THE PROPERTIES OF OXYNITRILASE

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INTRODUCTORY.

Oxynitrilase is the enzyme which catalyses the reaction between hydrocyanic acid and benzaldehyde.

$$C_6H_5CHO + HCN \rightleftharpoons C_6H_5CHOHCN.$$

That the mandelo-nitrile produced in this manner is optically active (dextro-rotatory), when emulsin influences the reaction, was discovered in 1908 by L. Rosenthaler (1). This was the first asymmetric synthesis accomplished through the agency of an enzyme. In the same year Feist (2) noted that when emulsin acted on amygdalin in the cold, dextro mandelo-nitrile resulted. This experiment was the first in which an enzyme produced an optically active nitrile by hydrolysis.

In 1909 Rosenthaler (3) came to the conclusion that the enzyme which catalyses the synthesis of mandelo-nitrile was not the same as the one which decomposes amygdalin. He called the synthetic enzyme σ-emulsin and the hydrolytic δ-emulsin, and claimed that both occur in ordinary emulsin along with amygdalase and glucosidase.

His theory of the hydrolysis of amygdalin by emulsin was that it took place in three steps, each of which was catalysed by a different enzyme in the emulsin (4).

I. Amygdalase hydrolyses amygdalin to α-glucose and mandelo-nitrile-glucoside.

II. Mandelo-nitrile-glucoside is hydrolysed by glucosidase to β-glucose and mandelo-nitrile.

III. Mandelo-nitrile is split up into benzaldehyde and hydrocyanic acid by δ-emulsin, or, as he now called it, δ-oxynitrilase.

He next turned his attention to the separation of δ- and σ- oxynitrilase. After trying the effects of a great many reagents on emulsin, he found that an emulsin solution, one-half saturated with ammonium sulphate and filtered, had lost its synthesising power, but still decomposed amygdalin. On dissolving in water the precipitate which the addition of ammonium sulphate had caused, the solution yielded optically active mandelo nitrile with hydrocyanic acid and benzaldehyde, but still showed hydrolytic properties. Similar results were obtained by the use of magnesium sulphate.

By these means Rosenthaler claimed to have isolated the so-called δ-oxynitrilase from the mixture of enzymes in ordinary emulsin, though he could never get his σ- oxynitrilase entirely free from δ-oxynitrilase. This last fact should have given him a clue to the real significance of his results. V.K. Krieble (5) by careful repetitions of Rosenthaler's work showed that in order to draw any conclusions as to the destruction of either of the properties (synthetic and hydrolytic) of oxynitrilase, it is necessary to follow the rates of reactions, and that no conclusion can be drawn by making only one determination, i.e., one point on the curve for a reaction. From

Krieble's results there is no evidence of the possible separation of a hydrolytic from a synthetic enzyme in a preparation of oxynitrilase, when treated according to the method employed by Rosenthaler.

Rosenthaler performed many experiments which proved that emulsin always hastens the rate of combination of hydrocyanic acid with aldehydes and ketones, and found that the enzyme changed the equilibrium points in these reversible reactions.

	Anisaldehyde HCN		Cinnamic aldehyde HCN	
Equilibrium without enzyme,	33.5%	Combination	39.85%	Combination
Equilibrium with emulsin,	73.5%	"	71.88%	"

If enzymes are to be regarded as catalysts, the above results must be incorrect, as it is manifestly impossible for a catalyst to change the equilibrium point of a reversible reaction. In fact, Krieble (loc.cit.) found that there is no variation in the equilibrium point of the reaction with and without enzyme even when samples of enzyme from widely diversified sources were used. Rosenthaler in all likelihood had not given the reactions without emulsin time to reach equilibrium. This again emphasizes the necessity of following the rate of reaction by taking points as the reaction proceeds, so that one may plot the curve of the rate and thus obtain a clear idea of the location of the equilibrium point, even though that point be not reached in the time of the experiment.

Rosenthaler (6) also showed that during the synthesis of

mandelo-nitrile the optical activity increases to a maximum and then decreases, and attributed the cause to the effect of the reverse reaction, the decomposition of mandelo-nitrile to hydrocyanic acid and benzaldehyde. Up to the present time no satisfactory explanation has been advanced for the decrease of optical activity. However, by following both the rate of chemical combination and formation of optically active nitrile simultaneously, that is, determining what per cent of the total nitrile formed is optically active as the reaction progresses, more interpretive results are obtained.

Rosenthaler (7) also investigated the effect of acid on the properties of the mixture of σ- and δ- oxynitrilase and states that the speed with which acid destroys δ-oxynitrilase is greater than the speed with which it destroys the σ- variety. By means of treating enzyme solutions with acid and neutralizing after a period of time, he claimed to have isolated σ- oxynitrilase free from δ-oxynitrilase.

His conclusions again cannot be accepted, since he bases them on one observation of the optical activity for an experiment. This cannot cover the possibility that his treatment of the enzyme has merely slowed up either the rate of combination (which he does not take into account at all) or that it has hastened it, and that the optical activity has already passed the maximum value and is on the down-grade of the curve. His opinion was that if in a given time the optical activity in an experiment with treated enzyme were not as high as that in a

standard experiment, then an actual amount of σ-oxynitrilase had been destroyed.

The necessity, therefore, if conclusive results are to be obtained, of following the rate of formation of optically active nitrile, the per cent combination, and their relative values, is apparent.

The following work has been performed on oxynitrilase, paying particular attention to the relation between the rate of formation of optically active nitrile (or enzyme action) and the rate of combination of benzaldehyde and hydrocyanic acid (or chemical action), when the enzyme catalyses the reaction.

EXPERIMENTAL.

I. Preparation of Reagents.

The properties of the enzyme, oxynitrilase, were studied with regard to its influence on the reaction between benzaldehyde and an exactly equivalent amount of hydrocyanic acid, under a wide range of conditions. It was of the utmost importance, therefore, that the reagents used should be pure.

For the preparation of benzaldehyde about 120 c.c. of commercial benzaldehyde, after being neutralized with a solution of sodium carbonate, were dried with sodium sulphate. The benzaldehyde was then separated in a separating funnel and distilled under a pressure of 20 millimetres, several capillary tubes being put into the distilling flask to prevent bumping.

The first fraction of the distillate was rejected since it contained a high percentage of water, in spite of the drying with sodium sulphate. The remainder was collected in an evacuated burette which, after the distillation was completed, was flooded with nitrogen gas. The benzaldehyde was kept in this burette in an atmosphere of nitrogen, to prevent oxidation, and portions drawn off as required for experimental purposes.

The hydrocyanic acid was prepared as follows:- 100 grams of potassium ferrocyanide were put into a double-necked distilling flask, together with 140 c.c. distilled water, and 70 grams concentrated sulphuric acid were slowly added from a

dropping funnel. The hydrocyanic acid formed was distilled, using a water condenser, into distilled water in a suction flask whose side-opening was connected by means of rubber tubing to a μ-tube containing a concentrated solution of potassium hydroxide in order to prevent any hydrocyanic acid from escaping. The end of the condenser was connected to a long adapter, the end of which dipped into the water in the receiver.

The distillation was carried on until the temperature in the distilling flask reached 100 degrees Centigrade. The solution of hydrocyanic acid thus obtained was redistilled as above. The solution finally obtained was about 10 per cent in strength and this was diluted to the strength required for the experiments.

As hydrocyanic acid polymerises on standing, it was necessary to redistill and standardize the solutions at intervals of about two weeks.

The enzyme oxynitrilase was extracted from peach leaves collected in June 1915 in Pennsylvania. They had been carefully sun-dried and preserved. The leaves were first ground in a dry coffee grinder and then pulverised in an agate mortar. They were kept in a stoppered bottle in the dark.

Ten-gram samples of ground leaves were weighed out and put into shaker bottles each containing 240 c.c. distilled water. Then the bottles and their contents were put on a shaking machine for three hours, during which time practically

all the enzyme was dissolved by the water. The contents of the bottles were filtered through absorbent cotton, using suction.

To each 100 c.c. of the filtrate 250 c.c. acetone were added. This precipitated the enzyme. Before filtering, the mixture was put into a centrifuge which was run at 1500 revolutions per minute for three minutes. By this means the precipitate was collected at the bottom of the tubes, and as most of the supernatent liquid could be poured off, the speed in filtering was greatly increased. The enzyme can hardly be filtered successfully without this process as it clogs the pores of the filter papers. The filtering was carried out as fast as possible as acetone, if left in contact with the enzyme for any length of time, greatly reduces its powers.

After pouring off most of the supernatent liquid, the residue was filtered through parchment filter papers, slight suction being employed. The precipitate on the filter papers was washed with a little alcohol and then a small amount of ether. The enzyme was scraped off the filter papers, ground in a mortar, dried over sulphuric acid and preserved in a weighing bottle fitted with a ground-glass stopper.

About 5 grams of finely powdered, greyish-white enzyme were prepared in this way from 240 grams of leaves.

II. Procedure.

Figure I. represents the reaction cell, which was a glass jar of 750 c.c. capacity, in the thermostat. As it was necessary to maintain an atmosphere of nitrogen in the cell during the reactions, owing to the fact that benzaldehyde oxidizes spontaneously in air, a mercury seal was devised for the stirring mechanism.

The stirring-rod F was rotated by a small electric motor connected to the pulley E. B is a glass tube firmly corked to the stirring-rod and which turns with it. As B dips into mercury, A, no gas can enter or leave the cell. G is a glass tube fitted with cork bearings at each end to ensure even rotation of the stirring-rod. The seal, stirring-rod and tubes were all permanently fixed in the rubber cork H which fitted tightly into the neck of the cell. This made the opening and closing of the cell very simple operations.

When starting an experiment, the required volume of distilled water was first run into the cell and then a definite amount of enzyme was added. The cell was then closed, by inserting the cork together with the stirring arrangements, and set up in the thermostat. The cell contents were stirred for 30 minutes to ensure a thorough solution of the enzyme and also to give the solution time to reach the temperature of the thermostat.

During this process, the tube C was connected to a nitrogen reservoir and the cell was flooded with nitrogen

which expelled the air through an opening in H not on the figure. When the cell had been thoroughly flooded, the nitrogen was turned off and the hole stoppered with a sealed glass tube.

The benzaldehyde was added through a small funnel inserted in a hole in H, and after another period of stirring, the equivalent hydrocyanic acid was added in the same manner. The experiment was considered as starting from the time at which the first drop of hydrocyanic acid entered the cell. It usually took 20 seconds to add all the acid.

Samples of the reacting solution were taken at any desired time by running nitrogen from the reservoir through the tube C into the cell. This forced some of the solution out through the tube D where 40 c.c. were collected in a small graduated beaker. When the cell solution had filled the beaker to the 40 c.c. mark, the nitrogen was shut off and the flow of solution stopped. Then 20 c.c. were pipetted from the beaker and used for determining the rate of combination while at the same time a 15 c.c. sample was taken for the optical activity.

The total time required for taking samples in the above manner was 30 seconds. The residual solution in the beaker was usually tested for acidity.

III. Method of Following the Rate of Combination.

As mentioned above, exactly equivalent amounts of benzaldehyde and hydrocyanic acid were used in each experiment. Since the benzaldehyde was of constant composition, being drawn from the stock preparation, and since 3 c.c. of benzaldehyde were used, this was made the basis of calculation.

The reaction between benzaldehyde and hydrocyanic acid takes place according to the following equation:-

$$C_6H_5CHO + HCN \rightleftharpoons C_6H_5CHOHCN.$$

That is to say, 106 grams benzaldehyde are equivalent to 27 grams hydrocyanic acid. Therefore the weight of benzaldehyde delivered by a 3 c.c. pipette was accurately determined, and the same pipette was always used to deliver the benzaldehyde required in the experiments. This weight was found to be 3.0879 grams benzaldehyde and this is equivalent to .7867 grams hydrocyanic acid. It was decided then to use 75 c.c. of a 1.049 percent solution of hydrocyanic acid in each experiment.

The determination of the percent combination at a given time was made by ascertaining the weight of hydrocyanic acid uncombined at that time. Then, knowing the total weight of acid in the cell, the percent combination was easily calculated. The sample taken for following the rate of combination was added to a 100 c.c. graduated flask containing 5 c.c. .2 Normal silver nitrate solution and 5 c.c. 4 Normal nitric acid.

The flask was then filled up to the 100 c.c. mark with distilled water, the contents filtered, and the amount of silver nitrate used up by the uncombined hydrocyanic acid was determined in 50 c.c. of the filtrate. This was done by titrating the excess silver nitrate with $\frac{N}{30}$ ammonium sulphocyanate, using a saturated solution of ferric alum (acidified with nitric acid) as an indicator. This method was suggested by Wirth (8).

Then, the value of .2N silver nitrate in terms of the ammonium sulphocyanate being known, the amount of silver nitrate combined with hydrocyanic acid was known, and this gave the weight of uncombined hydrocyanic acid present in the sample. From this the percent combination was calculated. The calculation was simple, as a factor which covered all experiments was worked out as follows:-

Total Volume of cell solution	=	600 c.c.
Sample taken	=	20 c.c.
Weight of hydrocyanic acid originally in cell	=	.7867 grams
c.c. ammonium sulphocyanate used in titration	=	8.56 c.c.

14.56 c.c. ammonium sulphocyanate are equivalent to 2.5 c.c. .2 N silver nitrate.

14.56 - 8.56 = 600 c.c. ammonium sulphocyanate which are equivalent to the number of c.c. silver nitrate used up by uncombined hydrocyanic acid in the sample.

29.12 c.c. NH_4CNS = 5 c.c. .2 N $AgNO_3$ = 1 c.c. N $AgNO_3$ = .027 grams HCN.

Therefore, 29.12 c.c. ammonium sulphocyanate are equivalent to .027 grams hydrocyanic acid.

Weight of uncombined hydrocyanic acid in sample

$$= 6 \times \frac{2 \times .027}{29.12} \text{ grams}$$

$$\text{Percent uncombined hydrocyanic acid} = 6.00 \times \frac{2 \times .027 \times 30 \times 100}{29.12 \times .7867}$$

$$= 6.00 \times 7.072$$

Therefore, after titrating samples, the number of c.c. ammonium sulphocyanate used was subtracted from 14.56 and the result multiplied by 7.072. This gives percent uncombined hydrocyanic acid, and by subtracting this from 100, the percent combined hydrocyanic acid, that is, the percent combination, was determined. This method was not only simple but possessed also the added merits of being very accurate and fast.

IV. Method for Following the Rate of Production of Optically Active Nitrile.

The devising of a method for following the rate of the production of optically active nitrile presented many difficulties and involved considerable experimentation before the process was perfected. Benzaldehyde and hydrocyanic acid combine to form mandelo-nitrile, a certain percentage of which, if an enzyme catalyses the reaction, is optically active. The rate of production of the optically active nitrile must, therefore, be followed by optical activity

measurements.

It is not practicable to read the rotation of the nitrile directly, owing to the fact that its specific rotation has never been accurately determined, and also because its optical activity is so low that the chance of error would be greatly increased.

The nitrile was, therefore, hydrolysed to mandelic acid and the rotation of the acid determined on the polaroscope. The hydrolysing agent employed was concentrated hydrochloric acid and the reaction takes place according to the following equation:-

$$C_6H_5CHOHCN + 2H_2O \longrightarrow NH_3 + C_6H_5CHOHCOOH$$

Mandelic acid is levo-rotatory and has a comparatively high specific rotation.

The actual hydrolysis may be carried out in several ways. The nitrile may be extracted with ether, the ether distilled off, the dry nitrile taken up in concentrated hydrochloric acid, hydrolysed to mandelic acid, and then the solution made up to a definite volume and read on the polaroscope. Or the sample may be treated with an equal volume of concentrated hydrochloric acid and the nitrile hydrolysed to mandelic acid. Then the solution may be evaporated to dryness, the mandelic acid dissolved in water, the solution made up to a definite volume and then the rotation of the mandelic acid determined. The objection to the latter is that on being evaporated to dryness, mandelic acid yields a thick

oily anhydride which does not give a good solution for reading on the polaroscope. Furthermore, neither of these processes gives constant results.

Finally the following method was adopted. The 15 c.c. samples were immediately added to 15 c.c. concentrated hydrochloric acid in glass-stoppered bottles. The concentrated acid instantly stops all reaction between hydrocyanic acid and benzaldehyde. The tightened stoppers were firmly tied with cord and then the bottles were suspended in a thermostat at 65 degrees Centigrade in order to hydrolyse the nitrile to mandelic acid. By experiment it was proved that with the above concentration of hydrochloric acid, complete hydrolysis was accomplished at 65 degrees Centigrade in 4 hours. In practice the samples were hydrolysed for about 15 hours (overnight) in order to eliminate any chance of incomplete hydrolysis. Owing to the tight fit of the stoppers, no concentration of the solutions took place. After completion of the hydrolysis, the solutions were filtered and read in a 2-decimetre tube on the polaroscope at room temperature.

From the rotation observed, the actual weight of mandelic acid present in 100 c.c. of the solution in the tube was calculated from the following formula:-

where c = number of grams mandelic acid in 100 c.c. of solution.

α = rotation of solution read in degrees.

l = length of the tube in decimetres.

S.R. = specific rotation of mandelic acid in the solvent present.

$$c = \frac{100 \times \alpha}{S.R. \times l}$$

Here c refers to the solution actually read, which was composed of 1 part cell solution to 1 part hydrochloric acid by volume. Therefore c is equal to the number of grams mandelic acid in 50 c.c. cell solution. Since the total volume of the cell solution was 600 c.c., the number of grams mandelic acid produced by hydrolysing the nitrile present is equal to 12c.

In an experiment in which 3 c.c. benzaldehyde and the equivalent weight of hydrocyanic acid are present, the weight of mandelic acid produced on hydrolysing the nitrile formed when 100% combination has taken place is 4.428 grs. By multiplying this by the percent combination found at this time by the method described above, the total weight of mandelic acid which would have been formed by hydrolysing the nitrile produced at this point is determined. Then by dividing the weight of optically active mandelic acid by the total amount of mandelic acid, the percent optically active mandelic acid, which is equal to the percent optically active nitrile produced at this point, is calculated.

It will be noted that the formula for determining the weight of optically active mandelic acid included as a factor the specific rotation of mandelic acid in the solvent present. It was, therefore, necessary to determine this by experiment.

Mandelic acid for this purpose was prepared by making 50 grams amygdalin up to 250 c.c. with 23.4 percent hydrochloric acid and heating at 65 degrees Centigrade for 3 hours. The mandelic acid produced was extracted three times with an equal volume of ether which was then distilled off. The mandelic acid was recrystallized twice from benzene and then filtered and washed with benzene.

The rotation of this mandelic acid at various concentrations in various solvents at different temperatures was determined.

I. .1000 grams mandelic acid dissolved in 25 c.c. of a mixture consisting of 15 c.c. water and 10 c.c. hydrochloric acid at 20 degrees Centigrade. Rotation = -1.30°.

II. .1000 grams mandelic acid dissolved in 25 c.c. solution of alcohol, water and hydrochloric acid (every 250 c.c. of which contained 100 c.c. hydrochloric acid, 125 c.c. water and 25 c.c. alcohol). At 20 degrees Centigrade the rotation = -1.30°.

III. .1000 grams mandelic acid in 25 c.c. hydrochloric acid solution (1 c.c. concentrated hydrochloric acid to 1 c.c. water). At 20 degrees Centigrade the rotation = -1.31°.

There was practically no change in rotation when the above solutions were read at 15 degrees and 20 degrees Centigrade. The alcohol solution (II.) was heated for 3 hours at 65 degrees Centigrade and then read again on the polaroscope. There was no change in rotation.

The above determinations were made in order to ascertain the effect of experimental conditions on the rotation of mandelic acid. It is evident that the presence of alcohol (10 percent by volume) and heating at 65 degrees Centigrade have no effect on the rotation of mandelic acid in hydrochloric acid solutions. The temperature (within reasonable limits) makes no appreciable difference so that solutions may be read at room temperature.

The specific rotation of the mandelic acid in hydrochloric acid solution (1 volume of water to 1 volume of hydrochloric acid) at low concentrations was determined.

4 percent solution, $\alpha = -1.31^{\circ}$ S.R. $= \frac{100 \times \alpha}{c \times l} = 163.75^{\circ}$

2 percent solution, $\alpha = -.66^{\circ}$ S.R. $= 165.00^{\circ}$

1 percent solution, $\alpha = -.33^{\circ}$ S.R. $= 165.00^{\circ}$

The mean specific rotation of our mandelic acid was therefore 164.58°. In order to provide for impurities in the mandelic acid employed in the above determinations, the specific rotation of the mandelic acid in aqueous solution was ascertained, and compared with that of pure mandelic acid as given by Landolt.

According to Landolt the specific rotation of pure mandelic acid in 2.45 percent aqueous solution at 20.6 degrees Centigrade is 153.76°.

Specific rotation of mandelic acid used in 2.45 percent aqueous solution at 20.6 degrees Centigrade is 150.6°.

The specific rotation of pure mandelic acid in hydrochloric acid solution was then calculated from the following proportion:- 150.61 : 153.76 :: 164.58 : specific rotation of pure mandelic acid under experimental conditions.

$$\text{S.R. to be used} = \frac{153.76 \times 164.58}{150.61} = 168.00$$

Our equation for determining the percent optically active nitrile produced at any time now becomes:-

$$c = \frac{1200 \times \alpha}{168 \times 2}$$

Where c is equal to the number of grams mandelic acid which would be produced by hydrolysing the total optically active nitrile in the cell solution, and α is the reading of the hydrolysed solution in degrees in a 2-decimetre tube.

$$c = \alpha \times 3.571.$$

Hence, in calculating the percent optically active nitrile produced at a certain time, the optical activity as read is multiplied by 3.571. The result is divided by the product of the percent combination at that point and 4.428.

For example.

At 10 minutes, percent combination = 33.8

$$\alpha = -.31^{\circ}$$

$$\text{Percent optically active nitrile produced at 10 minutes} = \frac{.31 \times 3.571 \times 100}{4.428 \times .338}$$

$$= 74 \text{ percent.}$$

That is to say, of the total amount of nitrile produced at this point, 74 percent was optically active.

V. Experiments.

Before performing any work with the enzyme, it was first necessary to make observations on the combination of hydrocyanic acid and benzaldehyde alone, in order that the method of procedure might be tested and also to make sure that experiments could be repeated and constant results obtained. It was decided to dissolve the benzaldehyde in 60 c.c. alcohol (10 percent of the total volume of cell solution) before adding it to the cell, because it was found that in order to make a homogeneous solution of 3 c.c. benzaldehyde in water, at least one hour's stirring was required, during which time there was danger of oxidation of the aldehyde due to traces of oxygen in the nitrogen gas. By using alcohol a homogeneous solution was obtained almost immediately, so that the hydrocyanic acid followed the benzaldehyde into the cell after an interval of five minutes.

Experiment 1. (Standard Experiment at 25 degrees Centigrade.)

Cell contents. 462 c.c. water, 3 c.c. benzaldehyde, 60 c.c. alcohol, and 75 c.c. hydrocyanic acid. Total volume 600 c.c.

Temperature. 25 degrees Centigrade.

Point	Time	Percent Combination	Point	Time	Percent Combination
1.	5 mins.	2.30	6.	180 mins.	22.75
2.	15 mins.	3.00	7.	7 hrs.	39.80
3.	30 mins.	5.95	8.	24 hrs.	65.00
4.	60 mins.	9.70	9.	48 hrs.	74.18
5.	120 mins.	18.40	10.	72 hrs.	75.80

Graph I., curve 1, shows percent combination plotted against time for this experiment. By repeating the experiment it was shown that the experimental conditions could be kept sufficiently constant to obtain concordant results.

The effect of an aqueous extract of the leaves on the reaction was then determined. The "enzyme solution" was made by extracting 20 grams of leaves with 240 c.c. water for 3 hours and filtering through absorbent cotton, using suction. 85 c.c. of the filtrate were added to the cell solution, the volume of water added being reduced in order to keep the concentration of the reaction mixture the same as in Experiment 1.

The optical activity obtained was high enough to warrant the use of leaf-extracts as the source of oxynitrilase for the reaction, but there are objections to the direct use of these solutions. A fresh extract must be made for each experiment, as it cannot be preserved for any length of time (owing to bacterial action), and the concentrations of the various extracts could not be relied upon to be the same, so that comparison of experiments would have been impossible. It was, therefore, decided to prepare a stock of dry enzyme so that a definite weight could be added to each experiment. The method of preparation of the enzyme is described above.

Experiments 2 and 3.

Experiments 2 and 3 were performed as standard runs at 25 degrees Centigrade, each containing 150 milligrams of stock enzyme.

Cell contents. 462 c.c. water, 3 c.c. benzaldehyde, 60 c.c. alcohol, and 75 c.c. hydrocyanic acid. Total volume 600 c.c.

Point	Time	% Comb. Expt.2	% Comb. Expt.3	α. Expt.2	α. Expt.3	%Opt.Act. Nitrile Expt.2	%Opt.Act Nitrile Expt.3
1.	2 mins.	9.14	-	-.06°	-	52.95	-
2.	5 mins.	17.30	20.13	-.15°	-.14°	69.95	56.10
3.	7 mins.	23.75	23.89	-.21°	-.22°	71.33	74.26
4.	15 mins.	39.87	38.77	-.37°	-.36°	74.85	74.90
5.	30 mins.	55.46	55.25	-.53°	-.52°	77.10	75.93
6.	45 mins.	62.06	62.07	-.58°	-.58°	75.40	75.37
7.	60 mins.	66.45	65.48	-.62°	-.63°	75.27	77.60
8.	120 mins.	70.00	70.77	-.65°	-.66°	74.90	75.23
9.	24 hrs.	71.80	74.45	-.38°	-.38°	42.70	41.18
10.	48 hrs.	74.20	80.50	-.21°	-.34°	22.83	34.15

Curve 2, Graph I., represents graphically the rate of reaction under the above conditions and from this it is apparent that the rate is much faster than without the enzyme (curve 1).

Curve 1, Graph II., represents the percent optically active nitrile plotted against percent combination for Experiment 2. The corresponding curve for Experiment 3

agrees very closely except for the first two points. The first points in all curves of this type are not to be relied upon as the optical activity read amounts to only .03° to .08° and an error of .01° (which is easily made) is about a 20 percent error. The curves as a whole are not as good as those for the rate of reaction, because in this case the errors involved are the sum of the errors in the reading of the optical activity and in the determination of the percent combination.

The combination of hydrocyanic acid and benzaldehyde when catalysed by oxynitrilase is a rather complicated reaction, because there are two combinations taking place simultaneously. These are the spontaneous or chemical combination, as shown in Experiment 1, and the combination due to the influence of the enzyme which is brought out by the figures for the percent optically active nitrile produced in Experiments 2 and 3.

From the curves showing the percent optically active nitrile produced it might be suspected that the enzyme is not as active at the beginning of the reaction as it is later on. However, this is probably due to the fact that the estimation of the percent combination is made by the loss of hydrocyanic acid. It is possible that this result is high because some of the hydrocyanic acid may have been lost by combination with the enzyme instead of precipitation by silver nitrate. It is a well-known fact that in many

cases the enzyme does combine with the substrate.

The effect of alcohol on the reaction was determined by performing an experiment in all respects identical with Experiment 2, except that no alcohol was added. In this case the enzyme was added to the required volume of water and stirred as usual for one half hour. The benzaldehyde was then added directly from the pipette and the mixture stirred for 15 minutes before the addition of the hydrocyanic acid.

Experiment 4.

Cell contents. 522 c.c. water, 3 c.c. benzaldehyde, and 75 c.c. hydrocyanic acid. Total volume 600 c.c. 150 milligrams enzyme were added.

Temperature. 25 degrees Centigrade.

Point.	Time.	% Combination	α.	% Optically Active Nitrile
1.	3 mins.	9.37	$-.09^{\circ}$	77.50
2.	7 mins.	20.25	$-.20^{\circ}$	79.65
3.	15 mins.	35.47	$-.35^{\circ}$	79.60
4.	30 mins.	52.51	$-.52^{\circ}$	79.87
5.	50 mins.	61.57	$-.63^{\circ}$	82.50
6.	60 mins.	64.47	$-.64^{\circ}$	80.10
7.	120 mins.	69.55	$-.69^{\circ}$	80.00
8.	24 hrs.	71.00	$-.46^{\circ}$	52.26

As shown by curve 3, Graph I., the speed of the reaction is slightly slower than that of the reaction

containing 10 percent alcohol, curve 2. On the other hand, the percent optically active nitrile formed plotted against percent combination (curve 2, Graph II.) produced a curve a little higher than that obtained in Experiment 2. That is, the chemical action was retarded in favour of the enzyme action.

The effect of doubling the weight of enzyme added was next tried.

Experiment 5.

Cell contents. 462 c.c. water, 3 c.c. benzaldehyde, 60 c.c. alcohol, and 75 c.c. hydrocyanic acid. Total volume 600 c.c. 300 milligrams enzyme added.

Temperature. 25 degrees Centigrade.

Point.	Time.	% Combination	α.	% Optically Active Nitrile. Expt.5	Expt.2
1.	2 mins.	18.3	$-.17^{\circ}$	74.9	52.95
2.	5 mins.	37.4	$-.35^{\circ}$	75.5	69.95
3.	10 mins.	52.0	$-.50^{\circ}$	77.56	-
4.	15 mins.	59.7	$-.59^{\circ}$	79.7	74.85
5.	30 mins.	68.1	$-.69^{\circ}$	81.73	77.10
6.	45 mins.	70.5	$-.67^{\circ}$	76.67	75.40
7.	90 mins.	72.85	$-.64^{\circ}$	70.9	-
8.	180 mins.	73.77	$-.58^{\circ}$	63.4	-
9.	24 hrs.	73.37	$-.24^{\circ}$	26.4	-

The rate of combination is faster than in standard experiments as shown by Graph I., curve 4. The percent optically active nitrile is not much higher than with standard concentration of enzyme. This curve is plotted on Graph II., curve 3.

The concentrations of benzaldehyde and hydrocyanic acid were then doubled and the reaction between them catalysed by oxynitrilase.

Experiment 6.

Cell contents. 384 c.c. water, 6 c.c. benzaldehyde, 60 c.c. alcohol, and 150 c.c. hydrocyanic acid. 150 milligrams of enzyme present. Total volume 600 c.c.

Temperature. 25 degrees Centigrade.

Point.	Time.	% Combination	α.	% Optically Active Nitrile
1.	2 mins.	6.10	-.06°	39.7
2.	5 mins.	10.25	-.17°	66.9
3.	10 mins.	17.70	-.36°	82.1
4.	15 mins.	25.30	-.56°	89.3
5.	30 mins.	42.55	-.86°	81.5
6.	45 mins.	52.50	-1.05°	80.9
7.	90 mins.	68.00	-1.36°	80.7
8.	180 mins.	75.50	-1.52°	81.2
9.	24½ hrs.	79.00	-1.12°	57.2
10.	48½ hrs.	79.25	-.81°	41.2

From Graph I., curve 5, it will be seen that the rate of combination is not greatly affected by doubling the concentration of the substrate, and curve 4, Graph II., shows that the percent optically active nitrile is but little higher than that produced in the standard experiment, curve 1.

When, however, the optical activity is plotted against time for Experiments 2, 5 and 6, as on Graph III., the significance of these experiments is more apparent. On Graph III., curve 1 represents the optical activity plotted against time for Experiment 2 (the standard), and curves 2 and 3 are similar curves for Experiments 5 and 6 respectively.

From the high optical activities obtained in Experiment 6, as compared with those obtained in Experiment 2, it is clear that in the latter reaction the enzyme was not working at its maximum capacity, due to the fact that the concentration of the substrate was too low for the amount of enzyme added. Curve 2 further substantiates this view. Here twice the weight of enzyme that was in Experiment 2 is working on the same amount of substrate, and yet the optical activity is at no point much higher than the maximum on curve 1, and the activity declines at an early point.

It is evident, then, from these curves that in the standard experiments the concentration of substrate is too low to give maximum results with the amount of enzyme added.

The same concentration was retained as standard, however, because in Experiment 6 the solution was not homogeneous even after the 15 minute point had been passed, and it was considered best to work with homogeneous solutions.

In order to study the effect of temperature on the reaction, an experiment was performed at 35 degrees Centigrade. This was in all respects the same as Experiment 2, except that the temperature was 10 degrees higher.

Experiment 7.

Point.	Time.	% Combination.	α.	% Optically Active Nitrile.
1.	2 mins.	10.9	$-.08^{\circ}$	59.2
2.	5 mins.	19.8	$-.17^{\circ}$	69.2
3.	7 mins.	27.2	$-.24^{\circ}$	71.15
4.	10 mins.	33.8	$-.31^{\circ}$	74.0
5.	15 mins.	41.85	$-.37^{\circ}$	71.3
6.	30 mins.	56.15	$-.44^{\circ}$	63.2
7.	45 mins.	62.00	$-.46^{\circ}$	59.8
8.	60 mins.	64.8	$-.49^{\circ}$	62.55
9.	90 mins.	66.6	$-.50^{\circ}$	60.5
10.	200 mins.	67.85	$-.41^{\circ}$	48.7
11.	$6\frac{1}{2}$ hrs.	68.85	$-.26^{\circ}$	32.8
12.	24 hrs.	69.55	0.00°	0.00
13.	48 hrs.	-	0.00°	0.00

From the above results it is apparent that at 35

degrees the rate of combination is very little different from that at 25 degrees. The enzyme action is decreased, as the percent optically active nitrile is never as high as it is at 25 degrees. This led to the idea that at lower temperatures, say zero degrees, the enzyme action might be much stronger.

Accordingly, several experiments were conducted at zero degrees Centigrade. The procedure was modified slightly as it was absolutely necessary that all the reagents should be at zero degrees when the experiment started. The required volume of water containing 150 milligrams of enzyme was stirred in the thermostat at zero degrees for 1¾ hours. The benzaldehyde was dissolved in alcohol and added while at room temperature. The hydrocyanic acid was measured into a small Erlenmeyer which was then tightly corked and put into snow and water for 1½ hours. The acid was added to the cell 5 minutes after the benzaldehyde.

The first experiment conducted at zero degrees contained no enzyme. Otherwise the cell contents were the same as for standard experiments.

Experiment 8.

Point.	Time.	Percent Combination.
1.	15 mins.	0.85
2.	30 mins.	1.15
3.	1 hr.	2.25
4.	2 hrs.	2.50
5.	4 hrs.	2.80
6.	6½ hrs.	5.60
7.	24 hrs.	10.60

It is clear from the above figures that at zero degrees the chemical combination is almost negligible.

The following experiment was a standard with enzyme, performed at zero degrees.

Experiment 9.

Point.	Time.	% Combination.	α.	% Optically Active Nitrile.
1.	15 mins.	9.4	$-.11^{\circ}$	94.4
2.	30 mins.	16.5	$-.21^{\circ}$	102.6
3.	50 mins.	25.4	$-.33^{\circ}$	104.8
4.	1¼ hrs.	34.1	$-.47^{\circ}$	111.15
5.	1¾ hrs.	43.6	$-.54^{\circ}$	99.9
6.	3 hrs.	56.5	$-.71^{\circ}$	101.4
7.	6 hrs.	70.8	$-.90^{\circ}$	102.5
8.	9 hrs.	76.8	$-.96^{\circ}$	100.8
9.	12 hrs.	79.5	$-.99^{\circ}$	100.4
10.	24 hrs.	83.7	-1.02°	98.3
11.	48 hrs.	85.22	-1.00°	94.6

According to the figures for the percent optically active nitrile formed, the chemical action has been altogether eliminated, as was anticipated from the results of Experiment 8. In Experiment 9 all the combination is due to enzyme action.

In all previous experiments the optical activity of the nitrile produced rose to a maximum value (usually attained at about 65 to 70 percent combination), and then dropped. This phenomenon has been noted by Rosenthaler and others, but up to the present no satisfactory explanation has been advanced.

That the drop is not due to enzyme action is proved by the last experiment, for since all the nitrile formed is dextro-rotatory (i.e., yields l-mandelic acid on hydrolysis), therefore the oxynitrilase extracted from peach leaves contains no l-oxynitrilase, and only synthesises and decomposes d-mandelo-nitrile. That the drop in optical activity is due to chemical action is further supported by the fact that where the percent optically active nitrile formed is low, the drop occurs soon in the reaction, whereas when it is high, as in the above experiment, the drop does not occur until a long time has elapsed.

The following may be the explanation of the drop in optical activity. At the beginning of the combination there are two reactions in operation at the same time, and

the velocities of the corresponding reverse reactions are too low to have much effect. Therefore, hydrocyanic acid and benzaldehyde are combining spontaneously (chemical reaction) to form racemic mandelo-nitrile, which is a mixture of equal parts levo- and dextro-nitriles. At the same time benzaldehyde and hydrocyanic acid are combining under the influence of the enzyme to form dextro-mandelo-nitrile. Thus at the start of the reaction an excess of dextro-nitrile is being formed and the solution is dextro-rotatory. When there is an appreciable amount of nitrile formed the reverse reactions set in and the decomposition of the nitrile begins to be noticeable. Now there are two reactions decomposing the nitrile. Under the influence of the enzyme only dextro-nitrile is decomposed, but the spontaneous reaction decomposes levo- and dextro-nitrile according to the ratio of their concentrations in the solution, that is, decomposes more dextro. That is, as the reverse reactions become stronger, the excess of dextro-nitrile decomposed becomes greater and greater and the optical activity of the solution drops.

At chemical equilibrium the enzyme synthesises and decomposes an equal number of molecules of dextro-nitrile. The chemical action, however, while it synthesises an equal number of dextro and levo molecules of the nitrile, decomposes more dextro-nitrile molecules than

it does levo-nitrile molecules, since there are more dextro-nitrile molecules present in the solution. The result is that at equilibrium the amount of dextro-nitrile decomposed is greater than the amount of levo-nitrile decomposed in the same time and the optical activity of the solution continues to drop, and finally a state is reached at which there is no optically active nitrile left.

The theory that the drop in optical activity is due to the fact that the enzyme is slowly killed during the reaction is disproved by the following experiment. A standard experiment was run at 35 degrees Centigrade until the optical activity decreased to a minimum. New hydrocyanic acid and benzaldehyde were then added and the temperature of the thermostat changed to zero degrees, at which temperature the enzyme is most active. If the optical activity rises again the enzyme cannot have been killed. The temperature was then changed to 25 degrees and it was shown that the optical activity again drops.

Experiment 10.

I. at 35 degrees.

Point.	Time.	Percent Combination.	α.
1.	3 mins.	17.1	$-.07^{\circ}$
2.	30 mins.	54.0	$-.39^{\circ}$
3.	60 mins.	66.2	$-.49^{\circ}$
4.	8 hrs.	71.5	$-.25^{\circ}$
5.	$18\frac{1}{2}$ hrs.	73.3	$-.09^{\circ}$

Before any additions to the cell were made, it was placed in a thermostat at zero degrees and the contents stirred for an hour and 20 minutes.

By this time 215 c.c. had been taken from the cell solution so that its volume was now 385 c.c. 2 c.c. benzaldehyde and 13 c.c. hydrocyanic acid solution containing .5425 grams hydrocyanic acid (the exact equivalent of the benzaldehyde) were then added. The benzaldehyde was put into the cell first and the stirring continued for 20 minutes before the addition of the hydrocyanic acid.

II. At zero degrees.

Point.	Time.	α.
6.	15 mins.	$-.15^{\circ}$
7.	60 mins.	$-.28^{\circ}$
8.	$6\frac{1}{2}$ hrs.	$-.72^{\circ}$
9.	$22\frac{1}{2}$ hrs.	-1.13°

This rise in the optical activity produced definitely proves that the enzyme is not harmed by catalysing the combination of hydrocyanic acid and benzaldehyde, and that, therefore, the theory that the drop in the optical activity in standard experiments is due to lessening of the powers of the enzyme will not hold. After standing for 24 hours in a thermostat at 25 degrees Centigrade the optical activity of a sample had dropped to -.93 degrees.

Unfortunately at this point in the investigation the

stock of benzaldehyde was exhausted. After preparing a fresh stock its acidity was found to be different from that of the old. 1 c.c. of the old benzaldehyde had required 2 drops of .1 Normal potassium hydroxide to neutralize it, whereas the new benzaldehyde was absolutely neutral to phenolphthalein. These determinations were made by dissolving 1 c.c. benzaldehyde in neutralized alcohol (faint pink to phenolphthalein) and titrating with .1 Normal potassium hydroxide until the same colour reappeared.

The difference in the two benzaldehydes appears to be negligible, but the following experiments show how sensitive the reaction is to changes in hydrogen-ion concentration.

Experiment 11.

Cell contents. 3 c.c. new benzaldehyde, 60 c.c. alcohol, 75 c.c. hydrocyanic acid, and 462 c.c. water. No enzyme added.

Temperature. 25 degrees Centigrade.

Point.	Time.	Percent Combination.
1.	5 mins.	7.35
2.	7 mins.	23.80
3.	15 mins.	41.70
4.	30 mins.	54.65
5.	45 mins.	63.65
6.	60 mins.	67.53
7.	120 mins.	74.90
8.	20 hrs.	77.20

The above results are plotted on Graph I., curve 7. The rate of combination is much faster than that plotted for the same experiment with the old benzaldehyde (curve 1, same Graph). The only difference between the two experiments is in the acid value of the two benzaldehyde preparations. The reaction is, therefore, more sensitive to hydrogen-ions than many indicators.

The next experiment is a replica of Experiment 2, except that the benzaldehyde preparations are different in the two runs.

Experiment 12.

Cell contents. 462 c.c. water, 3 c.c. benzaldehyde(neutral), 60 c.c. alcohol, 75 c.c. hydrocyanic acid, and 150 milligrams enzyme. Total volume 600 c.c.

Temperature. 25 degrees Centigrade.

Point.	Time.	% Combination	α.	% Optically Active Nitrile.
1.	2 mins.	39.20	-.10°	20.55
2.	5 mins.	63.20	-.17°	21.70
3.	7 mins.	69.45	-.18°	26.30
4.	15 mins.	76.60	-.19°	20.00
5.	30 mins.	78.35	-.15°	15.45
6.	45 mins.	79.20	-.11°	11.20
7.	1 hr.	79.00	-.10°	10.20
8.	2 hrs.	78.35	-.05°	5.30
9.	3 hrs.	78.60	-.03°	3.10
10.	6-2/3 hrs.	78.85	0.00°	0.00

Curve 8, Graph I., represents the rate of combination for this experiment. The speed of the reaction is much greater than that of the standard for the old benzaldehyde preparation. On the other hand, the percent optically active nitrile has been greatly reduced, in fact at 6 hours and 40 minutes the enzyme action has been eliminated entirely. Curve 2, Graph IV., shows the optical activity for this experiment plotted against time. Curve 1 on the same Graph is a similar curve for Experiment 2. Not only are the optical activities much lower on curve 2, but the drop sets in much sooner than on curve 1. All the samples taken from this experiment were yellow to paranitrophenol, the hydrogen-ion concentration being, therefore, below $10^{-6.7}$.

The following experiment is in all respects identical with Experiment 12, except for the fact that the benzaldehyde employed required one drop .1 N potassium hydroxide for the neutralization of 1 c.c.

Experiment 13.

Point.	Time.	% Combination.	α.	% Optically Active Nitrile.
1.	3 mins.	38.9	$-.16^{\circ}$	33.20
2.	$5\frac{1}{2}$ mins.	53.7	$-.22^{\circ}$	33.04
3.	7 mins.	59.5	$-.27^{\circ}$	36.60
4.	15 mins.	71.6	$-.34^{\circ}$	38.30
5.	30 mins.	77.15	$-.33^{\circ}$	34.25
6.	45 mins.	78.65	$-.29^{\circ}$	30.30
7.	60 mins.	77.5	$-.28^{\circ}$	29.15
8.	120 mins.	77.5	$-.25^{\circ}$	26.00
9.	180 mins.	77.5	$-.16^{\circ}$	16.65
10.	6 hrs.	79.15	$-.11^{\circ}$	11.20
11.	8 hrs.	79.05	$-.09^{\circ}$	9.20
12.	24 hrs.	79.05	0.00°	0.00

The rate of reaction is almost identical with that of the previous experiment but the percent optically active nitrile produced is greater. Increase in acidity, therefore, aids the enzyme action. Curve 3, Graph IV., represents the optical activities plotted against time. They are greater than those in Experiment 12 but still fall far short of those in Experiment 2 (curve 1). The drop is later than in Experiment 12, but far ahead of that in Experiment 2. Curves 5 and 6 (Graph II.) represent percent optically active nitrile plotted against percent combination for Experiments 12 and 13 respectively. By tests with

paranitrophenol, the hydrogen-ion concentration of the reacting solution was determined to be between $10^{-6.7}$ and $10^{-6.1}$. There is, therefore, but a minute difference between the conditions of this experiment and those of Experiment 12. The results indicate the extreme sensitiveness to small changes in hydrogen-ion concentration near the neutral point.

The effect of higher hydrogen-ion concentrations was studied by adding different volumes of .05N acetic acid to the reaction. The first addition was 5 c.c. of the acid, that being the volume necessary to bring about the first distinct change in colour of methyl orange in 600 c.c. distilled water.

Experiment 14.

Cell contents. 457 c.c. water, 5 c.c. .05N acetic acid, 3 c.c. benzaldehyde, 60 c.c. alcohol, 75 c.c. hydrocyanic acid, and 150 milligrams enzyme. Total volume 600 c.c.

Temperature. 25 degrees Centigrade.

Point.	Time.	% Combination.	α.	% Optically Active Nitrile.
1.	5 mins.	25.40	-.20°	63.50
2.	10 mins.	37.00	-.36°	78.50
3.	15 mins.	46.90	-.46°	79.10
4.	30 mins.	61.75	-.60°	78.35
5.	1 hr.	69.80	-.66°	76.25
6.	2 hrs.	72.35	-.66°	73.55
7.	3 hrs.	72.65	-.64°	71.05
8.	4-2/3 hrs.	73.35	-.58°	62.30
9.	8 hrs.	74.75	-.50°	53.95

The hydrogen-ion concentration of the cell solution was found to be between $10^{-5.2}$ and $10^{-6.1}$ as a result of tests with methyl orange and paranitrophenol. As before, increase in hydrogen-ion concentration has caused a slowing up of the chemical action and has hastened the enzyme action.

The amount of acid added in the next experiment was 22 c.c., that is, the volume required to make 600 c.c. distilled water acid (red to methyl orange).

Experiment 15.

Cell contents. 440 c.c. water, 3 c.c. benzaldehyde, 60 c.c. alcohol, 22 c.c. .05N acetic acid, 75 c.c. hydrocyanic acid, and 150 milligrams enzyme.

Temperature. 25 degrees Centigrade.

Point.	Time.	% Combination.	α.	% Optically Active Nitrile.
1.	5 mins.	17.60	-.14°	64.15
2.	10 mins.	24.40	-.27°	89.20
3.	15 mins.	33.00	-.36°	88.00
4.	30 mins.	47.45	-.54°	91.00
5.	1 hr.	61.10	-.68°	89.75
6.	3 hrs.	67.30	-.77°	92.30
7.	5½ hrs.	71.30	-.78°	88.20
8.	24 hrs.	71.35	-.55°	62.20

The rate of combination is much slower than in the previous experiment, which contained a little less than

one quarter the amount of acetic acid in this experiment. The percent optically active nitrile is higher in this experiment than in Experiment 14. The actual optical activity is, however, lower at the beginning of the experiment though it later on catches up to and reaches a higher maximum than that of Experiment 14.

The hydrogen-ion concentration of the cell solution was about $10^{-4.1}$. The results of Experiments 13, 14 and 15 clearly indicate that the enzyme action is very sensitive to small changes in hydrogen-ion concentration, that it is most sensitive between the hydrogen-ion concentrations $10^{-5.2}$ and $10^{-6.1}$, and that the most favourable condition for enzyme action is between these limits.

The final test applied to the enzyme was the effect of dialysis. This was carried out in the usual way, using a parchment membrane and stirring the enzyme solution throughout the process. 150 milligrams of oxynitrilase were dialysed for 17 hours and the water in the outer container was changed at the end of 4 hours and again after 16 hours. The enzyme solution remaining in the inner cell was used in the following experiment.

Experiment 16.

Cell contents. 462 c.c. water (including the dyalised enzyme solution), 3 c.c. benzaldehyde, 60 c.c. alcohol, and 75 c.c. hydrocyanic acid. Total volume 600 c.c.

Temperature. 25 degrees Centigrade.

The aldehyde used was neutral to phenolphthalein so that the results of the experiment are comparable to those of Experiment 12.

Point.	Time.	% Combination.	α.	% Optically Active Nitrile.
1.	2 mins.	29.9	-.13°	35.10
2.	8 mins.	54.95	-.35°	51.40
3.	15 mins.	68.25	-.45°	52.00
4.	30 mins.	74.45	-.49°	53.10
5.	45 mins.	75.40	-.48°	51.30
6.	60 mins.	76.24	-.44°	46.50
7.	120 mins.	76.65	-.42°	44.20
8.	180 mins.	76.80	-.39°	40.90
9.	6¼ hrs.	77.60	-.33°	34.30
10.	22 hrs.	77.60	-.18°	18.70

On comparing these results with those of Experiment 12, it is evident that dialysis removes something from the enzyme which catalyses the chemical action only or some substances which retard the enzyme action.

Experiment 17.

This experiment is an exact repetition of Experiment 16, except that the temperature was changed to zero degrees Centigrade.

Point.	Time.	%Combination.	α.	% Optically Active Nitrile.
1.	5 mins.	23.20	$-.19^{\circ}$	66.05
2.	10 mins.	38.20	$-.37^{\circ}$	98.30
3.	16 mins.	50.50	$-.45^{\circ}$	71.90
4.	30 mins.	66.10	$-.60^{\circ}$	74.90
5.	60 mins.	76.80	$-.74^{\circ}$	77.50
6.	120 mins.	83.75	$-.80^{\circ}$	77.00
7.	180 mins.	84.30	$-.82^{\circ}$	78.40
8.	7 hrs.	-	$-.79^{\circ}$	-
9.	$22\frac{1}{2}$ hours.	-	$-.80^{\circ}$	-

This experiment, as compared with the previous one, shows that at zero degrees the optical activity increases.

Conclusions.

As there are two reactions taking place simultaneously when oxynitrilase catalyses the combination of hydrocyanic acid and benzaldehyde, these being a chemical action and an enzyme action, it was necessary to devise a method for following the rate of the latter and a satisfactory method has been developed.

For a definite weight of enzyme there is a definite amount of substrate with which a maximum amount of optically active nitrile is produced. If the amount of substrate is less than the amount necessary for the maximum yield of active nitrile, doubling the amount of enzyme added has very little effect.

The enzyme action produces most nitrile at low temperatures, though at a slower rate than at higher temperatures, and as the temperature is raised the amount of nitrile produced by enzyme action decreases. At zero degrees the chemical action plays practically no part in the combination.

During the reaction the optical activity of the mandelo nitrile produced rises to a maximum and then drops. A theory to explain this phenomenon has been advanced.

The hydrogen-ion concentration has opposite effects on the enzyme and chemical actions. Increase in hydrogen-ion concentration hinders the chemical reaction and aids the enzyme action until a maximum for the latter is reached

between the hydrogen-ion concentrations $10^{-5.2}$ and $10^{-6.1}$. The reaction is more sensitive to hydrogen-ion concentrations than many indicators, being most sensitive between the limits mentioned.

On dyalysing the enzyme, something is removed from it which either catalyses the chemical action only or else hinders the enzyme action. Further experiments, however, will be necessary in order to draw any conclusions on this point.

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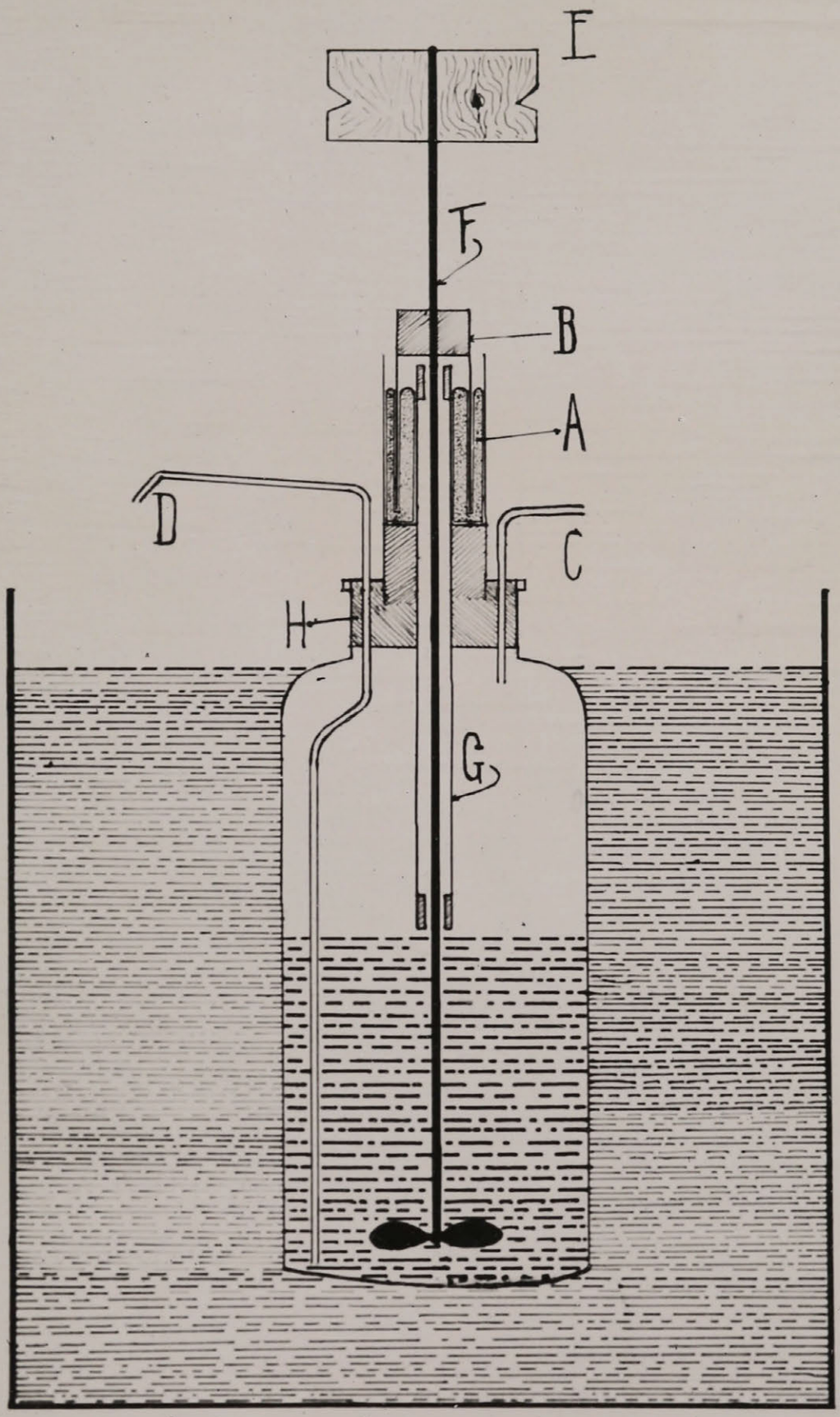
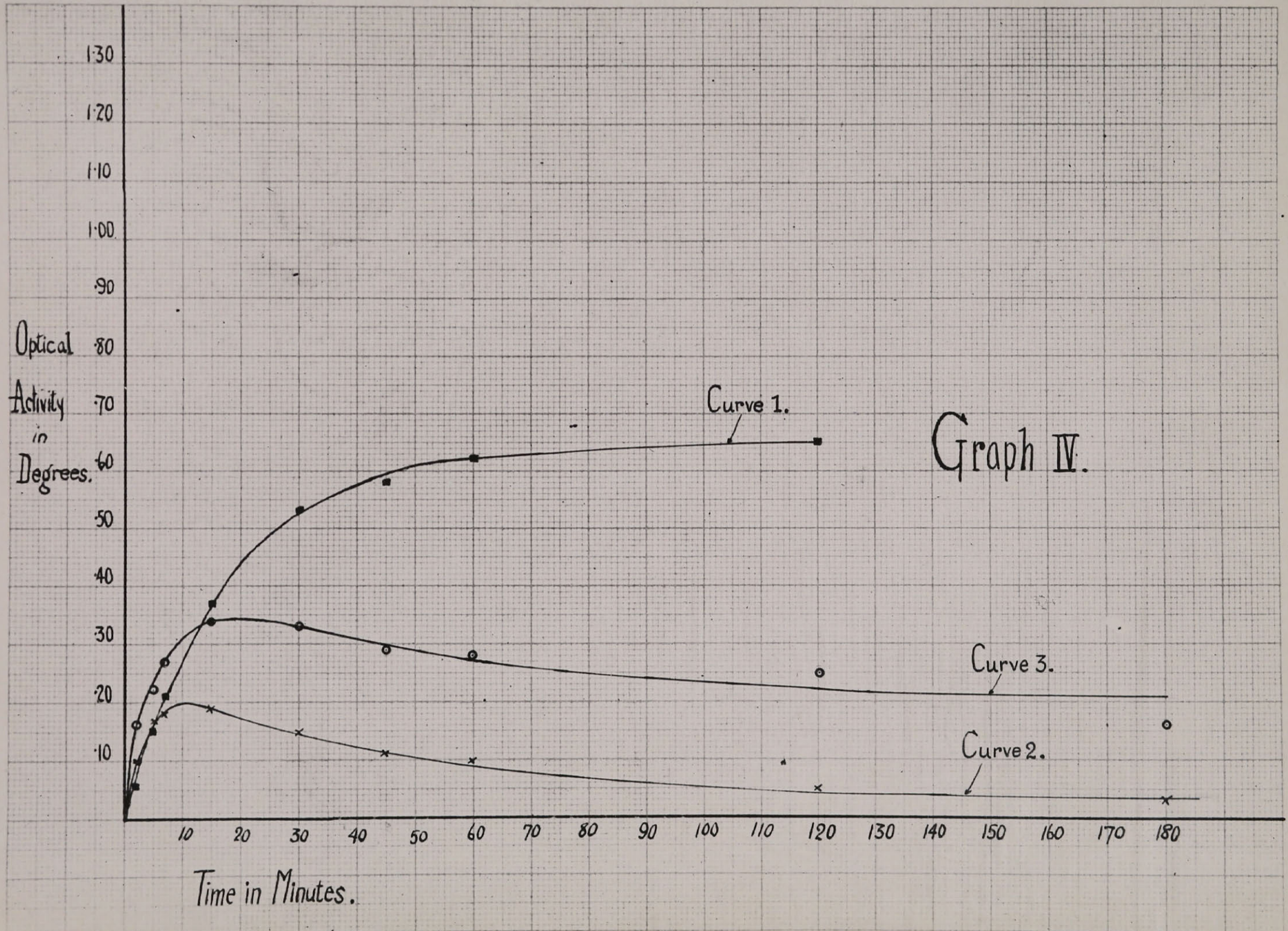
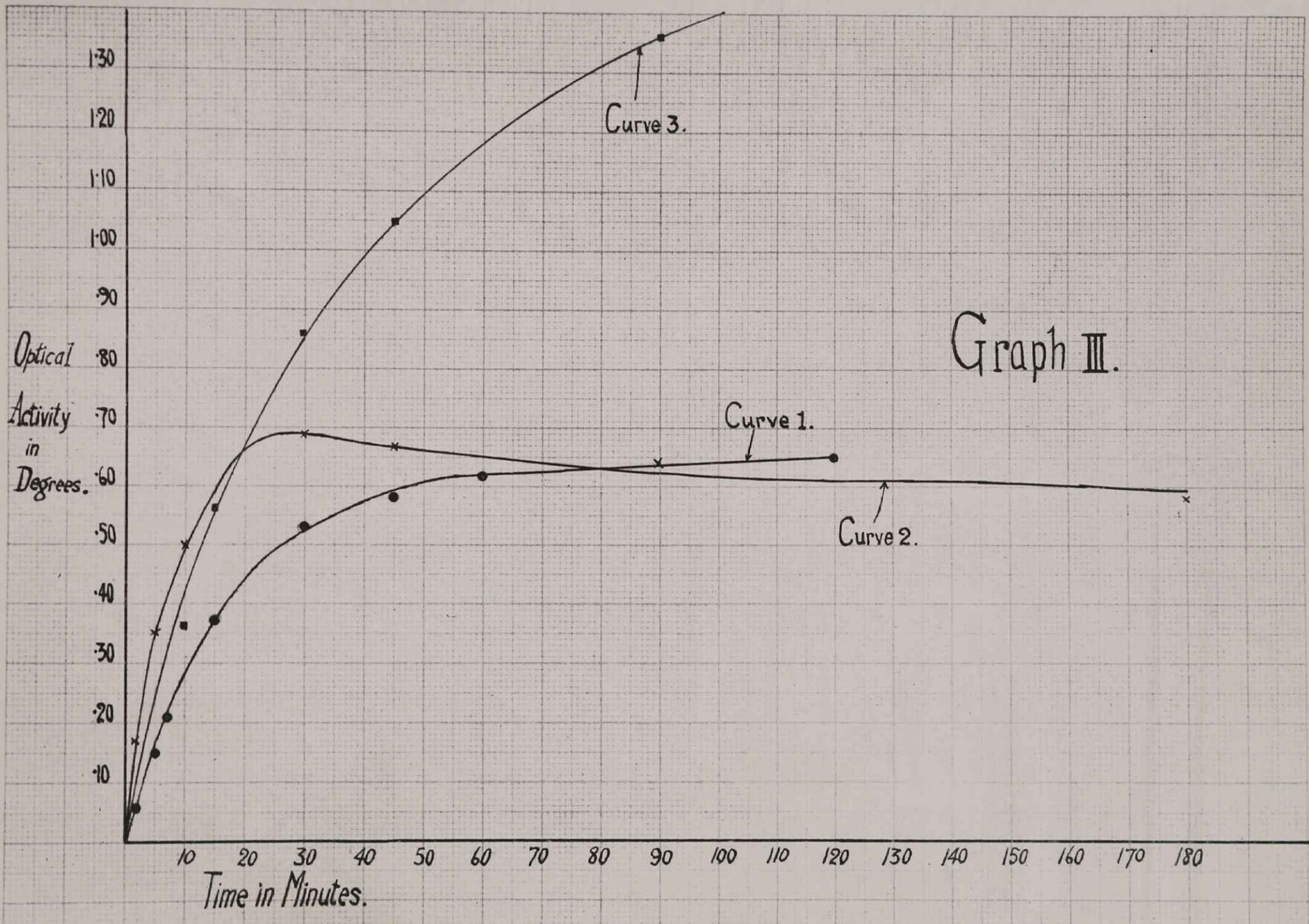


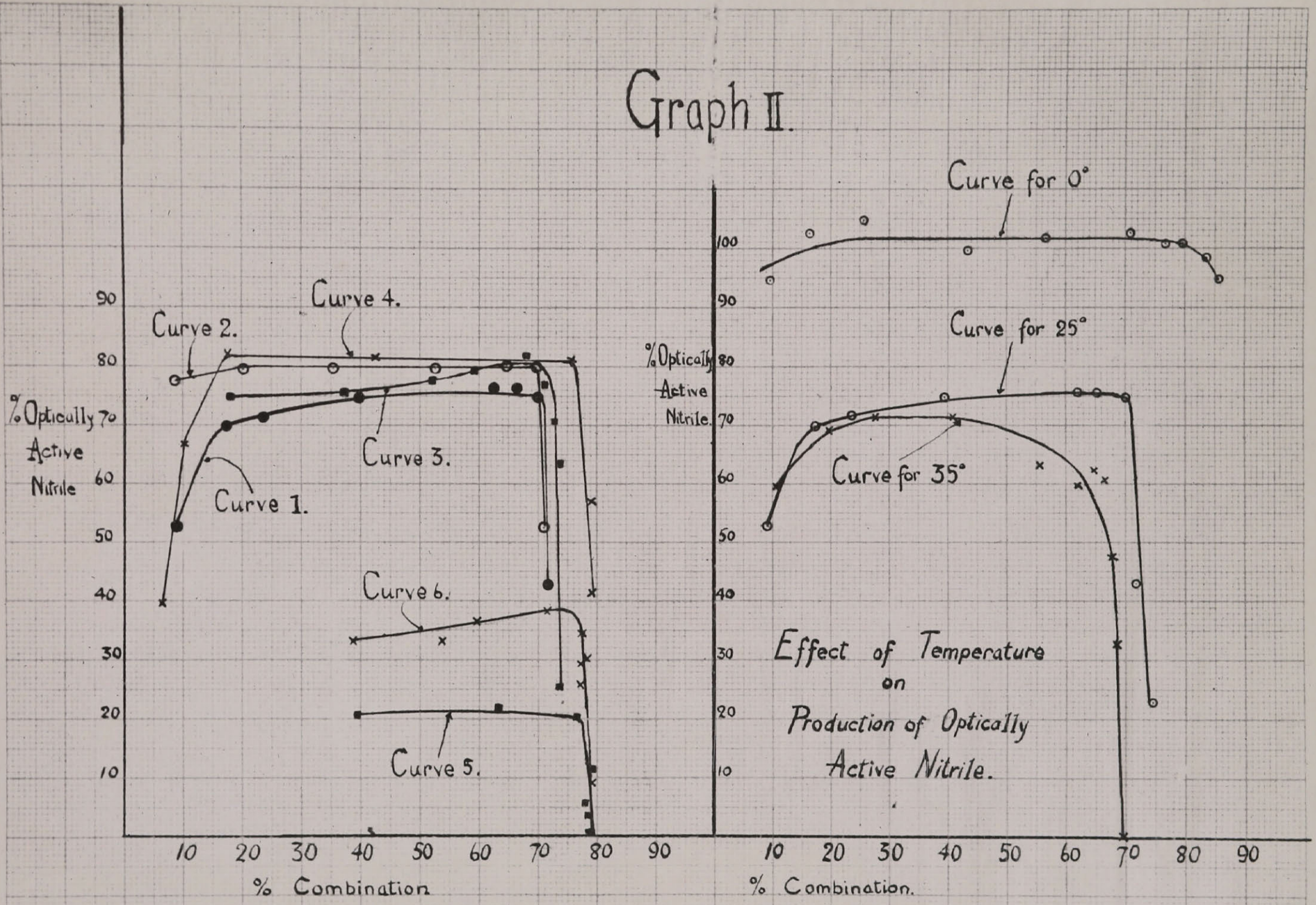
Fig. I.

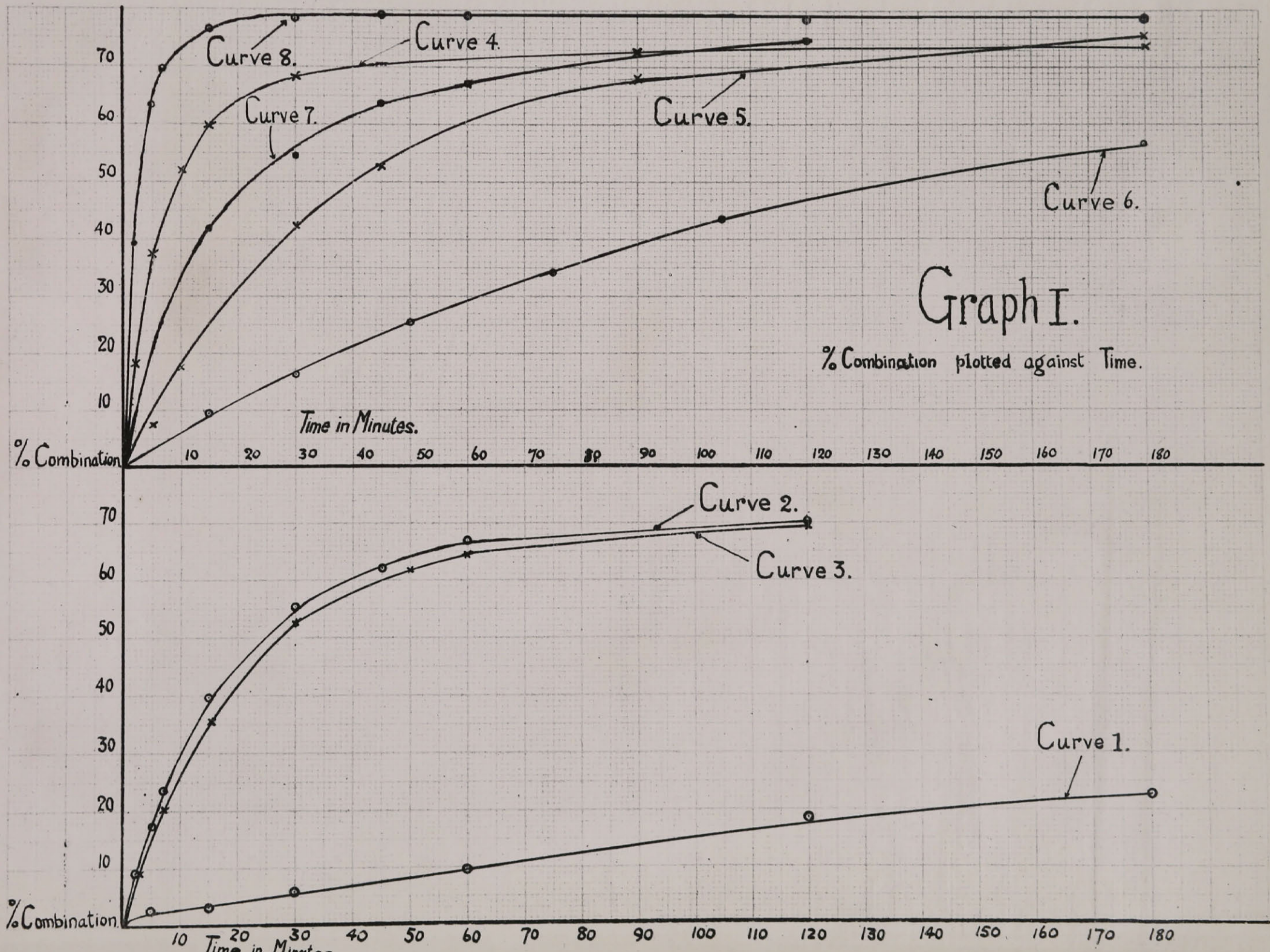

Optical Activity in Degrees.
1·30
1·20
1·10
1·00
·90
·80
·70
·60
·50
·40
·30
·20
·10
Curve 1.
Curve 3.
Curve 2.
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Time in Minutes.

Graph IV.


Graph III.
Curve 3.
Curve 1.
Curve 2.
Optical Activity in Degrees.
1·30
1·20
1·10
1·00
·90
·80
·70
·60
·50
·40
·30
·20
·10
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Time in Minutes.

Graph II.


Curve 1.
Curve 2.
Curve 3.
Curve 4.
Curve 5.
Curve 6.
% Optically Active Nitrile
% Combination
Curve for 0°
Curve for 25°
Curve for 35°
Effect of Temperature on Production of Optically Active Nitrile.
% Optically Active Nitrile.
% Combination.


Curve 8.
Curve 4.
Curve 7.
Curve 5.
Curve 6.
Graph I.
% Combination plotted against Time.
Time in Minutes.
% Combination
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
Curve 2.
Curve 3.
Curve 1.
% Combination
Time in Minutes.
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180