LXXXIX.—*The Fermentation of the Indigo-plant.*

By CYRIL BERGTHEIL.

THE fermentation which takes place when the indigo-plant is steeped in water in order to extract the dye has been the subject of several investigations, of which the following appear to be the most important.

In 1887, Alvarez described an organism under the name *Bacillus indigogenus*, which he isolated from an infusion of indigo-plant, and to which he ascribed the power of producing the fermentation (*Compt. rend.*, 1887, **115**, 286).

In 1896, C. J. van Lookeren and P. J. van der Veen expressed the opinion that the fermentation was due to an enzyme and not to a bacterium; but they neither succeeded in separating the enzyme nor in satisfactorily demonstrating its existence (*Landw. Versuchs-Stat.*, 1896, **46**, 249—288).

In 1898, Bréaudat, working with *Isatis alpina*, supported the latter view, and brought evidence to show that the production of indigo from that plant was due to the action of a hydrolysing enzyme on a glucoside contained in the plant. He also concluded that the oxidation of the substance produced by the hydrolytic cleavage of the glucoside, by which indigo is obtained, was brought about under the influence of an oxidase (*Compt. rend.*, 1898, **127**, 769).

In view of the important bearing of a thorough understanding of the fermentation process on the manufacture of indigo, it became necessary to re-examine the matter in some detail, and the work now described was carried out in India during the years 1902—1903 with that end in view.

Two specimens of *Indigofera* were used for the investigation—the

plant commonly grown in Bihar for indigo manufacture, and known until lately as *Ind. tinctoria*, but now classified by Prain as *Ind. Sumatrana*, and the Java or Natal variety, *Ind. erecta*. The former cannot be satisfactorily grown in Bihar during the cold weather, and the latter was therefore used. The two species were found to behave in exactly the same manner in all the comparisons made, the only difference being that more indigotin per unit weight of leaf can be obtained from *Ind. erecta* than from the ordinary plant. Only the leaves of the plant were used, since it is well known that practically no indigotin is obtainable from the stems or other parts.

On infusing the leaves in water at temperatures below 80°, the latter acquires a yellow colour with a green fluorescence, the time taken for this to set in varying with the temperature of the water used; simultaneously with the appearance of this colour, the infusion is found capable of giving a precipitate of indigo on being agitated with air. If the extract is made in boiling water, a clear solution is obtained, varying with concentration from light yellow to dark reddish-brown, which is incapable of giving indigo by agitation with air, but does so by the combined action of an acid and an oxidising agent. It is clear, therefore, that a different substance is extracted in the two cases, and it seemed probable that the substance obtained in the former case was derived from that obtained in the latter by fermentation.

Since the extract capable of giving indigo by agitation with air is obtained when the plant is infused in hot water and also in the presence of antiseptics, it seemed exceedingly improbable that the fermentation could depend on bacterial action. Nevertheless, the matter was first investigated from a bacteriological point of view, since it was found that the steeping vat was undoubtedly characterised by great bacterial activity, and many planters were of the opinion that bacterial action was essential to indigo production.

Bacteriological Investigation.

A sterile extract of the indigo-plant was made as follows. Forty grams of leaves were thrown into about 10 times their weight of briskly boiling water, and after boiling for 2—3 minutes the leaves were strained off, the extract poured into sterile test-tubes, and heated in an autoclave at 120° for three-quarters of an hour. After cooling, the tubes were kept at 32° for 24 hours and a subculture then made from one of them into a sterile tube containing nutrient broth, which was incubated at 32° for 24 hours. The broth remained perfectly bright and clear, and microscopic examination of a film prepared from it showed no micro-organisms. The extract of the indigo-plant was therefore sterile. The extract became somewhat darker after sterilisation and

a slight brown precipitate formed; it could, however, be kept for several months in tubes plugged with cotton-wool without undergoing any further alteration, and was found to retain the power of giving indigo by the combined action of an acid and an oxidising agent. It was observed that a tube left exposed to the air gradually assumed the yellowish-green fluorescence characteristic of the fermented extract, and acquired the power of forming indigo on agitation with air. This points to the existence of aërial organisms capable of producing the fermentation, and a microscopic examination of a film from a tube thus exposed was found to show the presence of bacteria of all descriptions. No organism having the specific property of producing indigo fermentation was, however, isolated from such a tube.

Forty grams of indigo leaves were completely steeped in water at about 30° and left for 12 hours. At the end of this time, the infusion was found to have assumed the characteristic greenish-yellow colour and to give copious indigo on atmospheric oxidation. A microscopic examination of a hanging drop from this infusion showed it to contain numerous kinds of bacteria in great quantity, and a film prepared from the same infusion showed many varieties of organisms, but a short bacillus with rounded ends, occurring frequently in pairs and in short chains surrounded by a capsule, seemed to predominate. A subculture was made from this infusion into one of the tubes of sterile indigo extract and incubated at 32°. After 20 hours, the colour of the extract in this tube had changed to yellowish-green and a scum of indigo had formed on the surface. A microscopic examination of a film from this tube showed similar organisms to that from the infusion with which it had been seeded.

It was thus shown that it is possible to set up fermentation in a sterilised extract of the indigo-plant by a very small amount of liquid transferred from an infusion of the plant in cold water. It was, however, possible that a small amount of an enzyme had been transferred to the sterile extract in the drop from the fermenting infusion, and that the bacteria were playing no essential part. To obviate this possibility, a subculture was made from a fermenting infusion into broth. The broth tube was incubated at 32° for 24 hours, after which it was very turbid; a subculture was then made into a second broth tube, which was incubated in its turn. Nutrient agar-agar plates were now made from this culture. Three distinct types of colonies were obtained, but semi-transparent surface colonies with a moist appearance and outline varying from circular to amœboid forms predominated. Microscopic examination of films from these moist colonies showed them to consist of the bacillus previously observed as predominating in infusions of indigo-plant. Subcultures were made from two of these colonies and from one of each of the other two

distinct types of colonies into sterile tubes of indigo extract which were incubated at 32° for 48 hours. None of them acquired the power of forming indigo by atmospheric oxidation. It seemed, therefore, that the fermentation produced in a sterile tube of extract when seeded direct from a fermenting infusion was due to a small amount of enzyme transferred, and not to micro-organisms.

It was thought possible, however, that organisms capable of producing the fermentation in the original infusion lost their power of so doing by prolonged culture in artificial media. To test this point nutrient agar-agar plates were made directly from an indigo infusion. Colonies of the same bacterium as that previously observed were again found to predominate, and on seeding from one of these directly into indigo extract, fermentation was found to have set up after 8 hours' incubation. Tubes of extract seeded from three other types of colony, which were found on microscopic examination to consist of organisms other than the predominant one, also showed slight fermentation after 14 hours. It was impossible that any of these fermentations could be caused by transferred enzyme, since only a minute portion of the growth from the surface of the colonies was taken in each case. On making repeated subcultures from a tube infected in the foregoing manner through a series of fresh tubes of extract, fermentation was always found to take place.

It is clear, therefore, that at least one organism capable of producing indigo fermentation is invariably found in large quantities in an infusion of the plant, and that it loses its power of so doing by continued culture in artificial media. The organism is a bacillus corresponding very closely with the description given by Alvarez of his *Bacillus indigogenus*, and is no doubt identical with it. It is probable that several bacteria occurring in indigo infusions are capable of producing the fermentation, and, from the fact that fermentation may be set up spontaneously in a sterile extract exposed to air, it is clear that there are also aërial organisms capable of doing so.

The Indigo Enzyme.

It was evident from the commencement of the investigation that the indigo fermentation could not be explained by bacterial action alone. The fact of its taking place in the presence of antiseptics strong enough to destroy bacterial life, and at a temperature equally destructive thereof, militated strongly against such an assumption, whilst the fact that the fermentation took place much more rapidly at a high temperature than at a low one seemed to indicate so strongly the presence of an enzyme that further bacteriological investigation

was abandoned, and attempts were made to prove the presence of an unorganised ferment.

A quantity of indigo leaves was reduced to pulp in a mortar, covered with water containing a little chloroform, and left in the incubator at 32° for 12 hours. At the end of this time, a scum of indigo had formed on the surface of the liquid. The pulp was squeezed through cloth and the turbid green liquid thus obtained was divided into two parts. The first part was treated with an equal volume of alcohol (sp. gr. 0·810), which produced a greenish-blue, flocculent precipitate and left the liquid clear and reddish-brown. The precipitate (ppt. A) was collected, washed with dilute alcohol, and transferred to a volume of water slightly less than half that originally taken for extraction; the mixture was shaken vigorously for an hour and then filtered, a clear yellow solution being thus obtained (sol. A). The filtrate from ppt. A was again treated with an equal volume of alcohol; this produced a white, gelatinous precipitate (ppt. B), which was collected and treated in a similar manner to ppt. A, giving a light yellow solution (sol. B). The second part of the original infusion was heated at 60—70° for an hour, when the greater part of the proteids present were thus coagulated and settled out as a heavy, gelatinous precipitate (ppt. C), leaving a clear reddish-brown liquid. The precipitate was collected, shaken up with water as before, and filtered, giving a yellow solution (sol. C). The filtrate from ppt. C was treated with an equal volume of alcohol, which produced a white, gelatinous precipitate (ppt. D). This was filtered off and treated in the same way as the previous precipitates, giving a light yellow solution (sol. D).

The fermentative power of the solutions so obtained was tested on an extract of indigo-plant prepared in the manner described in the bacteriological portion of this paper, except that the sterilisation in the autoclave was dispensed with, the extract being boiled for about ten minutes after straining from the leaves in order to destroy any possible enzyme or bacteria present, and used as soon as it was cold. Five c.c. of such an extract were poured into each of four test-tubes, and the same volume of one of the solutions to be tested was added to each. A fifth tube was prepared containing 5 c.c. of extract and 5 c.c. of water previously boiled and cooled. All were kept at 32° for 12 hours, at the end of which time the tube containing sol. A was strongly fermented and gave a large amount of indigo on shaking up with air. The other tubes were quite unaltered. The same experiments were now repeated, keeping the tubes at 40—50°; at this temperature, the change from brown to greenish-yellow took place in the case of sol. A in a very few minutes, and after an hour fermentation was far advanced. The remaining solutions had produced no perceptible change in the extract. It was found that if the sol. A

were boiled before being added to the extract it entirely lost its power of fermenting the latter.

It was evident, therefore, that there is a substance contained in the leaf of the indigo-plant which is soluble in cold water and can produce the indigo fermentation. Further, that this substance is precipitated on the addition of about 45 per cent. of alcohol to its aqueous solution, and is destroyed by heating at 100° in the presence of water; it also appears to be destroyed, or so far coagulated as to be insoluble in water, by heating its aqueous solution at 70°. It is apparently as much proteid in nature as the enzymes hitherto investigated, and, by reason of its power of producing fermentative change in the indigo complex occurring in the plant, should be classed among these substances.

The course of the action of this enzymic fermentation was studied in some detail, not only because it might possibly lead to improvements in the method of steeping used in indigo manufacture, but also because it had some theoretical interest. The change taking place under the influence of the enzyme is referred to in this paper as the "fermentation" for lack of a general term to distinguish enzyme action from fermentation under the influence of living organisms.

In the first place, attempts were made to obtain the enzyme in a more active form than in the foregoing sol. A. A dirty-white substance almost entirely soluble in water was obtained from this solution by precipitating with an equal volume of alcohol and drying the precipitate over sulphuric acid under diminished pressure. The solution was made up to the same volume as that from which the precipitate had been derived, and the comparative strengths of the solution thus obtained and of the original solution were determined by adding 5 c.c. of each to 5 c.c. of plant extract and keeping both at 40—50°. It was found that the colour change indicating the beginning of fermentation occurred much sooner in the case of the original solution than in that of the solution obtained by redissolving the precipitate. The enzyme, therefore, loses strength by the latter process. A second attempt to obtain a more active product was made by forming a precipitate of calcium phosphate in sol. A by adding a dilute solution of sodium phosphate followed by a solution of calcium chloride, and drying the precipitate over sulphuric acid under diminished pressure. The substance so obtained was extracted with water and the activity of the extract tested as described above; it was found to be considerably less than that of the original sol. A. It seemed, therefore, that the enzyme could not be obtained in a more active form than in sol. A by a process of reprecipitation, and it was ultimately decided to work with sol. A without attempting any process of purification. Such a solution of enzyme was accordingly used in all the earlier experiments, and was

found to be perfectly satisfactory so long as the tests were carried out on small quantities of extract, and colorimetric comparisons depended on to obtain the various data. However, in experiments in which larger quantities of extract were used and the indigotin obtained estimated volumetrically, it was found to be too weak to give sufficient indigotin for estimation in a reasonable period of time. It seemed probable that the enzyme was difficult of extraction owing to the presence of tannin in the leaves. This difficulty has been encountered by several investigators who have worked on enzymes occurring in foliage leaves; notably by Brown and Morris in dealing with diastase (Trans., 1893, 63, 604), and more recently by Mann, working on an enzyme occurring in the tea-leaf ("The Ferment of the Tea-leaf," 1901, and Abstr., 1903, ii, 388). Mann found that by pounding the leaves with hide-powder the tannin was fixed, and the enzyme could then be easily extracted in water.

This method was tried with indigo-leaves and gave very gratifying results, the solution obtained when the leaves were pounded with hide-powder before extraction being very much more active than that obtained when this substance was not used. Accordingly, in all the later experiments the enzyme solution was prepared as follows: the leaves were pounded to a pulp, the pulp mixed intimately with about a third of its weight of hide-powder, and the mass extracted with sufficient water containing a little chloroform to cover it for 12 hours. At the end of this time, the liquid was squeezed out of the pulp through cloth, and the enzyme precipitated with an equal volume of alcohol (sp. gr. 0·810). The precipitate was collected, washed with dilute alcohol, and transferred to a mixture of chloroform and water, with which it was shaken vigorously for an hour, allowed to remain for some hours, again thoroughly agitated, and finally filtered. A very active solution of enzyme could be obtained in this manner, and it was found that by adding a few drops of chloroform such a solution could be kept for several weeks without perceptibly losing strength.

Several attempts were made to obtain the substance yielding indigo, as it occurs in the plant, in a state of purity. This substance would seem to be extracted by steeping the plant in boiling water, since the extract on fermentation gives a solution having the same properties as that obtained by fermenting the plant direct by steeping it in cold water. The extract made in boiling water has only a slight action on Fehling's solution or phenylhydrazine, but the precipitation of indigotin from the extract, either by fermentation and atmospheric oxidation or by the action of an acid and an oxidising agent, is invariably accompanied by the production of a sugar, the presence of which in the solution may be shown by its reducing action on Fehling's solution and its power of forming an osazone with phenylhydrazine. The action

of an acid alone also leads to the production of a reducing sugar, although, in the absence of an oxidising agent, only a small quantity of indigotin is precipitated.

This indicates the derivation of indigotin from a glucoside, but numerous attempts to obtain a pure substance from the extract in boiling water all resulted in failure. The substance occurring in the plants dealt with appears to be much more stable than indican under the influence of heat (compare Schunck, *Mem. Manchester Phil. Soc.*, 1855, **12**, 177), since the hot water extract can be evaporated to a sticky, resinous consistency, and the residue dissolved in water repeatedly, without the solution losing its properties of giving indigotin either by fermentation or by the action of an acid and an oxidising agent. Indican, as described by Schunck, is very unstable under these conditions and would entirely decompose. It appears to be impossible, however, to obtain crystalline indican from the extract in the manner described by Hoogerwerf and ter Muelen (*Proc. K. Akad. Wetensch. Amsterdam*, 1900, **2**, 520). It was eventually decided to carry on fermentation experiments with the crude extract of the plant in boiling water. This course also had the advantage that the fermentations were carried on under conditions more nearly resembling those which obtain in the steeping-vat used in manufacture than if a purified product were employed.

Comparison of the Quantity of Indigotin obtained from an Extract of the Plant by the Action of an Acid and an Oxidising Agent, and that obtained by Fermentation.

With the view of estimating the total amount of indigotin obtainable from an extract of the plant by fermentation, it seemed advisable to ascertain whether the amount corresponded with that obtained from the same extract by the action of an acid and an oxidising agent, since, if an agreement could be shown to exist, the latter would provide a much more rapid method of arriving at the required result than that of carrying the fermentation to a conclusion and determining the indigotin after oxidation with air.

The best method for precipitating indigotin from an extract of the plant is that devised by C. Rawson in 1901 for the analysis of indigo-yielding plants. The extract is made strongly acid with hydrochloric acid, and a solution of ammonium persulphate is gradually added; the indigotin is precipitated in a finely crystalline form, and may be quantitatively estimated by collecting in an asbestos filter, washing the precipitate with boiling water, and, after thoroughly drying the filter with its adherent precipitate at a temperature not exceeding 70°, dissolving the precipitate in concentrated sulphuric acid. The solution

of indigotindisulphonic acid thus obtained is then diluted, cleared by the addition of barium chloride, and a known volume titrated with a standard permanganate solution in the manner described by Rawson (*J. Soc. Dyers and Colourists*, 1885, 1, 74; *J. Soc. Chem. Ind.*, 1899, 18, 251). This method of precipitating and estimating indigotin from an extract was frequently used in the course of the work, and will be referred to as the "persulphuric acid method."

The following method was employed for estimating the total indigotin obtainable from an extract by fermentation. The extract was made in boiling water in the manner already described, and, after boiling thoroughly for some minutes, was allowed to cool in a flask plugged with cotton-wool. About 5 oz. of leaves were used to a litre of water, and these relative quantities were adhered to throughout the investigation. A fresh extract was made for each set of determinations, since it was found that there was always a risk of premature fermentation being set up by aërial organisms when once the flask containing the extract had been unplugged.

Known volumes of the cold extract and an enzyme solution were mixed, the quantities of each being varied according to the number of determinations to be made and the activity of the enzyme solution used. The mixture was allowed to remain in a flask plugged with cotton-wool at the temperature of the air, and a small sample was taken out from time to time and the progress of the action checked by adding a drop of dilute ammonia; this reagent stopped further fermentation, and also considerably accelerated subsequent oxidation. Complete precipitation of all the indigotin obtainable by atmospheric oxidation was insured by shaking the test-tube containing the sample for five minutes. The precipitate was filtered off, the clear yellow filtrate was made acid with hydrochloric acid, and a few drops of ammonium persulphate solution were added, which precipitated the indigotin from the unfermented extract, producing a coloration varying from deep blue to pale green if the action was not finished, whilst the solution remained a clear yellow if fermentation was complete. No difficulty was experienced in bringing the action to completion in a reasonable period of time, providing that sufficient enzyme was added.

When the above test showed that fermentation was complete, one or more samples of known volume were taken from the fermented extract and, after adding a few drops of ammonia, were oxidised by shaking violently for ten minutes or so, the vessel containing the sample being opened from time to time to admit a fresh supply of air. The solution was then rendered strongly acid, boiled, and the precipitate collected on asbestos and the indigotin estimated in the same way as in the persulphuric acid method.

The following are details of the experiments the results of which,

expressed in indigotin obtained from 100 c.c. of extract, are summarised in the annexed table.

Experiment A.—The quantity of indigotin obtainable from two portions of extract of 100 c.c. each was determined by the persulphuric acid method (I and II); 220 c.c. of the same extract were fermented with 330 c.c. of a dilute enzyme solution, and fermentation being complete after 6 hours, two portions of 250 c.c. each were withdrawn, oxidised, and the indigotin estimated (III and IV).

Experiment B.—The indigotin obtainable from two portions of extract of 100 c.c. each was estimated by the persulphuric acid method (I and II); 325 c.c. of the same extract were fermented with 325 c.c. of an active enzyme solution and fermentation was complete in 1 hour. Two portions of 200 c.c. each were then withdrawn, oxidised, and the indigotin estimated (III and IV). The remainder of the fermented extract was then allowed to remain, and a third portion of 200 c.c. withdrawn after 20 hours from the start. This was oxidised and the indigotin determined as before (V).

Experiment C.—The indigotin from 180 c.c. of extract was determined by the persulphuric acid method (I); 900 c.c. of the same extract were fermented with 100 c.c. of enzyme solution and, after 20 hours, fermentation was complete. A portion of 200 c.c. was withdrawn, oxidised, and indigotin determined (II). The remainder was allowed to remain, and after 36 hours from the start a third portion of 200 c.c. was withdrawn, the indigotin obtained by oxidation being then estimated.

	Persulphuric acid method.		Fermentation.	
Experiment A...	I.	0·0840 gram.	III.	0·1016 gram.
	II.	0·0847 ,,	IV.	0·1008 ,,
Experiment B...	I.	0·044 ,,	III.	0·0697 ,,
	II.	0·0427 ,,	IV.	0·0690 ,,
			V.	0·0292 ,,
Experiment C...	I.	0·079 ,,	II.	0·096 ,,
			III.	0·0547 ,,

Thus there is invariably more indigotin obtained by fermentation than by chemical precipitation, so that a measure of the total amount of indigotin obtainable from an extract by fermentation cannot be arrived at in the latter manner.

It is to be noted, by a comparison of the figures obtained in No. V of Experiment *B* with Nos. III and IV of the same experiment, that a decrease in the amount of indigotin obtainable by atmospheric oxidation takes place if the fermented solution is allowed to remain after fermentation is complete. The same fact is illustrated by a com-

parison of Nos. III and II of Experiment *C*. The substance produced by fermentation seems to be unstable and to undergo some slow change whereby it loses the power of forming indigotin on oxidation. The fact that a loss of indigotin occurs if the plant is steeped too long in the manufacture is doubtless due to this circumstance.

The Progress of the Action with Time.

It has been shown by Adrian Brown in the case of invertase (Trans., 1902, **81**, 273), and by Horace Brown and Glendinning in the case of diastase (Trans., 1902, **81**, 388), that in solutions fermenting under the action of these enzymes a direct proportionality exists between the duration of the action and the quantity of fermentable substance transformed until a certain stage in the course of the action is reached, after which the proportionality ceases to hold.

It seemed of interest to ascertain whether such a proportionality could be found to exist in the course of the fermentation under consideration, and, if so, at what stage of the action it ceased.

As a preliminary to these and some of the succeeding experiments, it was necessary to show that boiling the indigo extract in the presence of dilute ammonia did not lead to the formation of indigotin or any substance insoluble in boiling water which would decolorise permanganate, and so introduce an error into the estimation of indigotin. An extract (200 c.c.) when boiled for an hour with 1 c.c. of dilute ammonia deepened in colour, but gave neither indigotin nor other precipitate.

Accordingly, the following method was pursued. A known volume of enzyme solution was added to a known volume of the plant extract and the mixture allowed to remain in a flask plugged with cotton-wool at about 30°, and samples of known volume were withdrawn from the flask at equal intervals of time, the amount being decreased as fermentation progressed, since in the more advanced stages sufficient indigotin for an accurate estimation could be obtained from a smaller sample than in the earlier stages. After adding 1 c.c. of dilute ammonia, the samples were oxidised with air, boiled, and the precipitate collected on asbestos, washed with boiling water, and the indigotin estimated as before.

The total amount of indigotin obtainable from the extract by fermentation was determined in a separate portion in the manner described under the last heading.

Experiment A.—A little more than 1800 c.c. of extract was fermented with 200 c.c. of enzyme solution. Samples were withdrawn at successive intervals of 45 minutes, the volumes taken being as follows: I. 500 c.c., II. 400 c.c., III. 300 c.c., IV. 300 c.c., V. 200 c.c.,

VI. 200 c.c. Ammonia was added, the sample oxidised, and the indigotin determined; 100 c.c. of extract were fermented separately to give the total indigotin obtainable.

Experiment B.—Nine hundred c.c. of extract were fermented with 150 c.c. of enzyme solution. The first sample was withdrawn after $3\frac{1}{2}$ hours and subsequent samples at intervals of one hour, the volumes taken being as follows: I. 300 c.c., II. 300 c.c., III. 200 c.c., IV. 200 c.c.; 100 c.c. of extract were fermented separately to determine the total indigotin obtainable.

The results of the preceding experiments are summarised in the following table:

Grams of indigotin obtained from 100 c.c. of extract.

No. of sample.....	I.	II.	III.	IV.	V.	VI.	Total obtainable.
Experiment A. ...	0·006	0·0104	0·015	0·0197	0·0235	0·0256	0·1175
Differences		0·0044	0·0046	0·0047	0·0038	0·0021	
Experiment B. ...	0·0105	0·014		0·0175	0·0192	—	0·102
Differences		0·0035		0·0035	0·0017		

The figures show that a proportionality between the duration of the action and the quantity of substance transformed exists in the early stages of the action, but that this relationship ceases to hold in experiment *A*, when a quantity between 0·0197/0·1175 and 0·0235/0·1175 (that is, between 16·8 and 20 per cent.) of the total action has taken place, and in experiment *B*, between the limits 0·0175/0·102 and 0·0192/0·102 (or 17·1 and 18·8 per cent.). Thus the proportionality between duration of action and the quantity of substance transformed holds until 17·1 to 20 per cent. of the total action has taken place.

The Influence of the Quantity of Acting Enzyme.

The following experiments were carried out in order to determine if a relation similar to the foregoing could be found to exist between the quantity of acting enzyme and that of the substance transformed.

Experiment A.—Known volumes of enzyme solution were added to each of 4 portions of extract of known volume, the relative quantities being so arranged that the quantities of enzyme acting on the unit volume of extract in the four experiments were in the ratio of $1 : 1\frac{1}{2} : 3 : 6$. The total volume was made the same in each case by adding water and the action allowed to proceed at the temperature of the air for 5 hours. At the end of this time, the fermented extracts were rendered ammoniacal, oxidised, and the indigotin estimated.

Experiment B.—The ratio between the volumes of acting enzyme solution and of extract were the same as in experiment *A*; the respective quantities and the total volumes were, however, decreased.

The action was allowed to proceed for $3\frac{3}{4}$ hours at the temperature of the air, after which the samples were oxidised and the indigotin estimated as before.

The total indigotin obtainable from 100 c.c. of extract was estimated in each experiment in the manner previously described.

The following table gives the details and results of the experiments:

Number of sample.	Extract.	Enzyme solution.	Water.	Indigotin obtained from 100 c.c. of extract.	Ratios between quantities of enzyme acting on the same vol. of extract.	Ratios between quantities of indigotin obtained from the same vol. of extract.	Total indigotin obtainable from 100 c.c. of extract.
				Experiment A.			
I.	450 c.c.	75 c.c.	0	0·011 gram	} 1 : 1·5	1 : 1·5	
II.	360 ,,	90 ,,	75 c.c.	0·016 ,,	} 1 : 2	1 : 1·6	0·098 gram
III.	270 ,,	135 ,,	120 ,,	0 026 ,,	} 1 : 2	1 : 1·15	
IV.	180 ,,	180 ,,	165 ,,	0·030 ,,			
				Experiment B.			
I.	360 c.c.	60 c.c.	0	0·008 gram	} 1 : 1·5	1 : 1·5	
II.	288 ,,	72 ,,	60 c.c.	0·012 ,,	} 1 : 2	1 : 1·9	0·1125 gram
III.	216 ,,	108 ,,	96 ,,	0·023 ,,	} 1 : 2	1 : 1·6	
IV.	144 ,,	144 ,,	132 ,,	0·037 ,,			

Thus, in both experiments the amounts of indigotin formed from unit volume of extract are proportional to the amounts of acting enzyme in samples I and II, but not in samples III and IV. So that the proportionality ceases to hold in experiment *A* when between 0·016/0·098 and 0·026/0·098 (that is, between 16·3 and 26·5 per cent.) of the total fermentation has taken place, and in experiment *B* between the limits 0·012/0·1125 and 0·023/0·1125 (that is, between 10·6 and 20·5 per cent.). By combining these two results, it is seen that the stage at which the proportionality ceases is reached when between 16·3 and 20·5 per cent. of the total fermentation is complete. This practically coincides with the point at which the proportionality between the time of action and quantity of indigotin formed was found not to hold.

The Influence of Temperature.

Difficulty was experienced in carrying out temperature experiments owing to the lack of gas and the consequent impossibility of making

use of a thermostat. The following determinations could therefore be made only with a degree of accuracy which was limited by the fluctuation of temperature in vessels placed on a water-bath heated by a spirit burner.

Determination of the Optimum Temperature.

In the preliminary experiments, which were carried out in test-tubes, several tubes were prepared containing 4 c.c. of plant extract, and three or four were placed in each of three beakers of water placed on a water-bath, which was heated as regularly as possible by a spirit burner. The beakers were separated from the bath by blocks of wood of varying thickness, according to the temperature required. Before the tubes were put in, the temperature of the water in the beakers was watched until a maximum was reached, which was kept as constant as possible. When the contents of the tubes had arrived at the same temperature as the surrounding water, 2 c.c. of a dilute enzyme solution (prepared without using hide-powder) was added to each tube, the time at which the enzyme was added and the temperature of the baths throughout the experiments being noted. An attempt was first made to obtain comparative measurements of the advance of fermentation by taking a tube from each bath after equal periods of fermentation, adding the same amount of ammonia to each, oxidising with air under the same conditions, and comparing the depth of the blue colour obtained in each case. It seemed probable that the optimum temperature for the action was higher than 40°, but it was found that above that temperature the depth of colour produced in this way was inversely proportional to the temperature after the fermentation had proceeded for a few hours; the effect of keeping tubes of fermented extract at temperatures above 40° for a few hours was accordingly tried, and it was found that the solution lost its power of forming indigotin on oxidation with air more or less quickly according to the degree of temperature at which it was kept. Evidently, therefore, the substance resulting from fermentation undergoes a more or less rapid change at an elevated temperature whereby it is no longer capable of giving indigotin on oxidation with air. The proposed method had therefore to be abandoned.

The effect of keeping tubes of dilute unfermented extract at temperatures above 40° for several hours was now tried, and it was found that no change in the quantity of indigotin which could be obtained by precipitation by the persulphuric acid method was apparent, however high the temperature. This was tested both by colorimetric comparisons and volumetric estimations of the indigotin precipitated from equal volumes of the same extract, which had been

kept at various temperatures. Accordingly the method devised for determining the point at which a fermentation is complete (p. 878) was used, and it was found that the depths of blue produced on adding the same quantities of hydrochloric acid and ammonium persulphate to the filtrates from the atmosphere oxidations were inversely proportional to the quantity of substance fermented. Care was taken that both the atmospheric oxidation and that of the filtrate with persulphuric acid were carried out under identical conditions in every case, and tubes were compared from each bath at equal intervals of time until one of them showed that the fermentation was complete.

The following table shows the temperatures between which the baths fluctuated in each experiment, the one in which the fermentation proceeded most rapidly being printed in bold figures in each case. The fourth column shows the limits between which the optimum temperature could be placed as a result of each experiment coupled with the results of those which had been made previously.

	Bath I.	Bath II.	Bath III.	Limits between which the optimum temperature lies.
Experiment A.....	32° (incubator)	40—45°	**50—55°**	45° upwards
Experiment B.....	40—45°	**50—55**	60—65	45—65°
Experiment C.....	42—49	**49—57**	60—63	49—63
Experiment D.....	**48·5—50**	55—56	59—63	49—56

Thus, the optimum temperature lies betweeen 49° and 56°.

Attempts were made to confirm this result by volumetric determinations of indigotin. It was thought improbable that correct results could be arrived at by estimations of the indigotin formed by atmospheric oxidation of the extracts fermented at temperatures above 40°, owing to the decomposition which the fermented extract had been found to undergo at such temperatures.

It seemed probable, however, that the required comparisons might be obtained by acting on the filtrates from the indigotin, derived by atmospheric oxidation of the fermented solutions, with hydrochloric and persulphuric acids under identical conditions. In the following experiments, the indigotin obtained in both ways was estimated.

Flasks containing 200 c.c. of plant extract were supported on wooden blocks on a heated water-bath in the same way as the beakers used in the previous set of experiments. When the temperature of the liquid in the flasks had reached a maximum and was fairly constant, 100 c.c. of an enzyme solution was added to each. The temperatures of the liquids were noted throughout the experiment, and at the end of five hours they were cooled rapidly, 5 c.c. of dilute ammonia added, and oxidised by shaking violently for 10 minutes. The solutions were

then neutralised exactly with hydrochloric acid, boiled, and the precipitate filtered off through asbestos, washed thoroughly with hot water, and the indigotin determined as in the previous experiments. The indigotin was precipitated from the filtrates by the persulphuric acid method, care being taken that it was carried out in exactly the same way in each case. Under these conditions the results are strictly comparable, although they do not correspond with the results obtained by fermenting the solutions and oxidising with air.

The following are the details of the experiments:

Experiment E.—The limits of temperature in the two flasks were: Flask I, 43—46°. Flask II, 49—51°.

The results of the indigotin estimations calculated on 100 c.c. of plant extract were:

	Flask I.	Flask II.
By fermentation and atmospheric oxidation.....	0·0069 gram	0·0084 gram
By the persulphuric acid method with the filtrate.....	0·045 „	0·038 „
Sum of the two estimations	0·0519 „	0·0464 „

Thus, the superiority of the higher temperature for fermentation is shown both by the relation between the quantities of indigotin produced by oxidising the fermented solutions and that between the quantities obtained by precipitating from the filtrates by the persulphuric acid method. Hence, the optimum temperature is above 46°.

Experiment F.—The limits of temperature in the three flasks were: Flask I, 49—51°. Flask II, 54—57°. Flask III, 57—59°.

The results of the indigotin estimations calculated as before were

	Flask I.	Flask II.	Flask III.
By fermentation and atmospheric oxidation	0·038 gram	0·035 gram	0·031 gram
By the persulphuric acid method with the filtrate	0·0152 „	0·0160 „	0·0176 „
Sum of the two estimations	0·0532 „	0·0510 „	0·0486 ,

Here, again, the comparative rates of fermentation under the three different conditions of temperature are shown both by the ratio between the quantities of indigotin formed by fermentation and atmospheric oxidation, and by the inverse ratio between the quantities derived from the filtrates by the persulphuric acid method. The conditions which obtained in flask I were clearly the best for fermentation. The optimum temperature is thus below 57°; it lies, therefore, between 46° and 57°.

The sum of the two indigotin estimations is recorded in each case in

order to illustrate the decrease in the total with rise in temperature. This indicates that more of the substance resulting from fermentation is destroyed the higher the temperature, and confirms the conclusion deduced in the preliminary temperature experiments. It is clear, however, that the decomposition is not sufficiently rapid to affect the relation between the estimations of indigotin obtained directly by oxidation of fermented solutions, provided that sufficient enzyme is added to produce sufficient indigotin for volumetric estimation in a moderately short time.

Accordingly, in the following two experiments, the indigotin was not precipitated from the filtrates from the oxidised solutions. The fermented solutions were oxidised with air in the presence of ammonia as before, but were not neutralised before boiling and filtering off the indigotin; 300 c.c. of extract were taken in each case and 10 c.c. of a strong enzyme solution added. The fermentations were allowed to proceed for $2\frac{1}{2}$ hours. The following table gives the details and results of the experiments, the quantity of indigotin being calculated on 100 c.c. of extract in each case, and the last column showing the limits between which it is possible to place the optimum temperature as a deduction from each experiment, coupled with the results already obtained in the same manner.

		Limits of temperature fluctuation.	Indigotin obtained from 100 c.c. of extract.	Limits between which the optimum temperature lies.
Experiment G	Flask I.	47—48°	0·0252 gram	46—53°
	Flask II.	52—53	0·0237 ,,	
Experiment H	Flask I.	47—48	0·0242 ,,	46—51
	Flask II.	50—51	0·0222 ,,	

Thus, the optimum temperature is found to lie between 46° and 51°. This temperature could not be determined more closely with the apparatus available. The volumetric determinations confirm the result arrived at in the preliminary experiments, and by coupling the conclusions arrived at by the two methods, the optimum temperature is found to lie between 49° and 51°. That is, it is very near to 50°.

Determination of the Temperature at which the Enzyme is Destroyed.

Test-tubes containing 2 c.c. of an enzyme solution were placed in a water-bath heated by a small paraffin lamp. In the first set of experiments, the lamp was so adjusted that the temperature of the water in the bath rose through about 7 degrees in 14 minutes, and a tube was withdrawn at the end of each 7 degree rise from 60° upwards. The contents of the tubes were allowed to cool, and then 4 c.c. of a plant extract added to each. After 8 hours, only the first tube with-

drawn—the temperature of which had not risen above 67°—showed the colour change indicating that fermentation had taken place, and gave a precipitate of indigo on agitation with air. The temperature at which the destruction of the enzyme takes place lies therefore between 60° and 74°.

In the second set of experiments, a tube was withdrawn at the end of each rise of 3 degrees from 59° to 74° and treated as before, a period of about 7 minutes being taken for each of these increments. In this case, all the tubes except the last withdrawn showed fermentation after 5 hours, that in the fourth (68—71°) being much inhibited. The point of destruction of the enzyme lies therefore between 68° and 74°.

Finally, the lamp used in heating the bath was so adjusted that a rise in the temperature of the water through 1 degree took 3 to $3\frac{1}{2}$ minutes, and tubes were withdrawn at the end of every degree rise from 68° to 73°. The intervals corresponding with these increments of temperature were:

Tube	I.	68—69°	3 minutes.	Tube	IV.	71—72°	$3\frac{1}{2}$ minutes.
„	II.	69—70	3	„	V.	72—73	$3\frac{1}{2}$ „
„	III.	70—71	$3\frac{1}{2}$ „				

After 6 hours, Tubes I, II, and III showed fermentation, whilst Tubes IV and V showed none; the fermentation was clearly inhibited in No. III. The temperature at which the enzyme is destroyed in neutral solution in $3\frac{1}{2}$ minutes is thus between 70° and 72°, or almost exactly 71°.

The Influence of Foreign Substances.

It was found that the influence of a foreign substance on a fermenting extract containing it could be clearly followed by comparing the course of the colour changes taking place in the solution with those in a second specimen of the same extract, fermenting under conditions differing only in the absence of the foreign substance the influence of which was to be determined. In the experiments now to be described, the change from the brown of the unchanged extract to the green of the fermented one took place in a very few minutes under normal conditions. In an experiment where no colour change was apparent in the same time, the fermentation was "inhibited," and if no change took place in an hour it was considered to be "much inhibited." If after 3 hours the colour was still unaltered, it was found that no action took place however long the mixture might be allowed to remain. In no case was the addition of a foreign substance found to accelerate the onset or course of the action.

Influence of Acids, Alkalis, and Neutral Salts.

The following substances were used in 5 per cent. solutions: hydrochloric acid, acetic acid, sodium carbonate, caustic soda, and sodium acetate.

Test-tubes were prepared containing 2 c.c. of plant extract and 2 c.c. of an enzyme solution, and 1 c.c. of one of the 5 per cent. solutions was added to each; as a check, 1 c.c. of water was added to a sixth tube, all being allowed to remain at the temperature of the air. Only the tube containing sodium acetate showed normal fermentation; the others remained unaltered. The solutions of acids and alkalis were now diluted 10 times and the experiments repeated with the diluted solutions exactly as before, so that now each tube contained 0·1 per cent. of a foreign substance. In this case, hydrochloric acid had a powerful inhibitory effect on the fermentation, and acetic acid a less powerful one, whilst both the alkalis stopped the action entirely. The acid and alkaline solutions were again diluted 10 times and the experiments repeated, each tube now containing 0·01 per cent. of the foreign substance. This resulted in a normal course of action in the case of the two acids, but inhibition in the case of the alkalis.

Finally, 0·1 per cent. solutions of the alkalis were obtained by diluting those used in the last set of experiments 5 times; 1 c.c. of each of these added to tubes prepared as before was found to have no effect on the fermentation.

The results may be summarised as follows:

Percentage quantity of foreign substance added.	Hydrochloric acid.	Acetic acid.	Sodium carbonate.	Caustic soda.	Sodium acetate.
1 per cent.	Destroyed	Destroyed	Destroyed	Destroyed	Normal
0·1 ,,	Much inhibited	Inhibited	Destroyed	Destroyed	Normal
0·01 ,,	Normal	Normal	Inhibited	Much inhibited	Normal
0·005 ,,	Normal	Normal	Normal	Normal	Normal

Thus, the alkalis have a more inhibitory effect on the action than the acids, and the caustic alkali than the carbonate. Hydrochloric acid acts more energetically than acetic acid, and sodium acetate up to 1 per cent. is without effect on the action.

To determine whether these effects of acids and alkalis on the fermentation were due to an action on the enzyme or on the glucoside, or to an actual inhibitory effect on the interaction of these substances, the following experiments were carried out.

Two tubes were prepared containing 2 c.c. of an enzyme solution and 2 c.c. of water. To one, 1 c.c. of 0·5 per cent. hydrochloric acid

was added (tube A), and to the other 1 c.c. of 0·5 per cent. caustic soda (tube B). After ten minutes, the hydrochloric acid was exactly neutralised with 0·5 per cent. caustic soda and the caustic soda with 0·5 hydrochloric acid, and then 2 c.c. of plant extract added to each. Also a tube containing 2 c.c. of plant extract and 2 c.c. of water was rendered acid (tube C), and a similar one made alkaline (tube D), and the acid and alkali neutralised after ten minutes precisely as before, 2 c.c. of enzyme solution being added to each after neutralisation. A check tube was prepared containing 2 c.c. of plant extract and 2 c.c. of enzyme solution, and made up to the same volume as the other four tubes. All were kept at the temperature of the air.

It was found that tube B fermented quite normally. In tube C, the action was much inhibited, but a slight fermentation took place after 3 hours. In tubes A and D the action was entirely stopped.

It was clear, therefore, that 0·1 per cent. of caustic soda was without effect on the enzyme, but that it was destroyed by the same quantity of hydrochloric acid, and that the plant extract was not subject to fermentation after the action of 0·1 per cent. of caustic soda, and only slightly so after the action of 0·1 per cent. of hydrochloric acid. On adding a solution of ammonium persulphate to tubes C and D (after acidifying the latter), a precipitate of indigotin was produced in each case, showing that the power of forming indigotin by chemical means is not destroyed in the plant extract by the action of the acid and alkali added, although the glucoside appears to undergo some change under their influence whereby it is no longer subject to fermentation by the enzyme.

Influence of Antiseptic Substances.

Tubes containing 2 c.c. of plant extract and 2 c.c. of an enzyme solution were prepared as before, and 1 per cent. of each of the following compounds was added: formaldehyde (commercial solution), chloral hydrate, phenol, chloroform, hydrocyanic acid, and boracic acid.

In each case, the action of the enzyme was inhibited. The comparative inhibitory powers of the substances as judged by the comparative rates at which fermentation was set up in the tubes is indicated by the order in which the names of the substances are placed in the foregoing list, formaldehyde having the greatest influence and boracic acid the least. In the cases of formaldehyde and chloral hydrate, not only did the action set in very slowly, but the compound resulting from the fermentation appeared to undergo change under their influence, the contents of the tubes becoming dark brown soon after the change to green had taken place, and giving no indigo when they were rendered

alkaline and oxidised with air. Under the influence of formaldehyde, the power of forming indigotin by the persulphuric acid method was also destroyed in the extract. This was not the case with any of the other compounds employed in these experiments. Boracic acid was almost without effect on the action.

Comparison with other Glucoside-splitting Enzymes

Emulsin and myrosin were selected as being the best known representatives of this class of enzymes, and solutions containing them were prepared as follows.

Emulsin.

Bitter almonds were divested of their outer coatings, ground as finely as possible, and extracted for 3 hours with twice their weight of cold water containing a little chloroform. The infusion was then filtered and 3 volumes of 85 per cent. alcohol added to the filtrate. The precipitate so produced was filtered off, washed with a mixture of equal volumes of alcohol and water, and then shaken vigorously with water for an hour. After allowing the bulk of the precipitate to settle, the supernatant liquid was filtered. A clear solution containing emulsin, but no amygdalin, was thus obtained.

A solution containing amygdalin was prepared by throwing finely-ground almonds into boiling water, boiling for a few minutes, and filtering. These operations were repeated, when a clear solution containing amygdalin was obtained. The emulsin was, however, entirely destroyed by the repeated boiling.

A solution containing the indigo enzyme and an extract of the indigo-plant in boiling water was prepared as before. Test-tubes containing the following mixtures were then prepared and allowed to remain at the temperature of the laboratory.

Tube I. Five c.c. amygdalin solution + 5 c.c. emulsin solution.
Tube II. ,, ,, ,, + 5 c.c. indigo enzyme solution.
Tube III. ,, ,, ,, + 5 c.c. water.
Tube IV. Five c.c. indigo extract + 5 c.c. emulsin solution.
Tube V. ,, ,, ,, + 5 c.c. indigo enzyme solution.
Tube VI. ,, ,, ,, + 5 c.c. water.

After 6 hours, the odour of benzaldehyde and hydrocyanic acid was very intense in tube I, whilst tube II was practically odourless. The contents of tube IV became slightly green, but, by comparison with tube V, it was evident that very little fermentation had taken place. On adding ammonia and shaking with air, only a slight blue colour was produced in tube IV, whilst a heavy blue precipitate separated

in tube V. The control tubes III and VI were quite unchanged. It was clear, therefore, that emulsin can produce the indigo fermentation, but is much less energetic than the enzyme prepared from the indigo-plant. It is very doubtful whether the indigo enzyme has the power of splitting up amygdalin. The characteristic odour accompanying its decomposition is not evident however long its solution remains mixed with a solution of indigo enzyme. This is contrary to Bréaudat's experience in dealing with a solution containing the enzyme which occurs in *Isatis alpina*. He found that this solution decomposes amygdalin in less than 24 hours.

These experiments were subsequently repeated with emulsin and amygdalin obtained from Kahlbaum and the foregoing results confirmed in each particular.

Myrosin.

A solution containing this enzyme was prepared from the seeds of a species of white mustard commonly grown in India (*Brassica Indica*?) in the same way as described for the preparation of a solution of emulsin from bitter almonds, and a solution containing sinigrin and no myrosin was obtained from black mustard seed in the same way as that described for the preparation of the amygdalin solution.

Tubes containing the following mixtures were then prepared and allowed to remain at the laboratory temperature.

Tube I.	Five c.c. sinigrin solution	+	5 c.c. myrosin solution.
Tube II.	,, ,, ,,	+	5 c.c. indigo enzyme solution.
Tube III.	,, ,, ,,	+	5 c.c. water.
Tube IV.	Five c.c. indigo extract	+	5 c.c. myrosin solution.
Tube V.	,, ,, ,,	+	5 c.c. indigo enzyme solution.
Tube VI.	,, ,, ,,	+	5 c.c. water.

After 6 hours, the odour of mustard oil was very strong in tube I, but in no other tube, whilst only tube V showed any sign of fermentation of the indigo extract. It is clear, therefore, that myrosin cannot produce the indigo fermentation nor can the indigo enzyme ferment sinigrin.

Summary.

It has been shown that, although there are several kinds of bacteria capable of producing the indigo fermentation, some of which are invariably present in an infusion of the plant, the action is in the main dependent on a specific enzyme occurring in the plant-cells. This enzyme acts on a glucoside also occurring in the plant-cells, producing a substance capable of giving indigotin by oxidation with air,

and a reducing sugar. The properties of this enzyme do not appear to be identical with those of any enzymes hitherto described, but it is best left unnamed until the glucoside on which it acts and the products of its action have been more closely identified. The action appears to take a similar course and to be characterised by similar variations with temperature and changes in the medium in which it takes place like that of other enzymes. No evidence of the existence of an oxydase in the indigo-plant was found, but a thorough examination of the plant for a substance of this nature is a matter for future investigation.