COZYMASE. A STUDY OF PURIFICATION METHODS.*

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Cozymase is one of the essential components of the complex enzyme mixture which effects alcoholic fermentation in the absence of living cells. The separation of the mixture into zymase and cozymase was first accomplished by Harden and Young[1] by means of ultrafiltration through a gelatin-impregnated Chamberland filter candle. The residue and filtrate as thus prepared possessed, separately, no fermentative action, but when mixed were found to produce a rapid fermentation. The active constituent of the residue was named zymase, while that constituent of the filtrate responsible for the reactivation of the residue was named cozymase.

The mechanism of the activation of the zymase was investigated by von Euler and Myrbäk,[2] who came to the conclusion that the cozymase was involved in that stage of the process in which inorganic phosphates are converted to carbohydrate esters. The same authors[3] have described a comprehensive and successful series of experiments on the purification of cozymase.

A complete knowledge of the properties and function of cozymase would be of extreme importance, as it should throw considerable light upon the mechanism of enzyme action as a whole, and should in addition have important bearing upon the entire question of carbohydrate utilization. It has been shown, for ex-

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[1] Harden, A., and Young, W. J., *J. Physiol.*, 1905, xxxii, p. i; *Proc. Roy. Soc. London, Series B*, 1906, lxxvii, 405.

[2] von Euler, H., and Myrbäk, K., *Z. physiol. Chem.*, 1924, cxxxix, 15.

[3] von Euler, H., and Myrbäk,, K., *Z. physiol. Chem.*, 1924, cxxxix, 281.

ample, by Meyerhof[4] that a substance, either identical with, or very similar to cozymase exists in most animal tissue, and the demonstration of a complete identity of the two would have important metabolic connotations.

In view of the importance of the field, as well as of the interest of one of us[5] in the problem of carbohydrate utilization, we decided to attempt an extension of the work of von Euler and Myrbäk on the purification of cozymase. At the beginning, however, we found that our yeast produced much less active enzyme preparations than those of von Euler and Myrbäk, as regards both the zymase and cozymase content, and also that the technique of the above authors with lead precipitation, which enabled them to secure an initial purification of from ten- to thirtyfold, was in our case practically useless.

We therefore abandoned our original intention and have instead studied the purification produced in our material by a variety of reagents. In the investigation we have repeated much of the work done by von Euler and Myrbäk, and several differences have been found, which appear difficult to explain solely upon the basis of the lower initial purity of our material. As certain of the experiments show distinct promise, we hope to be able to extend the work upon a material of considerably higher original purity, such as was employed by von Euler and Myrbäk.

Materials and Technique of Assay.

The yeast which we employed throughout was a bottom yeast supplied by the Eastside Brewery[6] in Los Angeles. It was obtained fresh, transported immediately to Pasadena, and used the same day.

For analyzing extracts for cozymase activity, the analytical procedure adopted was similar to that employed by von Euler and Myrbäk. When zymin (acetone-dried yeast) is washed with water the cozymase is largely removed. The washed material is therefore a source of zymase practically free from cozymase, and

[4] Meyerhof, O., *Z. physiol. Chem.*, 1918, ci, 165; 1918, cii, 1.

[5] Raymond, A. L., *Proc. Nat. Acad. Sc.*, 1925, vii, 622.

[6] I wish to express my gratitude to the many persons connected with the brewery, whose continued courtesy and assistance facilitated this research. A. L. R.

on addition of the latter is again capable of producing fermentation. Von Euler and Myrbäk found on adding cozymase extracts to such washed zymin that within reasonable limits of concentration the maximum rate of carbon dioxide production was proportional to the amount of extract added. Our experience in general confirmed this, as well as the fact that it is necessary to keep the phosphate concentration constant throughout. All solutions were adjusted to pH 6.3 to 6.5 before testing, but even in those cases in which all precautions were observed, we found variations of 5 to 10 per cent in certain analyses and our assays are therefore only reliable within these limits.

In order to prepare our zymin the procedure of Albert[7] was used. Although it appears quite simple, we found difficulty in preparing an actively fermenting zymin from our yeast. On substituting technical acetone for C.P. material, a completely inactive preparation resulted, as was the case when the acetone was added with stirring to the yeast instead of the reverse. Even those lots which were prepared by apparently identical procedures showed considerable differences in activity. The experimental work to be reported was, therefore, performed with two very similar lots which were, incidentally, the best we secured.

For assay purposes, the zymin was freed of cozymase by mixing with 8 parts of water and centrifuging. This washing was repeated twice more, and the zymin then produced almost no fermentation in the absence of added cozymase. The zymin was freshly washed as needed. Zymophosphate, prepared from the first zymin washes, was added to remove an induction period which resulted with the purer fractions.

The final concentrations in the fermenting mixture were:

Na_2HPO_4	0.013 M
KH_2PO_4	0.020 "
Glucose	10.0 per cent
Gentian violet	0.125 " "
Zymin (added)	10.0 " "

The gentian violet was added as an antiseptic and at the above concentration we observed no growths for the periods over which we worked (2 to 12 hours) and there was no appreciable inhibi-

[7] Albert, R., Buchner, E., and Rapp, R., *Ber. chem. Ges.*, 1902, xxxv, 2378.

tion of the enzyme action. The pH of the mixture was 6.4, and the total phosphate concentration was optimum for our yeast.

For determining the carbon dioxide formed an electrometric method[8] devised for the purpose was employed. Readings were made at 15 minute intervals, and the maximum rate was taken as a measure of the cozymase activity of the sample.

Typical fermentation curves are given in Fig. 1. In these the rates of carbon dioxide production are plotted as a function of

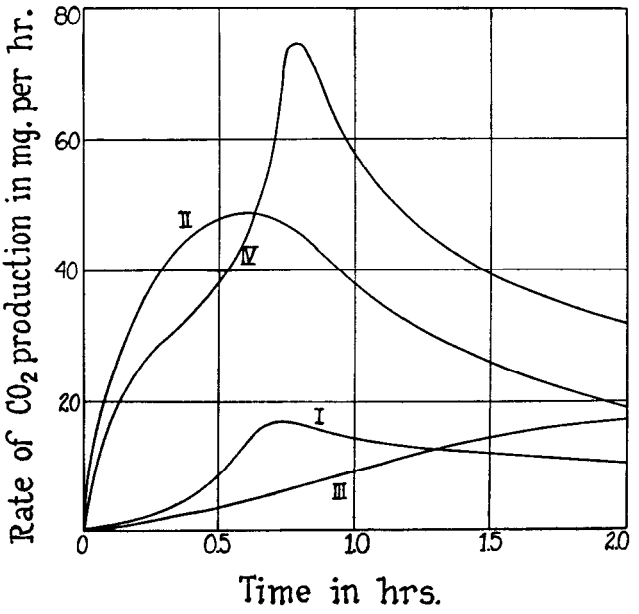

FIG. 1.

the elapsed time. Curves I and II were obtained with different quantities of cozymase, using the glucose-phosphate mixture described above, while Curves III and IV are the corresponding ones for runs employing twice the concentration of phosphate, other factors being unchanged. The longer periods before attainment of the maximum rate in the latter case are characteristic of high phosphate concentrations, as are the larger maximum rates for the same cozymase concentration.

[8] Raymond, A. L., and Winegarden, H. M., *J. Biol. Chem.*, 1927, lxxiv, 189.

For convenience in reporting our results, we have defined a unit as being that quantity of cozymase required to produce a maximum rate of fermentation of 1 mg. of carbon dioxide per hour. We have also used von Euler's activity coefficient, which is defined as the maximum rate of carbon dioxide production in cc. per hour, divided by the solid content of the sample expressed in gm. The symbol Aco was suggested by von Euler to designate this unit and has been employed throughout this paper.

Extraction of Cozymase from Yeast.

Our cozymase solutions were prepared by extracting yeast with hot water, a procedure which has been shown by others to destroy the zymase activity without appreciably impairing the cozymase. Fresh and washed yeasts were both investigated, but as the only difference between the two extracts lay in the fact that those from the washed yeast contained slightly less solids, the latter were not extensively employed.

In order to obtain an efficient extraction, various factors were examined with a view to determining the optimum yield of cozymase, without regard to the purity of the resultant product. Batches of 10 to 20 liters were prepared by adding yeast to water at 90–92°, stirring 2 to 3 minutes, and cooling as rapidly as possible by immersing the metal container in running water.

The effect of concentration of the yeast upon the yield was studied and it was found that the efficiency of the extraction increased largely with decreasing concentration down to 0.1 gm. of fresh yeast per cc. of solution. More dilute extracts were not examined, as solutions of much lower activity required too extensive evaporation before they could be used.

Acidity was also examined with regard to its effect on the yield, glacial acetic acid and sodium carbonate being added to the hot water to vary the acidity. The pH was determined and the solution neutralized to pH 6.4, and tested. The results are included in Table I.

Electrodialysis.

No attempts appear to have been made toward purifying cozymase by means of electrodialysis. As the method offers great possibilities with those substances which migrate under the influ-

ence of an electric field, we decided to investigate the results with our material.

A cell was built consisting of seven 50 cc. compartments, in a row, and separated by parchment paper partitions. There were placed in the compartments in order: I, copper sulfate solution (1 M) and a copper electrode; II, potassium chloride (0.1 M); III, potassium chloride (0.1 M) adjusted to the desired pH; IV, cozymase solution similarly adjusted; V, VI, and VII were duplicates of III, II, and I respectively. The potassium chloride solution in II and VI prevented contamination of that in III and V.

To see if the pH of the solution affected either the magnitude

TABLE I.

Reagent.	HOAc		Control.	Na_2CO_3	
Concentration, *gm. per liter*.....	5	1		1	5
pH of extract.....	3.8	4.6	6.0	7.0	10.0
Activity, *units per cc*.....	3.6	3.6	4.7	2.9	2.5

TABLE II.

Experiment No.	pH	Activity (units per cc.).		
		Anode.	Center.	Cathode.
IV	2.0	0.2	1.4	0.3
II	4.4	0	2.7	0
I	6.4	0.2	3.2	0.2
III	9.7	0	2.3	0

or direction of migration of the activity, a set of four experiments was performed covering a pH range of 2 to 10. A potential drop of 4 volts per cm. was maintained in the cozymase solution and 1000 to 1800 coulombs passed in 40 to 60 minutes. In the case of Experiment III, the apparatus was kept in the ice box to decrease the destruction due to the alkalinity. The pH did not change appreciably during the run. The activities of the solutions are given in Table II. In no case therefore was an appreciable amount of cozymase transferred to the potassium chloride compartments, although tests showed the parchment paper was permeable to the cozymase over the entire pH range employed.

In view of the fact that the paper was permeable to the cozymase and yet practically no transference occurred in an hour, it seems justifiable to conclude that the cozymase carries no appreciable fraction of the current over the pH range examined. Whether this is due to its being present at low concentration, or to its having a low degree of ionization, or to both, cannot be determined from the above data. However, the possibility of purifying crude solutions by this means seems to be definitely excluded.

Alcohol Precipitation.

As alcohol has been suggested for purifying cozymase, we performed a number of experiments with this reagent. We found that an alcohol concentration of 40 per cent produced a fine flocculent precipitate and left the majority of the activity in the

TABLE III.

	Yield.	Aco.	
		Dry.	Ash-free.
	per cent		
40 per cent precipitate	11	15	29
80 " " "	51	36	42
80 " " filtrate	61	31	44
Original		26	36

solution, but that 80 per cent alcohol, on the other hand, produced a less satisfactory separation and frequently occasioned considerable losses of activity. In no case, however, was there any significant purification.

A typical experiment in which the concentration was first made 40 per cent and then 80 per cent is given in Table III. The yields are expressed as the percentage of the original cozymase present in the different fractions. The failure of the activities to add up to 100 per cent is presumably due either to varying phosphate concentrations in the different fractions, or else to the removal of some inhibiting material. The phenomenon was frequently observed.

Lead Precipitation.

Due to the great success of von Euler and Myrbäk in using lead precipitation as a means of purification, we attempted to repeat

their experiments with this reagent. The procedure evolved by them consisted in a precipitation at pH 6, which left the active material in the filtrate, followed by precipitation of this active material at pH 10.

We employed both washed and unwashed yeast and prepared an extract exactly as they described. Precipitation at pH 6 gave a mixture very difficult to filter, which was turbid after centrifugation. Practically none of the activity was to be found in the precipitate. Solid determinations indicated only very slight increase in the Aco. Von Euler and Myrbäk, on the other hand, obtained threefold purification and reported no mechanical difficulties.

At pH 10 we found the activity to be again almost entirely in the filtrate. This is contrary to the results of the above authors, who found precipitation to begin at pH 8.5 and be complete at pH 10. We also duplicated our own results with pH 6 filtrates to which additional lead acetate had been added, indicating that the difference in the alkaline precipitation was not due to insufficient lead. To ascertain whether we were dealing solely with a concentration effect, we evaporated a sample of our material to $\frac{1}{6}$ volume *in vacuo* and repeated the experiment. Again the activity was to be found almost completely in the filtrates, both at pH 6 and 10.

In order to determine the effect of higher pH on the precipitation of the active material, we added lead acetate in different concentrations and tried pH values of 10.5 and 11. The behavior was quite variable and appeared to depend upon the particular batch of material employed. One lot, for example, made 0.005 M with lead acetate, gave a precipitate containing 79 per cent of the active material at pH 10.5 and 72 per cent at pH 11.0, while another, using lead ion concentrations of 0.005, 0.01, and 0.015 M, at pH 10, 10.5, and 11 gave a maximum precipitation of 27 per cent. It is possible that the differences between von Euler's results and our own regarding the precipitation at pH 10 are dependent upon the same factors that must be operating in the cases cited. In any event, the lead is not a satisfactory precipitant until these apparent anomalies are understood.

In those cases in which the lead did precipitate the active material, the purity was considerably increased. The most effective

purification which we secured, however, was but sevenfold increase over the original material. In view of the fact that similar precipitations in the hands of von Euler and Myrbäk produced ten- to thirtyfold increase over their original, the above results do not appear particularly encouraging.

In view of these facts, as well as the one mentioned above that the precipitation is not at all satisfactory at pH 6, we decided to modify von Euler's first procedure as follows. Batches of cozymase solution were prepared by adding 2½ kilos of fresh yeast to 15 liters of water at 90–92°. The solutions were filtered immediately and cooled. The cooled, filtered solutions were placed in a large glass container capable of holding 25 to 30 liters and a hot concentrated lead acetate solution was added to the extent of 7.5 gm. of lead acetate per liter. The pH was then adjusted to 9.0 by the addition of 6 N NaOH with constant stirring, and the mixture was allowed to settle for 1 or 2 minutes. The supernatant portion should be perfectly clear and the mixture should filter rapidly, giving a light yellow, crystal-clear solution. When this was not the case, additional portions of 0.5 gm. per liter of lead acetate were added and the pH readjusted after each addition to 9.0. In no case was it necessary to add more than 8½ gm. per liter of lead acetate to produce perfect precipitation. The phosphate content[9] of the solution dropped from an average of 0.35 mg. per cc. to about 0.007 mg. per cc. which accounts for a large portion of the added lead.

These final solutions, after neutralization and removal of lead, contained from 90 to 100 per cent of the activity of the original extract but solid determinations showed only slight changes in Aco.

Solutions prepared in this way and still containing a small amount of lead, were quite stable. Unlike the original boiled yeast extracts, they exhibited little tendency to develop mold or bacterial growths, and lost less than 50 per cent of their activity on being kept in the ice box for 2 or 3 weeks. As mentioned above, inorganic phosphates were almost completely removed by the lead precipitation, and therefore did not interfere with additional reagents. For these reasons, we adopted this precipitation as

[9] Determined by the method of Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, 1924, lxi, 63.

standard and the further work described in this paper was largely done on such lead-treated solutions, from which excess lead was not removed.

Mercury Precipitation.

The results with mercury as a precipitant for cozymase were particularly interesting, as they showed certain peculiarities that do not appear to have been reported previously in biological purifications. On adding $Hg(CN)_2$, $HgCl_2$, and $Hg(NO_3)_2$ of the same concentration to the lead-treated solution, there were observed, respectively, no, slight, and heavy precipitation. This is in exact correspondence with the degree of ionization of these salts.

More quantitative experiments were performed with $HgCl_2$ and $Hg(NO_3)_2$. To samples of cozymase solution $HgCl_2$ solution was

TABLE IV.

$HgCl_2$ concentration.	Yield.	Aco.	
		Dry.	Ash-free.
M	*per cent*		
Original.		20	31
0.0025	25	75	139
0.005	42	125	185
0.0075	65	117	192

added to concentrations of 0.0025, 0.005, and 0.0075 M and the pH was adjusted to 7.8 to 8.0. The addition of a few drops more of $HgCl_2$ solution produced no precipitation or opalescence in the filtrates, indicating complete precipitation in each case. It was therefore of great interest that not only did the yield of active material carried down by the precipitate increase with increasing concentration of $HgCl_2$, but the Aco likewise progressively increased. The data are given in Table IV.

The above experiment was then repeated with $Hg(NO_3)_2$ at the same concentrations. The clear solutions, after centrifuging off the precipitate, were tested with more of the reagent and further precipitation was found to occur with the 0.0025 and 0.005 M concentrations, but not with the 0.0075. In this case, the yields increased as before, but the purity underwent a constant decrease.

In experiments at pH 6.5 the results were exactly similar to those at the higher pH, but the yield was in each case lower.

The results confirm the experiment of von Euler to the extent that $HgCl_2$ to complete precipitation in neutral or acid solution is a poor precipitant. They are, however, quite different in their final interpretation, for they indicate $HgCl_2$ to have considerable possibilities in the purification, if used in larger quantities than necessary for complete precipitation.

The experiments are also of importance in connection with biological purifications employing mercury, as they indicate important differences resulting from different salts of this element, and serve as well to invalidate complete precipitation as a reliable guide.

Silver Precipitation.

Silver, like mercury, was found to be a fairly satisfactory precipitant for cozymase. Our preliminary experiments indicated

TABLE V.

Concentration, *M*	0.0	0.025	0.005	0.0075
Yield, *per cent*		48	61	63
Aco (ash-free)	30	173	206	203

approximately neutral solutions to be satisfactory and the effect of concentration was therefore investigated at pH 7.2 to 7.3. One experiment is listed in Table V.

Attempts to increase the above yields by altering the acidity met with failure. Occasional batches, however, gave yields of 75 to 85 per cent at the above pH. As the Aco was likewise fairly high, as will be observed above, it would appear that silver might perhaps be profitably employed.

Removal of Metals as Sulfides.

In the case of the three metals described above, lead, mercury, and silver, it was frequently observed that the loss in total cozymase was very great. This was assumed to be due to the fact that when the metals were removed as sulfides, as a preliminary to the tests of activity, some of the cozymase was adsorbed on

the precipitated sulfides. An experiment was undertaken to examine this possibility.

To pairs of samples of cozymase solution the above three ions were added to a concentration of 0.0075 M, and the pH was adjusted in one of each pair to 10 and in the other to 5. Without removing any precipitate, the solutions were saturated with hydrogen sulfide, filtered, and the hydrogen sulfide removed *in vacuo*. The solutions were then tested and the losses in activity thus determined. Losses of from 10 to 20 per cent were observed in the acid solutions and from 0 to 10 per cent in the alkaline.

In purer samples the above phenomenon was of even greater magnitude. With silver, in particular, we had great difficulty and in a few cases losses of as high as 60 and 80 per cent occurred. As the precipitation of the sulfides is best accomplished in acid solutions and as it is here that the greatest losses occur, this factor must be given close attention. We avoided the difficulty as best we could by precipitating the sulfides from dilute solutions in which case the losses were much lower. The matter is of interest because of its general bearing on the problem of biological purifications by means of the heavy metals.

Cadmium.

Cadmium hydroxide was precipitated by adding $CdCl_2$ to the cozymase solution to the desired molality, adjusting to pH 10.5 to 10.7, centrifuging off the precipitate, neutralizing both portions, and removing the cadmium as a sulfide. Preliminary experiments had shown practically no losses by adsorption on cadmium sulfide. Not only were the yields invariably low, but also almost no purification resulted, and this was true at both lower and higher acidities.

Iron.

As precipitation of iron hydroxide in the cozymase solutions was found to be difficult, the hydroxide was freshly prepared, washed, and added to the cozymase solution to 0.05 molal. The solution was made alkaline to pH 10.1 to 10.2, and shaken for 10 to 20 minutes. On centrifuging off the precipitate, neutralizing, and testing, 88 per cent of the original activity was found to remain in the filtrate, while the precipitate was inactive.

Aluminum.

The use of aluminum hydroxide as an adsorbent for the cozymase activity has been discussed by von Euler. He found that the hydroxide was a quite effective adsorbent at pH 10, and deduced the Aco of the material to be quite high. He did not, however, utilize the method.

On original extracts, untreated with lead, we found aluminum hydroxide to be quite ineffective. Aluminum sulfate was added to aluminum ion concentrations of 0.005, 0.01, and 0.015 molal, and then NaOH to pH 10. The precipitates were centrifuged off and both portions neutralized. Table VI gives the distribution of the cozymase in per cent of the original. However, in repeating the experiment, with the exception that a lead-precipitated

TABLE VI.

Concentration, *M*	0.005	0.01	0.015
Precipitate, *per cent*	3	7	25
Filtrate, *per cent*	90	61	59

TABLE VII.

Concentration, *M*	0.005	0.01	0.015
Precipitate, *per cent*	77	103	75
Filtrate, *per cent*	33	4	0

solution was employed, more promising results were secured, as indicated in Table VII.

The difference in the behavior of the two solutions may perhaps be accounted for by the absence of phosphate in the lead-precipitated material, as aluminum phosphate, which is surely formed in the original material, may interfere.

The problem of recovering the active material from the hydroxide still remained. Two methods were examined and both found to be useful. The first consisted in dissolving the precipitate in a minimal quantity of 6 N hydrochloric acid, diluting, and neutralizing. In the second method the precipitate was suspended in water and adjusted to pH 5.9 to 6.1 and kept in the ice box for 12 to 24 hours. As the mixture became more alkaline on standing, it was readjusted once or twice during the period.

Both methods were found to give filtrates containing the major portion of the active material. The second procedure, moreover, resulted in a fairly pure product, the Aco being increased, for example, in one experiment from 30 to 275 (ash-free) by precipitating at pH 10 and reversing the adsorption at pH 6.0. Repeating the adsorption and reversal once more caused an increase to only 335.

Miscellaneous Reagents.

The remaining reagents which we employed were examined only casually and will, for this reason, be only briefly mentioned.

Barium, in accordance with the statements of Von Euler, was found to produce no precipitation of the active material, even in a solution as alkaline as pH 10.5.

Tannic acid was likewise found to precipitate none of the active material from dilute solutions, and concentrated solutions were not investigated. The filtrate from the tannic acid precipitation was noted to develop only faint precipitates with either phosphotungstic or silicotungstic acids.

Silicotungstic and phosphotungstic acids were both tried, but without particular success. Considerable loss of activity in general resulted with no very great increase in the purity of the cozymase preparation.

We desire to thank Professor A. A. Noyes for grants and facilities which allowed us to pursue these studies, and Professor R. C. Tolman for his constant interest and encouragement.

SUMMARY.

Electrodialysis and a number of reagents have been examined with regard to their applicability to purifying cozymase extracts.

Lead salts were found to give variable results. The experiments of von Euler and Myrbäk could not be duplicated on the available cozymase extracts.

Mercuric chloride and nitrate were found to effect different results. The experiments appear to have considerable bearing on biological purifications with this element.

Mercury, silver, and aluminum were found to be most useful.