acohol to ether was changed. The filtered extract (180 cc.) was poured into 4 volumes of 1 : 1 alcohol-ether mixture. After standing for 30 minutes only a small amount had settled out, and so 280 cc. of ether was added, which produced a precipitate of the usual appearance. In Preparation 5, as a result of Preparation 4, four and one-half volumes of 4 : 5 alcohol-ether mixture was used. The liquid became turbid but no precipitate formed until 2 additional volumes of ether had been added. The pancreatin used here was a different sample which was probably responsible for the different concentrations required. Two points seem to be established with respect to this precipitation; that ether is essential to the coagulation of the precipitate at this point, and that the exact concentration necessary for effective precipitation varies somewhat with different samples of pancreatin.

The viscous precipitate from the alcohol-ether mixture (Precipitate 1) was dissolved in 40 cc. of thrice distilled water and poured into 200 cc. of absolute alcohol. The resulting precipitate (Precipitate 2) was an amorphous solid but no longer viscous. It should be noted that a precipitate is now obtained with alcohol alone, although the presence of ether was necessary in the first precipitation. (See discussion on p. 1905.)

In each case an attempt was made to disperse Precipitate 2 in 250 cc. of 50% alcohol, preparatory to dialysis. At first a dispersion was obtained which was apparently uniform but, as has been reported,[1] whenever this was put to dialyze a precipitate settled out ("sac precipitate"). An investigation of this phenomenon disclosed the fact that, when all

[1] Sherman and Neun, THIS JOURNAL, 40, 1138 (1918).

lumps are broken up and Precipitate 2 disappears, the liquid contains a finely divided suspension, which will coagulate and precipitate if stirred for a few minutes. It was first noticed that this new precipitate (Precipitate 3) apparently ceased to come down after the first hour of dialysis. It was then found that if the precipitate were allowed to form in the beaker for 20 minutes before being placed in the sac and then filtered off, very little came down during the dialysis. Finally, prolonged stirring (15 minutes) failed to produce complete dispersion and when this mixture stood 45 minutes in the ice-box it became very turbid. Filtration of this suspension through hardened paper gave a clear liquid which did not become turbid on standing for 15 minutes in the ice-box. From these data it seems that Precipitate 2 cannot be dispersed completely in 250 cc. of 50% alcohol even though all the solids present were previously dissolved in less than 200 cc. of the same solvent. This point has not been investigated in the earlier work in this laboratory due to the fact that the object has always been to produce a highly active amylase and so this dispersion was made as quickly as possible to avoid deterioration. While the fine white suspension is only slightly visible, the loss of salts as dialysis begins hastens the coagulation, consequently the precipitate appears to form from a clear solution as dialysis proceeds. In all cases this precipitate[1] was filtered out before proceeding further.

This filtrate (250 cc.) was dialyzed against 3 successive 2-liter portions of 50% alcohol; the first for 16 hours, the second for 8 hours, and the third for 16 hours; in all 40 hours. The inner solution (Mother Liquor 3) measured 300 to 450 cc. and was filtered from Precipitate 3 unless that had already been removed. Some variations were made in this dialysis. In the first experiment a thin collodion sac was used and a test of the dialysates showed that they were both amylolytically and proteolytically active. In order better to avoid mechanical defects a heavier sac was made for the second dialysis. In the third experiment a medium sac was used and in all 3 cases some of the enzyme was found in the dialyzates. No maltose was present during dialysis in Experiment 1, 5 g. in Experiment 2, and 2 g. in Experiment 3. The effect of the maltose in reducing deterioration of amylase was not conclusively determined but does not seems very great.

Thirty cc. of the clear Mother Liquor 3 was removed for the determination of activity and solids and the remainder poured into an equal volume of a 1 : 1 alcohol-ether mixture, and allowed to stand for one hour. The mixture was then centrifuged and the precipitate (Precipitate 4) removed from the tubes with a steel spatula, and dried in an evacuated desiccator in the ice-box. In Preparation 3, Mother Liquor 4 was turbid and was

[1] As previously noted, this precipitate, formerly referred to as "sac precipitate" or "sac settlings," is very active proteolytically.

allowed to stand overnight when it was centrifuged again (Precipitate 5). To Mother Liquor 5 was added more alcohol and ether and the mixture centrifuged. This gave Precipitate 6.

In Preparations 4 and 5 dialysis was omitted and the relative proportions of alcohol, ether and water were varied somewhat in view of our experience with Preparations 1 and 3. While a moderately active product was obtained, it was not so active as the best preparations which have been made in this laboratory. However, the time required to make a preparation was reduced from 3 days to one.

The amylolytic activity was determined in all cases by the method of Sherman, Kendall and Clarke,[1] except that in the case of the liquid dispersions the activity was calculated to one cc. and not to one mg.

Proteolytic activity was determined by a variation of the method given by Sherman and Neun.[2] The 100 cc. of digestion mixture was taken from the bath and poured into 25 cc. of a solution containing 200 g. of crystallized sodium sulfate per liter and then 5 cc. of 0.5 *M* hydrochloric acid was added. This mixture was then made up to 200 cc., filtered, and aliquots taken for the determination of nitrogen. The method of making the casein substrate was modified as follows in order to avoid some of the usual difficulties, especially the formation of lumps. 8 cc. of molar sodium hydroxide was added to 200 cc. of distilled water and the solution heated to 40°. 10 g. of casein was finely powdered and added slowly to the solution with constant strirring. The dispersion was then heated rapidly to 80° or 90° and filtered through a fluted filter paper. This gave

TABLE II.—DISTRIBUTION OF THE SOLIDS AND ENZYMIC ACTIVITY EXPRESSED AS PER CENT. OF THE TOTAL.

	Expt. 1.		Expt. 2.		Expt. 3.	
	Solids.	Amylolytic activity.	Solids.	Amylolytic activity.	Solids.	Amylolytic activity.
Residue	62.5	19.3	40.0	22.2	48.8	18.7
Mother liquor 1	4.3	0.7	5.0	0.1	6.4	2.1
Mother liquor 2	12.2	0.0	13.5	0.0	13.9	0.0
Mother liquor 4	2.0	0.6	1.3	4.3	1.6	0.2
Dialyzate 1	7.8	3.7	2.5	1.2	4.4	1.6
Dialyzate 2	1.8	1.5	0.6	0.6	1.7	0.5
Dialyzate 3	1.6	1.4	0.5	0.9	1.3	1.3
Precipitate 3	0.1	0.1	1.0	0.2	1.0	0.4
Precipitate 4	0.9	5.7	0.9	4.7	2.4	14.5
Washings	10.1	0.3	..	..	..	..
Total	102.9	33.2	64.3	34.2	81.2	39.3
Loss	...	66.8	35.7	65.8	18.8	60.7
Mother liquor 3	2.9	8.0	6.4	19.5	5.7	34.7

NOTE.—The "washings" given in this table consisted of some 50% alcohol used in the first experiment to wash the residue from the first filtration.

[1] *Loc. cit.*

[2] Sherman and Neun, THIS JOURNAL, 38, 2203 (1916).

a clear dispersion which is made up to 500 cc., and 50 cc. used for each determination.

The distribution of the solids and enzymic activity of the pancreatin during its purification is shown in Table II.

Proteolytic activities of most of the fractions were also determined.

Discussion.

The phenomena observed in the various preparations show that Precipitate 3 ("sac precipitate") does not come down on account of any effect of the sac nor primarily as a result of the dialysis, but is formed by the coagulation of particles which are present as fine suspension. This coagulation will take place if the suspension is allowed to stand, but it is accelerated by dialysis. The fact that this fraction is insoluble in 250 cc. 50% alcohol while it was formerly dissolved in less than 200 cc. of that reagent is inexplicable as a case of true solution but perfectly regular when considered as a colloidal phenomenon, since substances present in the earlier dispersion are absent in the latter, having been removed by the intervening precipitations. This fraction, as has been stated in earlier papers, is a very active protease; in fact, it is the most active protease preparation of which we have knowledge. In this connection it should also be noted that the first product (Precipitate A) obtained by Sherman and Neun (*loc. cit.*) through alcoholic precipitation after the usual dialysis was a more active protease than the second one (Precipitate B). These results indicate that the protease fraction of pancreatin is more easily coagulated than the amylase. If this is so, a larger proportion of the protease should remain behind during dialysis. The protease not only dialyzes but also precipitates during the dialysis and so the activity of this precipitate must be combined with that of the inner solution in order to permit a proper comparison of the relative rates of dialysis of the amylase and protease originally present. Compared in this way, it was found that in each of our 3 experiments the protease remained in the dialyzing sac in larger proportion than did the amylase. It seems then that in the dispersed state the protease dialyzes more slowly and, therefore, presumably consists of larger particles than the amylase.

From 3 to 7% of the original amylase was found in the dialyzates. This amounts to from 10 to 20% of the total amylase accounted for, or on the average about $^1/_5$ of the amount remaining in the sac after dialysis. Such slow dialysis is enitrely consistent with the classification of the enzymes as colloidal substances, since it has often been found that some colloids do dialyze through collodion membranes.

The average of the first 3 experiments gave the amylase content of the residue as 20.0% of the original; Mother Liquors 1 and 2, and Precipitates 3, 7.1%; Mother Liquor 3, 20.0%. At the end of the dialysis, therefore, there remained 20% of the original total amylolytic activity,

and 27.2% was present in the fraction separated before the end of the dialysis, in all 47.2%, leaving a deterioration of 52.8%. Of the 20.0% remaining after dialysis 10% was recovered in Precipitate 4 and Mother Liquor 4 and 10% deteriorated. We see then, that of the original amylolytic activity 62.8% was lost by deterioration and 37.2% was found in the fractions separated. Precipitate 3 is obtained from a volume of from 300 cc. to 500 cc. and Precipitate 4 from a volume of 600 cc. to one liter. This precipitation brings down about 35% of the solids present which represents from 25 to 70% of the amylolytic activity and from 30 to 80% of the proteolytic activity present. When the activity of the enzyme left in the Mother Liquor 4 is taken into account, the deterioration in the last precipitation amounts to from 20 to 60% of the amylase and from 6 to 50% of the protease present. The 2 main factors in this loss are the heating of the centrifuge and the fact that the precipitate cannot be removed quantitatively from the centrifuge tubes. In these experiments liquid air was not used for cooling the centrifuge, as in some of the work of Sherman and Neun, and the higher temperatures here encountered presumably increased the loss due to deterioration in solution and lowered the activities of the final precipitates.

It seems that precipitation with absolute alcohol is efficient in freeing an aqueous dispersion of the enzyme. Whether it precipitates nearly all of the enzyme or inactivates that which remains in solution is not certain, but from the results recorded in the previous paragraph it seems probable that the enzyme is fairly well precipitated and that the deterioration is no more largely due to the alcohol than to other causes, such as increased temperature. Alcohol cannot be used exclusively as a precipitant because it will not precipitate the first extract unless at least 50% of ether is present. The way in which the ether functions is not known since ether alone will not give the precipitate. The exact concentration of ether necessary varies with the composition of the original pancreatin and the thoroughness of the extraction.

The present experiments afford no convincing evidence as to whether or not amylase changes to protease. The difference in the rate of deterioration here observed may be due either to such a change or to greater instability of the amylase.

Summary.

1. Alcohol up to 5%, or alcohol-ether mixture up to 8% of the volume of the substrate did not materially affect the activity of the pancreatic amylase. Higher concentrations were not tested, as these are much above the quantities introduced with the enzyme in the experiments here described.

2. When 50% alcohol extracts of pancreatin were precipitated by alcohol-ether mixture as in our usual purification process, the residual

solution (Mother Liquor 1) contained about 1% of the active amylase and 2.5% of the solids of the original pancreatin, or about 5% of the solids originally extracted.

3. When the alcohol-ether precipitate was dispersed in water and mixed with absolute alcohol, 25% of the solids originally extracted remained in the filtrate (Mother Liquor 2) but this filtrate showed no amylolytic activity.

4. From 10 to 20% of the amylolytic activity accounted for was found in the dialyzates.

5. In the purification experiments here described about 50% of the amylolytic activity was lost by the end of the dialysis, about 45% in the final precipitation and separation, and about 5% was found in the final precipitate. By the use of liquid-air cooling it has been possible in other experiments to diminish the losses in the later stages of the process and considerably increase the proportion of active amylase recovered in the final product.

6. The precipitate which forms in the inner solution during dialysis (Precipitate 3, "sac precipitate") shows very high proteolytic and little or no amylolytic activity. This material, which was originally extracted by 50% alcohol, now precipitates from this solvent. The explanation of this fact is probably to be found in the removal, through the intervening purification process, of some substance or substances whose presence interferes with the coagulation of the protease or stabilizes its dispersion.

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NEW BOOKS.

Cours de Chimie a l'Usage des Etudiants P. C. N. et S. P. C. N. PAR R. DE FORCRAND. Deuxième Edition, Paris, Gauthier-Villars et Cie. 1918. Tome I, pp. VIII and 438; Tome II, pp. 528. 14 × 22 cm. Price of Vol. I, 15 francs.

The first edition of this book was reviewed in THIS JOURNAL, **27**, 790 (1905). The second edition has veen very considerably enlarged but retains the same general scope. The book is intended for use in the instruction of students who are candidates for the "Certificat d'études supèrieures de physique, chimie et sciences naturelles (or S.P.C.N.). It is intended to give the work covered, in 3 lessons a week for one academic year. If the material of this book is fairly mastered in that period it must represent far more intensive work than is done by American students. The first 75 pages give the fundamental principles, laws and nomenclature of the science. There are some advantages of such a method of approach, but a beginner would certainly find these pages difficult. About