[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF MCGILL UNIVERSITY.]

ENZYMES: THE SYNTHETIC AND HYDROLYTIC OXYNITRILASE. PART II.

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Rosenthaler[1] has recently published a series of papers which seemed to indicate that emulsin contains a synthetic as well as a hydrolytic enzyme for benzaldehyde-cyanhydrin. The contention that the hydrolytic enzyme is distinct from the synthetic is such a fundamental one in enzyme chemistry that it seemed worth while to investigate it still further, using quantitative methods. Bayliss,[2] has repeated certain of Rosenthaler's experiments and came to the conclusion that Rosenthaler's contention is not well founded. Bayliss' experiments, however, are not strictly parallel to those of Rosenthaler's and his conclusion is, therefore, hardly justified. Rosenthaler used emulsin which he subjected to various treatments and then studied its synthetic action on hydrocyanic acid and benzaldehyde, and its hydrolytic action on benzaldehydecyanhydrin.

$$C_6H_5CHO + HCN \rightleftarrows C_6H_5CHOHCN.$$

Bayliss used glucose and glycerol to study the synthetic action and the corresponding glycerol glucoside to study the hydrolytic action. These reactions are quite distinct from each other. Rosenthaler's is one of addition while Bayliss' is a condensation reaction between an alcohol and glucose with the loss of water. This point seems important as it has not yet been proved that the oxynitrilase in emulsin is also the enzyme which splits glucose from the benzaldehydecyanhydrin in the glucoside amygdalin. In fact it seems very unlikely that oxynitrilase has this power, as the preparation of the enzymes from the leaf of *Prunus Serotina* does not hydrolyze salicin to glucose and the alcohol saligenin,[3] or amygdalin into glucose or a disaccharide and the alcohol benzaldehydecyanhydrin. Whether it splits glucose from Fisher's mandelonitrile glucoside has not yet been determined. As Rosenthaler[4] did not intend that his methods of treatment would distinguish the hydrolytic from the synthetic enzyme in any other case except the oxynitrilases, it is necessary to restrict oneself to the chemical reaction which he used, namely, the synthesis and hydrolysis of benzaldehydecyanhydrin. Unfortunately, the cyanhydrin reaction is a very tedious one to study. We spent considerable time in trying to develop methods of analysis in order that we might follow the rate of combination. The first method we used was to stop the reaction,

[1] *Biochem. Z.*, **28**, 408 (1910); **50**, 486 (1913); *Arch. Pharm.*, **251**, 56 and 85 (1913).

[2] *J. Physiol.*, **46**, 236 (1913).

[3] Krieble, THIS JOURNAL, **35**, 1643 (1913).

[4] *Biochem. Z.*, **59**, 498.

extract the nitrile present, and then hydrolyze it with hydrochloric acid to mandelic acid. The mandelic acid was extracted with ether and then the ether was boiled off and the mandelic acid dried to constant weight. As the mandelic acid was contaminated with benzoic acid it was necessary to dissolve it in water and make it up to a definite volume and then titrate an aliquot part in order to calculate the amount of pure mandelic acid. The method did not prove satisfactory, as mandelic acid forms an internal anhydride when evaporated in presence of traces of hydrochloric acid. The second method was more satisfactory and depended on the fact that when a nitrile is hydrolyzed with hydrochloric acid all the nitrogen is changed to ammonium chloride. The nitrile was extracted with ether and then hydrolyzed with hydrochloric acid as before. The hydrochloric acid solution was then made up to a definite volume and an aliquot portion transferred to a Kjeldahl distillation flask. It was made strongly alkaline and distilled into standard acid. From the amount of standard acid used one could calculate the amount of nitrile in the original solution. Before we tried this method very extensively, we came across a method devised by Wirth[1] which proved so very satisfactory that we used it exclusively in our later experiments. The method depends on the fact that dilute acid arrests the synthesis or hydrolysis of mandelonitrile completely. The amount of free hydrocyanic acid can then be estimated with the aid of silver nitrate and ammonium thiocyanate.

Another difficulty in studying this reaction is its sensitiveness to minute traces of acid. In our earlier experiments we prepared the benzaldehyde for each experiment by shaking up a suitable amount of the laboratory stock with bicarbonate of soda solution, then extracting it with ether, drying it over anhydrous sodium sulfate and then distilling, collecting the ether separately. We did not always, however, use the benzaldehyde at once. Sometimes it was prepared several hours before we actually used it. In such cases the aldehyde would be kept in a small stoppered Erlenmeyer flask. We found that we could not get concordant results in this way and we finally traced the difficulty to the fact that the acidity of the aldehyde varied sufficiently to change the rate. We found, for example, that freshly distilled benzaldehyde required about 2 cc. of $N/8$ sodium hydroxide solution for neutralization, but if it was allowed to stand over night in a stoppered bottle it frequently required 8 to 10 cc. of $N/8$ sodium hydroxide solution. We finally prepared a large amount of pure benzaldehyde by treating it with aqueous bicarbonate of soda as already described and then distilling it under reduced pressure directly into a buret. When we were finished with the distillation we flooded the apparatus with nitrogen, exhausted it, and then filled a second time. The buret was disconnected from the distillation apparatus and connected to a large

[1] *Arch. Pharm.*, 249, 382–400 (1911).

reservoir of nitrogen. The tip of the buret was continually kept immersed in water. When benzaldehyde was required the tip was washed and dried and, after running out 5 or 6 drops, the amount required for the experiment was withdrawn. In this way we had benzaldehyde for our experiments which did not vary more in acidity than 0.1 cc. of $N/8$ sodium hydroxide for 1 cc. of benzaldehyde.

The hydrocyanic acid was prepared by treating potassium ferrocyanide with sulfuric acid in a distilling flask and then boiling off the hydrocyanic acid and collecting it in distilled water. The aqueous acid was again placed in a distilling flask and the acid boiled off into distilled water. This operation of boiling off the hydrocyanic acid into distilled water was frequently repeated to keep it as pure as possible from decomposition products. The aqueous solution was always clear and colorless.

We spent much time in trying to prepare pure mandelonitrile but we frankly admit that we did not succeed, nor were we able to buy a pure specimen. We hope in a separate communication at a later date to give our results on the preparation of the nitrile. The nitrile which we used was made in the following way: 28 g. benzaldehyde, 87 cc. of 9.2% HCN and 120 cc. water were put into a glass stoppered bottle. Before closing, the bottle was flooded with nitrogen. It was shaken in a shaking machine for nearly 6 days when the theoretical amount of hydrocyanic acid for pure nitrile had disappeared. The oily layer was separated in a separating funnel. It was clear and colorless, but it was not pure nitrile, because when it was hydrolyzed it yielded only about 82% of the theoretical amount of ammonium chloride. When 1 cc. was added to 168 cc. of water it yielded only 13.7% of free HCN while if it had been pure it should have yielded 19% of free HCN on reaching the equilibrium point.

In our experiments we studied the rate of reaction and the equilibrium point between hydrocyanic acid and benzaldehyde in the presence of oxynitrilase to determine whether there was any difference in the enzyme obtained from various sources and also in the enzyme obtained from the same source, but by various methods and subjected to certain treatments. It is obvious that if there is a synthetic and a hydrolytic enzyme for a certain reaction there must be a difference in the equilibrium point. This point has been particularly emphasized by Bayliss.

Experimental.

The first point we investigated was to examine oxynitrilase from various sources. We extracted wild-cherry leaves (*Prunus Serotina*), and peach leaves collected in 1914. We also tried emulsins, one sample we extracted from the kernels in peach stones, another from sweet almonds, and the third was a preparation bought from Kahlbaum.

The experiments were carried out in a 225 cc. wide-mouthed bottle. It was closed with a cork which had three holes in it. Through one the

stirring rod passed, in the second we inserted a glass tube to pass nitrogen gas, and the third was used to pipet out definite amounts at various intervals of time. It was closed when not in use with another cork. The experiments were conducted in the following manner: 124 cc. of water was put into the bottle with 50 cc. of the enzyme solution and 1 cc. (1.0285 g.) of benzaldehyde. The bottle was put into a bath kept at 25° and the contents of the bottle stirred. Before the stirring was started, however, nitrogen was passed through the flask for several minutes to insure that all the air was expelled. After a half hour, 25 cc. of HCN solution was added (containing 0.262 g. HCN) and the rate followed by pipeting out 20 or 25 cc. into a 100 cc. flask containing 5 cc. of $N/5$ $AgNO_3$ and 5 cc. $4N$ nitric acid. The flask was filled to the mark, the solution filtered, throwing away the first portion of the filtrate, and then titrating 50 cc. of the filtrate with a $N/30$ ammonium thiocyanate solution. Nitrogen was circulated through the bottle for thirty minutes after the hydrocyanic was added when the equilibrium was very nearly reached. Stirring was discontinued at this point, too, as the solution had become perfectly homogeneous.

The method is not altogether satisfactory. In the first place, the amount of benzaldehyde used does not completely dissolve in the amount of water used, so that the solution is not homogeneous at the beginning of the experiment. Consequently there is always the possibility of not withdrawing equimolecular quantities of aldehyde and acid with the pipet. There is also the possibility that a certain amount of the aldehyde becomes oxidized during the first half hour before the hydrocyanic acid is added.

TABLE I.

	Millimoles of free HCN per liter.	
Prunus Serotina leaves extract from 2 g..	9.1	
Prunus Serotina leaves acetone ppt. from extract of 2.5 g.....	9.7	
Peach leaves acetone ppt. from 2 g.....	9.2	Total millimoles of HCN added per liter 48.5. Temperature 25° C.
1 g. emulsin (Kahlbaum).....	10.0	
1 g. emulsin from sweet almonds.....	9.0	
1 g. emulsin from peach kernels.....	10.0	

The emulsin experiments were not so satisfactory, as 25 cc. of the solution gave such a bulky precipitate in the 100 cc. measuring flask that one could hardly tell when the liquid was exactly at the mark.

From these results one sees that the equilibrium point does not vary more than the experimental error and certainly shows no evidence of the presence of a synthetic or hydrolytic oxynitrilase. It is conceivable, however, that if there are two enzymes they always occur in the same ratio to each other. We, therefore, tried various lengths of time of ex-

traction and also various amounts of water in the extraction to see if a solution could be obtained which gave a different equilibrium point. All the following experiments were carried out in the wide-mouthed bottle already described, at 25°.

Experiment 1.—2.5 g. wild-cherry leaves were extracted for 3 hours with 60 cc. of distilled water. The mixture was filtered and 50 cc. of the extract added to 124 cc. of distilled water and 25 cc. of hydrocyanic acid (0.262 g. HCN). When the temperature inside the bottle had reached 25°, 1 cc. (1.0285 g.) benzaldehyde was added. At the time indicated, 10 or 20 cc. were withdrawn and analyzed. The second line represents millimols of free hydrocyanic acid per L.

Minutes.....	13	52	134	313	1140	1800
	41.2	23.9	14.4	10.7	9.4	9.3

Experiment 2.—In this experiment the same amount of a similarly prepared enzyme solution was used but the method of the experiment was slightly changed. The enzyme solution and distilled water were put into the bottle, then it was flooded with nitrogen and 1 cc. benzaldehyde added. After $^1/_2$ hour of stirring the 25 cc. hydrocyanic acid were added.

Minutes.....	11	30	102	279	1020
	35.4	26.1	15.9	9.9	9.1

Experiment 3.—In this case, the 2.5 g. of leaves were extracted for 14 hours. Method same as in Experiment 1.

Minutes.....	11	29	85	234	464	1260	3300
	42.5	33.5	20.8	13.0	9.3	9.3	9.1

Experiment 4.—2.5 g. were extracted for 24 hrs. Method same as Experiment 1.

Minutes.....	9	36	145	320	1080
	40.6	33.7	16.8	11.8	9.7

Experiment 5.—In this experiment, 2.5 g. of wild-cherry leaves were extracted with 120 cc. of water for 3 hours and 100 cc. of the extract used instead of 50. Only 74 cc. of distilled water were used instead of 124. The method was same as Experiment 2.

Minutes.....	12	30	90	300	1080
	34.7	26.8	14.8	9.9	9.1

Experiment 6.—In this case, 5 g. of leaves were extracted with 60 cc. and 50 cc. used. Method as in Experiment 2.

Minutes.....	12	30	90	300	1080
	35.9	23.5	13.2	11.1	9.1

We carried out about 20 experiments with the extract of the same stock of ground leaves in which the nitrile was extracted and hydrolyzed and in every case the mandelic acid was found active, the percentage of active acid varying from 40 to 50% of the total mandelic acid produced.

Where the extract was boiled, the mandelic acid was inactive. This proves that the leaf actually contained active oxynitrilase.

In the third series of experiments we repeated some of the methods which Rosenthaler gave for separating the hydrolytic enzyme from the synthetic enzyme. We did not use emulsin as a source of our oxynitrilase because it does not produce a very active nitrile[1] and contains only a small amount of oxynitrilase. The rate for 1 g. of emulsin is approximately the same as for 75 mg. precipitated from the extract of 2 g. of peach leaves with acetone. We, therefore, used the extract from peach leaves. The benzaldehyde contained from 0.15% to 0.2% benzoic acid when titrated in 10 cc. alcohol with $N/8$ alkali, using phenolphthalein as an indicator. The following table gives the results tabulated in millimoles per liter:

TABLE II.

	5 min.	10 min.	15 min.	30 min.	24 hrs.	48 hrs.
1. Blank	45.7	44.9	43.8	40.5	..	9.6
2. Precipitated enzyme	26.1	17.6	16.4	14.3	9.2	No change
3. Precipitated enzyme	25.5	19.4	16.7	14.3	11.6	9.0
4. Precipitated enzyme boiled	39.7	36.5	32.2	27.6	14.6	...
5. Enzyme treated with HCN	25.5	18.6	16.1	13.9	9.8	9.1
6. Enzyme treated with benzaldehyde	20.4	16.5	14.2	12.9	9.8	9.3

The method was the same as described in Expt. 2 of the last series, *i. e.*, the enzyme solution and water were put into the bottle, the bottle placed in the water bath and flooded with nitrogen. 1 cc. of aldehyde was then added and the contents stirred for $^1/_2$ hour before the 25 cc. of hydrocyanic acid were added.

Experiment 1 gives the rate at which benzaldehyde and hydrocyanic acid combine by themselves at this concentration.

The enzyme in Experiment 2 was prepared by adding 5 g. of ground peach leaves in a bottle and 120 cc. of water. The bottle was closed and placed in a shaking machine for 3 hours. The leaves were filtered through cotton wool placed on a Buchner funnel. To 100 cc. of the filtrate 250 cc. of acetone were added, shaking the beaker containing the extract during the addition. After a few minutes a light precipitate formed. This was filtered on a quantitative filter paper placed in a 4-inch Buchner funnel. Only slight suction was used in order not to draw the precipitate through the filter. The enzyme on the paper was washed first with about 20 cc. of 95% alcohol and then with an equal amount of ether. When we were ready to use it, we placed the filter paper in the bottom of a large beaker and added 75 cc. of water. The beaker was swirled occasionally for several hours. The solution was rinsed out with enough water to make the volume up to 100 cc. 50 cc. was used for each experiment and contained about 80 mg. of enzyme. The second 50 cc. was boiled for five

[1] Krieble, THIS JOURNAL, **35**, 1643 (1913).

minutes and then made up to 50 cc. again and used in Expt. 4. This experiment shows that the boiled enzyme solution contains certain substances which act as catalysts. They catalyze the synthesis of the dextro nitrile as well as the laevo, as the resulting nitrile is always inactive.

The second 50 cc. of the enzyme solution prepared in Experiment 3 was used for a duplicate experiment which was carried out under exactly the same conditions, but was stopped at the end of an hour and a half and the nitrile extracted with ether. The ether was boiled off and the nitrile hydrolyzed with strong hydrochloric acid. It had a volume of 25 cc. after hydrolysis and was laevo active 9.05° in a 2 dm. tube at room temperature.

Rosenthaler had found that, if 1 g. of emulsin in 100 cc. of water was treated with 5 g. of benzaldehyde for 2 hours, the resulting emulsin retained its synthetic activity but the hydrolytic property had dropped to half value under the conditions of his experiment. The activity was measured by the optical activity of the nitrile after a definite interval of time. No attention was paid to the rate of reaction or the equilibrium point. We prepared our extract as in the previous experiment, then to 100 cc. we added 5 g. of our pure benzaldehyde in a glass-stoppered bottle just large enough for the experiment. It was put into a shaking machine and rotated for 2 hours. The enzyme was precipitated with 250 cc. of acetone, then filtered out and washed with alcohol and ether. It was dissolved and made up to 100 cc. in the usual way, half of it being used to study the rate of reaction and get the equilibrium point and the other half to get the optical activity of the nitrile. The second experiment was stopped at the end of one and one-half hours. The nitrile was extracted and hydrolyzed with hydrochloric acid. It had a volume of 25 cc. and was laevo active 9.30° in a 2 dm. tube at the room temperature. Benzaldehyde has, therefore, no deteriorating effect on oxynitrilase so far as its synthetic action goes.

Rosenthaler also found that hydrocyanic acid partly destroyed the hydrolytic power of emulsin on mandelonitrile but had no effect on its synthesis. He treated 1 g. of emulsin with 1.35 g. of hydrocyanic acid in 100 cc. of water for 2 hours. We used 120 cc. of water containing the same concentration of hydrocyanic acid (1.35%) for the extraction of 5 g. of peach leaves for 3 hours. The enzyme was precipitated and redissolved in the usual way. Half of it was used to study the rate of reaction and the equilibrium point. The results are given in Expt. 6 in Table I. The second half was used to determine the activity of the nitrile produced. After hydrolysis the volume of the solution was 25 cc. and it read 8.85° laevo in a 2 dm. tube at the room temperature.

The above series of experiments were repeated on the hydrolysis of the nitrile. Unfortunately, we did not succeed in preparing this substance

in the pure state, as we have already said. The samples we bought were even worse than our own, so we used the best we had. This nitrile dissociated so slowly in water that no perceptible change was noticeable after several hours. The blank experiment was carried out as follows: 196 cc. of water and 2 cc. (2.121 g.) of nitrile were put into the wide-mouthed bottle, placed in the water bath at 25° and stirred for 30 minutes in order to get the solution saturated with nitrile. 3 cc. of 0.01 *N* alkali were then added and rate of dissociating followed by pipeting out definite amounts at various intervals of time.

TABLE III.

	5 min.	15 min.	30 min.	45 min.	1½ hrs.	1¾ hrs.	4 hrs.	20 hrs	44 hrs.
1. Blank	1.56	2.25	..	3.15	3.66	..	5.30	6.42	6.1
2. Precipitated enzyme	3.66	4.42	..	6.14	..	6.22	..	6.22	6.22
3. Enzyme treated with HCN	3.91	4.94	..	6.09	..	6.29	..	6.42	6.42
4. Enzyme treated with benzaldehyde	3.77	4.68	5.96	..	..	6.09	..	6.32	6.32

The enzyme preparations for Experiments 2, 3, and 4 in Table III were prepared in the same way as for the Experiments 2, 5, and 6 in Table II. In these experiments, the 50 cc. enzyme solution was included in the 195 cc. of water which was put into the bottle at the beginning of the experiment. 2 cc. of nitrile was then added and the contents stirred for 30 minutes. Then 3 cc. of 0.01 *N* sodium hydroxide was added and the rate followed as usual. The figures in the table represent millimoles per liter. A duplicate experiment was carried out to Nos. 2, 3, and 4, which was extracted with ether at the end of 2 hours, after the addition of alkali. The ether was boiled off and the nitrile hydrolyzed. The volume of the hydrolyzed solution was 25 cc. in every case and the activity for the three experiments was +1.12°, +1.55°, +1.6°, respectively, in a 2 dm. tube at the room temperature.

It is apparent from the above results that benzaldehyde and hydrocyanic acid have absolutely no injurious effect on the hydrolytic power of oxynitrilase as the rate of reaction and equilibrium point are exactly the same in every case. The amount of active nitrile is even slightly higher where the enzyme was treated with aldehyde and hydrocyanic acid than where it was not.

We do not consider that Rosenthaler's method is very well adapted for the study of hydrolysis. He suspends the nitrile in water and then passes a current of air through the suspension, extracting whatever nitrile is left at the end of 24 hours. It is very likely that he does not always arrest the reaction at the same point as the amount of nitrile may easily be a variable quantity when the current of air is not actually measured in each experiment. It is also evident that more or less of the benzaldehyde formed is oxidized to benzoic acid which retards the reaction. Conse-

quently, one cannot draw any very valid conclusions from his results as he only has one point on the curve.

Rosenthaler has suggested several other methods for separating the synthetic from the hydrolytic enzyme; for example, by treating emulsin with acid and then neutralizing with alkali, by partial precipitation with ammonium sulfate and copper sulfate. We have repeated some of these experiments but we cannot interpret the results correctly until we have made a further study of the effect of inorganic salts on the rate of reaction. There is, however, no evidence of a change in the equilibrium point though there is a marked change in the rate of the reaction.

Conclusions.

1. It is necessary to prepare a stock of pure benzaldehyde and to keep it over nitrogen in order to obtain comparable results in the synthesis of mandelonitrile.

2. There is no evidence of a variation in the equilibrium point in the synthesis of mandelonitrile, even though the oxynitrilase is obtained from widely diversified sources.

3. As far as investigated, there is no evidence of the possible separation of a hydrolytic from a synthetic enzyme in a preparation of oxynitrilase when treated according to methods suggested by Rosenthaler.

We are continuing the study of the general properties of oxynitrilase and expect to repeat all the other methods suggested by Rosenthaler for the separation of the hydrolytic from the synthetic enzyme.

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ON THE REACTION OF THE PANCREAS.

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It is well recognized that the so-called pancreatic juice has a distinct alkaline reaction which has been expressed in different terms. The degree of alkalinity varies with the condition of the alimentary tract with reference to presence of food and with other factors as well. With lowered food ingestion the alkalinity decreases, as has been shown by the investigations of Glaessner,[1] Schumm[2] and others. The latter found the reaction of the human pancreatic juice from a temporary fistula to be equivalent, in the mean, to about 0.6% Na_2CO_3. The alkalinity found by Glaessner was, apparently, much less, and similar values have been reported by Ellinger and Cohn.[3] Observations made by Wohlgemuth[4]

[1] *Z. physiol. Chem.*, 40, 465.

[2] *Ibid.*, 36, 292.

[3] Ellinger and Cohn, *Z. physiol. Chem.*, 45, 28.

[4] Wohlgemuth, *Biochem. Ztschr.*, 39, 302.