The two arrows are introduced to indicate the direction from which hydrolytic attack may be supposed to proceed: a molecule open to attack in such manner would, doubtless, be far less stable than one in which only a single contiguous oxygen centre serves to direct the attack.

The hydrolysis of melibiose and of raffinose by emulsin lactase will be considered in a later communication; the hydrolysis of melibiose by acids and by melibiase will also be dealt with separately.

Studies on Enzyme Action. XII.—*The Enzymes of Emulsin.*

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Authors' title slip :—D. Q.
Subject slips :—
D 1850 Amygdalin, hydrolysis by emulsin to amygdonitrileglucoside.
D 8014 Emulsin (almond)—presence in, of three enzymes.
Q 1240 Amygdalase, presence of, in almond emulsin.
D 8012, Q 1230 } Lactose, hydrolysis of, by glucolactase.
D 6300 Hydrogen cyanide (from amygdalin).
D 6350 Glucose (from amygdalin)].

Emulsin has frequently been the subject of discussion in this series of studies: thus the rate at which it hydrolyses milk-sugar was considered in No. II (vol. 73, pp. 507, 515) and its action contrasted with that of Kephir-lactase, which was shown to act more rapidly than emulsin. In No. III (vol. 73, p. 518) it was pointed out that whilst Kephir-lactase is controlled by galactose and scarcely at all by glucose, emulsin is controlled by glucose and only to a minor extent by galactose. These conclusions were confirmed by experiments with the methyl-glucosides and galactosides (p. 523). In No. V (vol. 74, p. 188) the question was discussed whether emulsin proper hydrolyses milk-sugar or whether, as Bourquelot and Hérissey have contended, emulsin contains a small proportion of lactase: against this assumption it was argued that the curve was not of the form to be expected if only a small quantity of lactase were present; that whereas Kephir-lactase was controlled by galactose alone, emulsin was most retarded by glucose and only to a slight extent by galactose, also that the curves for emulsin differed in

character from those for lactase. Attention was directed, however, to Pottevin's statement that *Aspergillus niger* contains an enzyme capable of hydrolysing β-glucosides, but not β-galactosides nor milk-sugar. In No. X (B, vol. 79, p. 360) some of the statements made in earlier studies were rectified and it was announced that evidence had been obtained that the activity of ordinary emulsin is due, as the French workers had contended, to the presence of a distinct enzyme capable of hydrolysing β-galactosides, on which emulsin proper has no action, this enzyme affecting only β-glucosides.

While admitting this to be the case, however, we may point out that the conclusions we have to bring forward involve merely a different reading of the facts: the facts have been correctly advanced on both sides.

The explanation of the difference in the interpretations arrived at is simply that Kephir-lactase and emulsin-lactase are distinct enzymes—the one being a *galacto-lactase*, the other a *gluco-lactase*: the former being controlled by galactose, the latter by glucose. Probably, therefore, hydrolysis is effected in consequence of the attachment of the one enzyme to the glucose section and of the other to the galactose section of the biose.

In No. IX (B, vol. 79, p. 350) of these studies, proof was given by Messrs. Caldwell and Courtauld of the existence in yeast of a specific enzyme, *amygdalase*, capable of hydrolysing the bioside amygdalin into amygdonitrile-glucoside and a single molecule of glucose:

$$C_6H_5CH(CN)O—C_{12}H_{21}O_{10} + H_2O \rightarrow C_6H_5CH(CN)O—C_6H_{11}O_5 + C_6H_{12}O_6.$$

But amygdalin is resolved by emulsin into two molecules of glucose and one of the benzylidenecyanhydrol; this latter being unstable in presence of water decomposes into hydrogen cyanide and benzaldehyde. If both junctions were severed by one and the same enzyme, the effect produced would be one entirely *sui generis*, so far as our knowledge extends. From this point of view, the behaviour of emulsin is of special importance and has engaged our attention during several years past.

By systematically studying the action of acids on amygdalin, Messrs. Caldwell and Courtauld were led to discover that the two glucose sections of the molecule are more readily separated from one another than is the benzylidenecyanhydrol; they succeeded in verifying this conclusion by isolating Fischer's glucoside—as the compound of the cyanhydrol with glucose is termed—from the product obtained on partially hydrolysing amygdalin.

We have endeavoured to follow the course of change under the influence of emulsin. The analytical difficulties experienced at first were very serious, and trustworthy results were obtained only after long study of the methods.

Eventually, however, we succeeded in determining both hydrogen cyanide and glucose in a fairly satisfactory manner, although not with the degree of accuracy we desired.

The results of such determinations having shown that apparently the production of glucose was in advance of the production of hydrogen cyanide to a not inconsiderable extent during the earlier part of the change, it appeared probable that the action of emulsin was comparable with that of acids and that Fischer's glucoside was the main initial product of change: we therefore endeavoured to isolate this substance when the action was about half complete.

The neutral solution was carefully evaporated on the water bath and the syrup ultimately obtained was then extracted with ethylic acetate in the manner described by Caldwell and Courtauld.* The ultimate product was identical in crystallographic character, melting point and solubility with Fischer's glucoside and the melting point of a mixture of the two substances was that of the glucoside; it also gave an acetate indistinguishable from that prepared from Fischer's glucoside.

There can, therefore, be practically no doubt that besides *gluco-lactase*, emulsin contains a *β-glucase* capable of hydrolysing substances such as β-methylglucoside, together with a third enzyme, *amygdalase*. That gluco-lactase is present in addition to amygdalase is proved by the fact that the former may be destroyed by heating, whilst the latter persists.

In view of this result, we are justified in laying emphasis on the statement made in No. X of these studies:—"As the investigation is extended, the evidence becomes more and more convincing that the action which an enzyme exercises is specific; in other words, that it is limited to compounds of a particular type—to a greater extent, indeed, than is recognised in earlier communications of this series." In fact, it is difficult to overrate the value of enzymes as diagnostic agents.

In No. VII (B, vol. 76, p. 592) of these studies, dealing with the synthetic action of enzymes, evidence was adduced tending to prove that emulsin and maltase give rise respectively to maltose and "isomaltose." In view of the results now brought forward, it is clear that the nature of the synthetic processes must be regarded as uncertain until definite proof is obtained that this or that enzyme is the one concerned in the formation of a particular biose. At present we see no reason, however, to doubt the validity of the arguments previously adduced, the results obtained on repeating the experiments having uniformly served to confirm the early observations.

But in extending the inquiry painful experience has convinced us that it

* 'Chem. Soc. Trans.,' 1907, vol. 91, p. 670.

is full of pitfalls and difficulties: it was always obvious that the enzymes were mixtures, and the evidence constantly forced upon us that the products are mixtures has been in no sense a surprise. To carry the investigation to a successful issue, we have not only to overcome the difficulties incident on the preparation of considerable quantities of the enzymes in suitable condition, but also to devise special methods of separating, purifying and definitely characterising the products.

Preparation of Emulsin.—Emulsin being an article of commerce, a dried solid preparation obtained from Merck, of Darmstadt, was made use of in the experiments referred to in the earlier communications of this series. This was always found to be active towards both amygdalin and milk-sugar. A similar preparation obtained from Kahlbaum in 1906 was almost worthless, being without action on milk-sugar even at 36° and acting only slowly on β-methylglucoside and salicin. We were led by the observations of these differences to prepare fresh emulsin from almonds, in the expectation that it would be much more active than a dried preparation; this proved to be the case.

The almonds—either the sweet or bitter variety may be used—were reduced to a pulp by passing them between rollers; the pulp was then pressed to remove as much as possible of the oil and afterwards macerated during about 24—48 hours with about two or three times its weight of water containing a little toluene. The most active preparations are obtained by extracting at a moderately low temperature, about 10°—20°. The cloudy liquid obtained on filtering the extract was mixed with two drops of acetic acid per 100 c.c., to precipitate part of the albuminoids present; the relatively clear liquid obtained, after separating the precipitate by filtration, was then mixed by degrees with about an equal volume of alcohol. The precipitate thrown down by the alcohol was allowed to subside and then washed by decantation with alcohol; finally, it was removed to a filter-paper. The adhering alcohol having been absorbed as far as possible by means of filter-paper, the moist solid was shaken up with distilled water containing toluene, the amount of water being varied according to the strength of the extract desired. After remaining in contact with the solid during 48 hours, at 37°, the solution was filtered and the residue again extracted with water. The extract thus obtained is a colourless, clear liquid, admirably suited for polarimetric work.

Lactoclastic Power of "Emulsin."—In comparative experiments made with such a solution, at 15° and 36°, with milk-sugar and β-methylglucoside, the glucoside underwent hydrolysis to a considerable extent at the lower temperature, whereas the milk-sugar was but slightly attacked; at the higher temperature, both were hydrolysed comparatively rapidly.

On heating the extract at 45° during three hours it was entirely deprived of the power of hydrolysing milk-sugar; it retained its activity as a hydrolyst of β-methylglucoside, amygdalin and salicin, however, not only after 20 hours' heating at 45°, but even when heated during several hours at 55° in presence of these hydrolytes; the enzyme was practically destroyed by heating at about 59°.

The preparation obtained by macerating almonds with water at about 57° was found to be active towards the three glucosides at 38°, but when heated to 62° it became inactive.

By macerating almonds with water at about 0° and then again macerating the extracted paste with a further quantity of water at 45°, extracts were obtained from which "emulsins" were prepared in the manner described. Both preparations hydrolysed milk-sugar at 38°; only that made at the lower temperature, however, produced perceptible hydrolysis at 15°—an indication that the gluco-lactase had been preferentially extracted at the lower temperature.

Cane-sugar, maltose and β-methylglucoside were not in the least affected by these preparations.

A solution containing 5 per cent. of lactose was completely hydrolysed at 38° in the course of a week, when presumably the enzyme was in equilibrium with the products of change; a volume of the sugar solution equal to that first taken was then added: it was found that hydrolysis took place as before and became complete, showing that the enzyme had retained its activity. Extracts prepared in the manner described, if maintained sterile by means of toluene, have been found to retain their activity almost unchanged during many months.

A typical series of values obtained with emulsin, prepared by extracting almond paste at about 0°, is contained in Table I.

Whereas, in the experiments recorded in No. II of these studies, made with Merck's relatively inactive emulsin, a series of decreasing values were obtained, the values recorded in the upper part of Table I form an increasing series. The change follows a straight-line law during at least seven hours until about 30 per cent. of the sugar is hydrolysed. Viewed in the light of the explanation formerly given, which has found general acceptance,* the results are easily explained and significant: obviously only a very small quantity of a highly active enzyme was present—so much so, indeed, that the magnitude of the active system remained constant during a considerable period, until as much as 30 per cent. of the hydrolyte had been attacked.

Additional proof that this explanation is admissible is given in the lower

* Compare Bayliss, 'Archives des Sciences Biologiques,' 1904, vol. 11, suppl., p. 261.

Table I.

5 grammes milk-sugar per 100 c.c. X = percentage amount hydrolysed.

Time.	10 c.c. enzyme.		20 c.c. enzyme.	
	X.	K.	X.	K.
hours.				
2	4·4	0·00975	8·3	0·0018
3	6·8	0·0102	12·1	0·0184
5	13·2	0·0113	21·4	0·0209
7	18·0	0·0123	31·9	0·0238
10	20·8	0·0101	42·3	0·0239
24	40·5	0·0094	61·0	0·0170
27	42·5	0·0089	64·5	0·0166
75	70·3	0·0070	83·3	0·0104

Time.	40 c.c. enzyme.		60 c.c. enzyme.	
	X.	K.	X.	K.
hours.				
2	15·2	0·0358	18·5	0·0444
3	18·5	0·0296	22·7	0·0373
5	21·1	0·0251	36·5	0·0394
7	33·5	0·0253	44·9	0·0380
9	42·2	0·0264	52·5	0·0360
10	44·0	0·0252	53·5	0·0332
24	73·4	0·0240	76·0	0·0258

part of the table, in which are recorded the results of experiments made with four and six times the amount of enzyme used in the experiment just described. It will be noticed that when the fourfold amount was used, the quantity of enzyme was such that the hydrolyst no longer had unlimited choice, but was forced to compete for the sugar molecules: being dependent on the proportion of unhydrolysed molecules, the rate of change followed the simple mass-action law, as shown by the approximately constant value of K after about three hours.

Using the fourfold and sixfold amount of enzyme, the rate of change is such that the influence of the products of change soon becomes of consequence; a decreasing series of values is therefore obtained. On comparing the values found, it is obvious that at first, when the amount of enzyme used was doubled, the rate of change was doubled; subsequent additions, however, did not produce the proportionate increase in the rate of change.*

* The experiments were all carried out at 38° in precisely the manner already explained (No. II). Jena-glass vessels were used throughout, with the exception of the pipettes. The data given are quoted as illustrations of the results obtained in a number of similar experiments.

Amount of enzyme.	Velocity constant K.
10 c.c.	0·0107
20	0·0212
40	0·0279
60	0·0385

While the evidence secured is sufficient, we consider, to prove the existence of a lactase in almonds distinct from emulsin proper (β-glucase), it in no way serves to distinguish the enzyme from that in Kephir grains, the action of which was considered in Nos. II and III. The proof on which we rely as a means of differentiating the two enzymes is that the one is controlled by glucose, the other by galactose. The effect of these hexoses on Kephir-lactase was considered in No. III. Results are now recorded in Table II which illustrate the behaviour of emulsin-lactase prepared in the manner described above; they afford complete confirmation of those obtained four years ago, which were then properly interpreted as proof of the non-identity of the two enzymes, although it was not then realised that emulsin proper (β-glucase) was incapable of hydrolysing milk-sugar. Such evidence, in our opinion, is a clear indication that the hydrolysis of milk-sugar is consequent on the attachment of the enzyme in the one case to the galactose section and in the other to the glucose section of the biose; we may point to this conclusion as a striking illustration of the diagnostic value of what may be termed the *control method* which has been developed in the course of these studies and as being also an interesting extension of our conception of the manner in which enzymes may affect hydrolysis.

The Amygdaloclastic Activity of Emulsin.—Although the individual substances which are formed when amygdalin is hydrolysed—hydrogen cyanide, benzaldehyde and glucose—may be estimated more or less easily when apart, serious errors arise when the ordinary analytical methods are applied to their determination in admixture.

Ripper's method* of determining benzaldehyde gives low results, even with benzaldehyde alone, the values varying with the dilution. It is altogether vitiated by the presence of hydrogen cyanide. All attempts we have made to fix the cyanide as an insoluble or non-volatile cyanide have been unsuccessful, some always passing over during the removal of the benzaldehyde by distillation.

The determination of benzaldehyde in the form of a difficultly soluble hydrazone is unsatisfactory, chiefly on account of the difficulty of securing its conversion completely into hydrazone and because of the readiness with which it is oxidised.

Liebig's method of estimating hydrogen cyanide cannot, as a rule, be applied directly to the solution in which hydrolysis has taken place, as the emulsin is liable to interfere with the determination of the end point, by rendering the solution turbid. The method

* 'Zeit. anal. Chem.,' 1902, vol. 41, p. 61.

Table II.

5 grammes milk-sugar per 100 c.c. 40 c.c. enzyme extract* + 5 grammes glucose or galactose.

Time.	Amount hydrolysed.		
	No addition.	Glucose.	Galactose.†
hours.	grammes.	gramme.	grammes.
2	0·35	0·05	0·36
5	0·85	0·10	0·88
10	1·225	0·37	1·23
24	2·025	0·75	1·96

Time.	Amount hydrolysed.		
	No addition.	No. 1 galactose.	No. 2 galactose.
hours.	grammes.	grammes.	grammes.
19‡	1·59	1·58	1·60
21	1·71	1·68	1·69
27	1·86	1·83	1·87

5 grammes milk-sugar per 100 c.c. 30 c.c. enzyme extract§ + 3 grammes glucose.

Time.	Amount hydrolysed.	
	No addition.	Glucose.
hours.	grammes.	gramme.
2	0·169	0·095
5	0·412	0·175
7	0·530	0·220
10	0·730	0·280
24	1·120	0·572

* From bitter almonds.

† Purified material.

‡ These results illustrate the character of the action during the later stages of hydrolysis, those above during the earlier; the two samples of galactose were from different sources: both were purified by careful recrystallisation. The two sets of observations were made at intervals three months apart.

§ From sweet almonds.

which was adopted by Tamman,* involving the precipitation of the cyanide by excess of silver nitrate and the subsequent determination of the excess, has frequently given impossible results in our hands: apparently the benzaldehyde interferes.

* 'Zeit. physiol. Chem.,' 1892, vol. 16, p. 286.

Ultimately we were led to devise a method which not only renders possible the estimation of hydrogen cyanide with a fair approximation to accuracy, but also permits of a considerable number of determinations being taken in hand in rapid succession—a matter of some importance. The solution (10 c.c.) in which hydrolysis has taken place is added to a decinormal solution of silver nitrate (20 c.c.) previously mixed with an equal bulk of a decinormal solution of sodium acetate. The mixture is then heated (5—10 mins.) on a water bath. A bulky precipitate of silver cyanide is thus formed, which may be set aside until it is convenient to complete the analysis. The precipitate, having been filtered off and washed with boiling water, is transferred to a beaker with the aid of about 50 c.c. of water; it is then dissolved in 5 c.c. of strong ammonia, the precipitate adhering to the filter being removed by means of 5 c.c. of ammonia, diluted with 25 c.c. of water, and the beaker in which precipitation is effected also washed out with 5 c.c. ammonia and 30—40 c.c. of water. The combined solutions are mixed with 4 c.c. of a normal solution of sodium chloride and poured through a thistle funnel into a round-bottomed Jena flask containing a solution of 25 grammes of tartaric acid. The liquid is raised to the boiling-point and distilled during 15 mins. in a current of steam, the distillate being received in a flask containing a slight excess of potash. The solution is then titrated with a solution of silver nitrate. A small amount of benzoic acid often distils over, but this does not appear to interfere with the titration.

To determine the amount of glucose produced during hydrolysis, the standard method described by Brown, Morris and Millar,* involving the use of Fehling's solution, was adopted, as it was found that neither amygdalin nor hydrogen cyanide had any reducing action on Fehling's solution and that emulsin had a very slight action, the effect of which was determined and allowed for. The procedure is as follows:—

10 c.c. (or 20 c.c. at the end of the first hour) of the liquid in which the action has been stopped by adding a drop of sulphuric acid are introduced into a Wurtz flask and the sulphuric acid neutralised by adding a previously determined quantity of N/5 potash; the liquid is then diluted to about 125 c.c. and distilled by passing in steam during about 30 minutes. The liquid is next rinsed into an evaporating basin, evaporated to a small bulk, transferred to a 50-c.c. flask, cooled and diluted to 50 c.c. The required quantities (25 c.c.) of each of the two Fehling's solutions are mixed in a 400-c.c. wide Jena glass beaker with such a quantity of water that addition of the sugar solution will make the volume up to 100 c.c.; this beaker is heated in a boiling-water bath during 5 mins., then removed and 25, 15 or 10 c.c. (according to its strength) of the solution in which glucose is to be determined is added. The beaker is then replaced in the water bath, covered with a clock-glass and heated during exactly 12 mins. The precipitated cuprous oxide is collected on an asbestos filter in a Soxhlet tube, washed with about 200 c.c. of boiling water, then with 10 c.c. of alcohol and dried in a water oven. The oxide is oxidised by heating in a current of air and then reduced to copper by heating in a current of purified dry hydrogen, cooled and weighed. The initial oxidation is necessary, in order to prevent volatilisation of part of the copper, which occurs when the cuprous oxide is heated directly in a current of hydrogen, probably owing to the presence of organic matter.

Using Fischer's glucoside as test material, which is resolved simply into glucose, benzaldehyde and hydrogen cyanide by emulsin, the amounts of hydrogen cyanide found have been uniformly low—on the average from one to two units below the calculated percentage values—so that the process is certainly open to improvement and simplification; we hope to improve it.

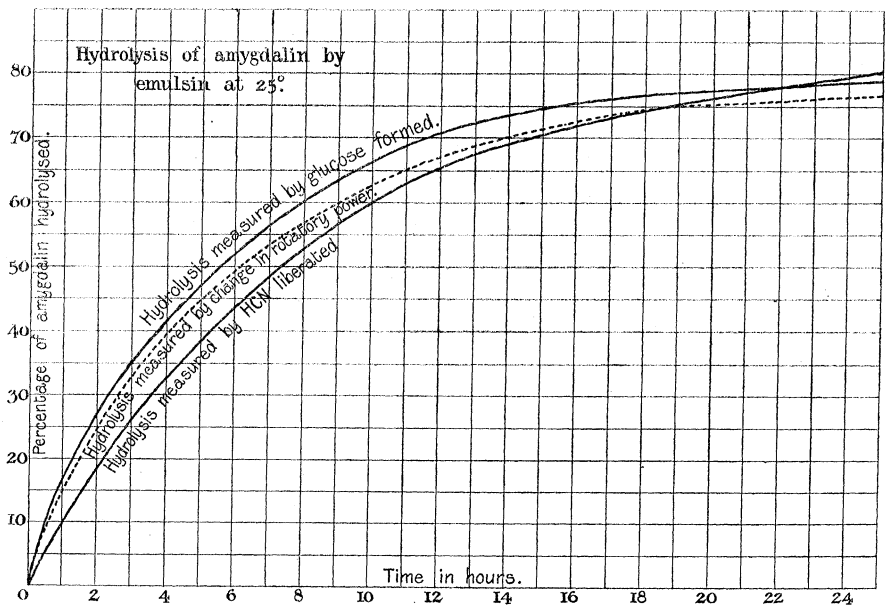
In numerous experiments in which the proportions of reducing sugar and

* 'Chem. Soc. Trans.,' 1897, vol. 71, p. 281.

hydrogen cyanide formed on digesting amygdalin with emulsin have been determined, the extent to which hydrolysis takes place, as measured by means of the cyanide, has always been found lower than that measured by the amount of sugar produced—assuming this to be glucose—to an extent considerably beyond the probable error affecting the determinations. The following series of percentage values may be quoted in illustration:—

Time.	Rotatory power.	Hydrogen cyanide.	Glucose.
hours.			
1	14·6	9·9	16·7
3	33·0	26·7	35·5
5	44·7	38·6	46·1
7	53·7	48·5	56·7
9	59·9	56·9	63·9
11	65·5	63·3	69·0
25	76·8	80·7	79·1

The experiment is one of the few in which it was found to be possible to determine the hydrogen cyanide by direct titration. It will be seen that the difference increases as the action proceeds, and then diminishes; the change indicated by the variation in rotatory power also rises to a maximum, and then diminishes. The relationship is more obvious in the following diagram.



The following values were obtained in an experiment in which the precipitation method was used in determining the cyanide:—

Time.	Glucose.	HCN.
hours.		
2	38·3	31·0
3	48·0	41·0
4	54·4	49·5
5	60·8	55·75
6	65·6	61·25
24	94·5	93·0

An emulsin which had been heated during three hours at 55°, although weaker, produced a smaller proportion of cyanide as compared with that of glucose than the unheated extract—

Time.	Glucose.	HCN.
hours.		
17·5	77·6	68·8
40·5	89·0	74·5

The amount of Fischer's glucoside we have separated from partially hydrolysed material has been small—at most 3 or 4 per cent. of the weight of the amygdalin used; apparently there is no great difference between the rates at which this glucoside and amygdalin are hydrolysed by the β-glucase. In some cases we have even failed to isolate a crystalline product and have had reason to suspect that the glucoside has been in part converted during its manipulation into the stereoisomeride.