1. Studies of the chemistry of carbazole and fluorene.

2. The dehydrogenating enzymes of milk.

Thesis for the degree of Ph.D. in the Faculty of Science

of

Glasgow University.

Submitted by:

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The work contained in the first part of this thesis was carried out in the Organic Chemistry Department of Glasgow University during the sessions 1927-28 and 1928-29, under the supervision of Dr. S. H. Tucker. It is regretted that, from lack of time, the main part of the work - the synthesis of 1 1'- dicarbazyl was not completed, but work on that subject is still in progress, and it is hoped that the synthesis will be accomplished.

During session 1929-30 the work contained in the second part of the thesis was carried out, under the supervision of Herr Geheimrat H. Wieland, in the chemical laboratories of the Bavarian Academy of Science in Munich, affiliated with Munich University.

I offer my most sincere thanks to Dr. S. Norwood Tucker and Herr Geheimrat Prof. Dr. H. Wieland for the encouragement and help they have given in the carrying out of this work. I also thank the Department of Scientific and Industrial Research and the Carnegie Trust for research grants, which made this work possible.

1. Studies of the chemistry of carbazole and fluorene.

Part 1. Page.

Synthesis of 1:1'- dicarbazyl.

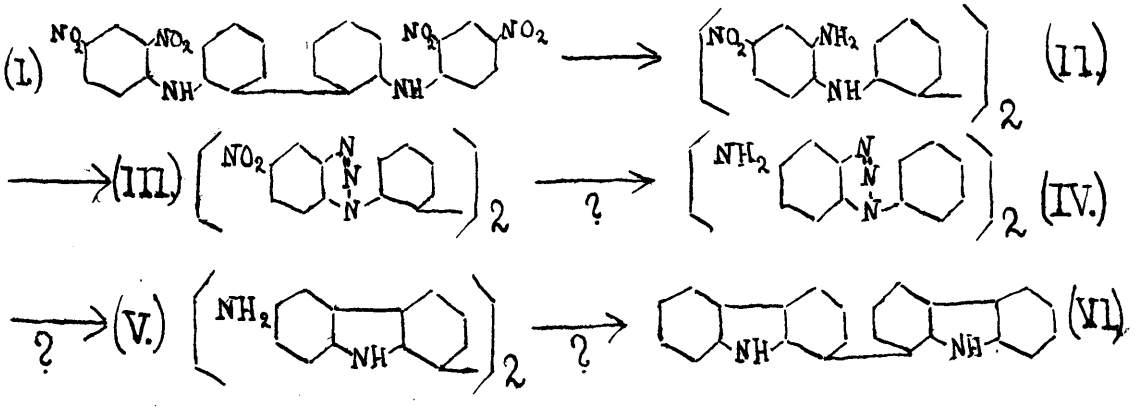
The oxidation of carbazole gives two dicarbazyls, m.p. 220° and 265° respectively, and also an amorphous compound [1].

The dicarbazyl of m.p. 220° has been shown to be NN'- dicarbazyl [2]. The dicarbazyl, m.p. 265° may be 1:1'- dicarbazyl, and synthesis of 1:1'- dicarbazyl will prove if this is so.

The method used for the synthesis of 3:3'- dicarbazyl [3] was tried using 2:2'- diaminodiphenyl in place of benzidene. Under no condition of experiment could a condensation product of 2:2'- diaminodiphenyl and O-chloronitrobenzene be isolated.

Method A.

A synthesis has been attempted according to the scheme [4].



1.) Perkin & Tucker, J.C.S., 119, 216 (1921).

2.) Tucker & McLintock, J.C.S., 1927, 1214.

3.) Tucker, J.C.S., 1926, 3033.

4.) Compare Ullmann, Abs., 1904, 728.

The compounds (1), (11) and (111) were obtained, but on reduction of (111) a pure product could not be isolated, which is due to the instability of the amino compound to atmospheric oxygen.

Experimental.

NN'-Di-(2:4-dinitrophenyl)- 2:2'-diaminodiphenyl. (1).

2:2'-diaminodiphenyl (20g.), 1-chloro-2:4- dinitrobenzene (75g.) and dry potassium carbonate (30g.) were heated together with vigorous stirring, on an oil bath at 140° for four hours. (The potassium carbonate prevents the decomposition of 2:2'-diaminodiphenyl which takes place very readily at 140° in the presence of hydrochloric acid, to give carbazole and ammonia.) Whilst the melt was still hot, benzene (250c.c.) was added and when cool, the insoluble portion was filtered off and washed with more benzene until the insoluble portion was free from 1-chloro-2:4-dinitrobenzene.

The residue was now washed several times with water to remove potassium chloride and potassium carbonate, and there resulted a fairly pure condensation product, which melted at 220°-222°. (Yield 45g : 80%). This was crystallised from benzene, in which it is nearly insoluble in the cold, but reasonably soluble when hot, and from which it crystallises in yellow orange crystals, m.p. 228°. This compound is insoluble in alcohol and ether, soluble in chloroform, benzene, toluene, acetic acid and acetone, (Found: N, 16,3. $C_{24}H_{16}O_8N_6$ requires N.16.3%.)

Recent work on the condensation of 2:2'- diaminodiphenyl and 1-chloro-2:4-dinitrobenzene has given different results to these described above [1]. Two compounds m.p. 177°-178° and 216°-217° are described. The compound m.p. 177°-178° is held to be NN'-Di-(2:4-dinitrophenyl)-2:2'-diaminodiphenyl, but this does not agree with the work described above, where the m.p. of this compound was found to be 228°. The conditions used varied considerably as in the above experiments a larger excess of 1-chloro-2:4-dinitrobenzene was used, potassium carbonate was present and the experiments were carried out on an oil bath.

Work on the condensation of 2:2'-diaminodiphenyl and 1-chloro-2-nitro-4-cyanobenzene has shown there to be three compounds formed (Page 12):- a carbazole derivative, a compound from the condensation of one molecule of 2:2'-diaminodiphenyl with one molecule of 1-chloro-2-nitro-4-cyanobenzene, and a compound from the condensation of one molecule of 2:2'-diaminodiphenyl with two molecules of 1-chloro-2-nitro-4-cyanobenzene. Three such compounds are of course possible with 1-chloro-2:4-dinitrobenzene, and it is therefore possible that the compound m.p. 177°-178° is not as described but is formed by the condensation of one molecule of 2:2'-diaminodiphenyl with one molecule of 1-chloro-2:4-dinitrobenzene. It must be noted that this compound has a similar nitrogen content.

1.) Le Fevre, J.C.S., 1929, 737.

Its melting point also appears to agree with this structure, as the melting point is lower than the melting point of the carbazole derivative, which was the case in the analagous compounds from the 2:2'-diaminodiphenyl and 1-chloro-2-nitro-4-cyanobenzene.condensation.

NN'-Di-(2-amino-4-nitrophenyl)-2:2'-diaminodiphenyl.

The above tetra nitre compound (30g.) (1) was carefully powdered and added to a mixture of rectified spirits (150 c.c.) and ·88 ammonia (50 c.c.) in a flask fitted with a reflux. Hydrogen sulphide was now passed through the mixture until it was saturated, and then the flask was heated on the water bath for 15 minutes, allowed to cool, again saturated with hydrogen sulphide and then again heated. Reduction took place and the product of reduction dissolved in the alcohol, giving a deep red solution. Later the alcohol became saturated and the reduced compound separated as a dark red solid. The intermittent passing of hydrogen sulphide and heating was repeated six times, after which the contents of the flask were poured into water, which precipitated all the reduced compound. The solid was now filtered off and the amine extracted as the hydrochloride by repeated treatment with 10% hydrochloric acid.

From the hydrochloride solution the free base was precipitated with ammonia, and the free base was filtered off, dried on pourous plate, and then taken up in benzene from which it crystallised on cooling in small deep red crystals. It was recrystallised from benzene. It gives small bright red crystals, m.p.125° (Yield 12g.: 45%).

This compound is very readily soluble in benzene, acetone, acetic acid, chloroform; soluble in alcohol, Methyl alcohol and ether. Benzene is the only solvent from which it crystallises satisfactorily. (Found: N 18.5, $C_{24}H_{20}O_4N_6$ requires N 18.4%).

Di-(5-nitro-1:2:3-benztriazolyl)-2:2'-diphenyl.

The above compound (10g.) (11) was dissolved in acetic acid (50 c.cs) and coarsely powdered sodium nitrite (3g.) was added then the whole was heated to boiling. On allowing to cool a very little of a brownish yellow compound crystallised out. On pouring into water the remainder of the compound was precipitated. This compound was crystallised from methyl alcohol in which it is only slightly soluble (1 in 100). It does not crystallise satisfactorily from any solvent, since it comes out as an almost amorphous, yellow powder. By repeated crystallisation from methyl alcohol a compound which melted about 140° was finally obtained.

This compound is very soluble in acetic acid and acetone; soluble in benzene and chloroform; and sparingly soluble in methyl and ethyl alcohol. Heated on a spatula it explodes. (Yield 9.5g.:90%) (Found: N, 23.1. $C_{24}H_{14}O_4N_8$ requires N. 23.4%)

Several attempts were made to reduce (111) to give (1V). In all cases the reduction appeared to go but in no case could a pure product be got. Among the methods tried were, using tin/

/tin and hydrochloric acid, zinc and acetic acid, a method using iron and hydrochloric acid [1], and a method using sodium polysulphide.

Using all the above methods on almost black solid was got which could not be induced to crystallise from any solvent, although it was soluble in most solvents. This solid was also soluble in hydrochloric acid, and so probably was the desired compound.

Attempts were made to acetylate this base, and a compound was got which crystallised in pale yellow crystals from acetic acid. This compound appeared to be the acetyl derivative. On filtering off, however, it almost immediately turned black in the air, probably due to oxidation.

Since the acetyl derivative is so unstable to atmospheric oxygen, it is only to be expected that the base should also be unstable, as acetyl derivatives are as a class much more stable than the bases from which they are derived. This instability must be due to the large number of nitrogen atoms in the molecule. It is just possible that the reduction may also have reduced the -N-N- group in the molecule, which certainly would make the compound very easily oxidised.

Method B.

The difficulties encountered in Method A were due to the presence of the extra nitro groups. These were present only to render the chlorines sufficiently reactive to make the condensation/

1.) R. Winton West, J.C.S., 1925, 194.

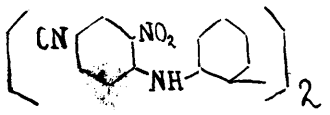
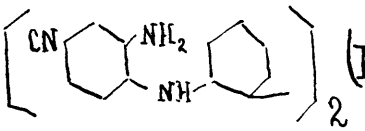
/condensation possible. It has been shown how difficult it is to get rid of these groups. It is apparent that if it were possible to use a compound in place of the 1-chloro-2:4-dinitrobenzene, which has a more easily removed group than a nitro group, in the 4 position, and a group which would activate the chlorine sufficiently to make the initial condensation possible, then we should have an easier synthesis.

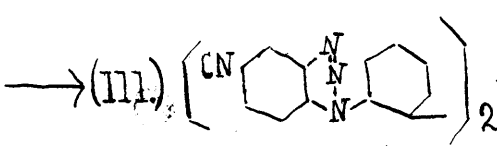
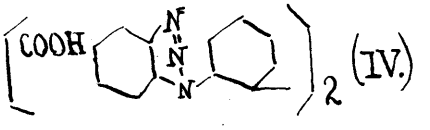
1-chloro-2-nitro-4-carboxybenzene was tried, but in spite of repeated attempts, no condensation product could be isolated.

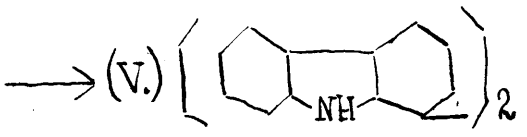
According to Brewin and Turner [1] cyano groups activate the chlorine atom, and it was found that 1-chloro-2-nitro-4-cyanobenzene condenses with 2:2'-di-aminodiphenyl to give three distinct products. One of these compounds is formed by the condensation of one molecule of 2:2'-diaminodiphenyl with one molecule of 1-chloro-2-nitro-4-cyanobenzene, one by the condensation of one molecule of 2:2'-diaminodiphenyl with two molecules of 1-chloro-2-nitro-4-cyanobenzene, and the third by the loss of ammonia from the first named compound.

The following scheme for the synthesis of 1:1'-dicarbazyl was suggested:-

1.) J.C.S., 1928, 332.

(I.)  (II.) 

 (III.)  (IV.)

 (V.)

The compounds (I), (II), (III) and (IV) were obtained but the final step could not be carried out, in spite of repeated attempts and variation of conditions of experiment. It is difficult to account for this failure, but the presence of the carboxyl groups in the molecule may be the cause.

Compound (III) when heated gave off a gas and a colourless compound resulted which was not the starting material, and was therefore apparently 1:1'-di-5-cyanocarbazyl. The yield of this compound was so small that this method is unpractical.

Compound (I) when hydrolised with concentrated hydrochloric acid gave a compound which was apparently NN'-di-(2-nitro-4-carboxylphenyl)-2:2'- diaminodiphenyl. When hydrolised with sodium hydroxide in alcohol solution, there resulted two compounds - 1-hydroxy-2-amino-4 carboxylbenzene (due to fissure of the molecule) and NN'-di(2-amino-4-carboxylphenyl)-2:2'-diaminodiphenyl.

Attempts to remove the carboxyl groups from NN'-di-(2-nitro-4-carboxylphenyl)-2:2'-diaminodiphenyl and NN'-di-(2-amino-4-carboxylphenyl)-2:2'-diaminodiphenyl were not successful. It thus appears that the great difficulty throughout the synthesis is the removal of the carboxyl groups. In addition, the passing to the carbazole nucleus appears to be much more difficult than in analagous compounds, which must be due to sterical reasons. Heating of 1:1'-di-5-carboxycarbazyl, which could be obtained by hydrolysis of the product of heating of 2:2'-di-(5-cyano-1:2:3-benztriazolyl)-diphenyl, may give 1:1'-dicarbazyl, and this method appears to be the only possible means which has not been attempted.

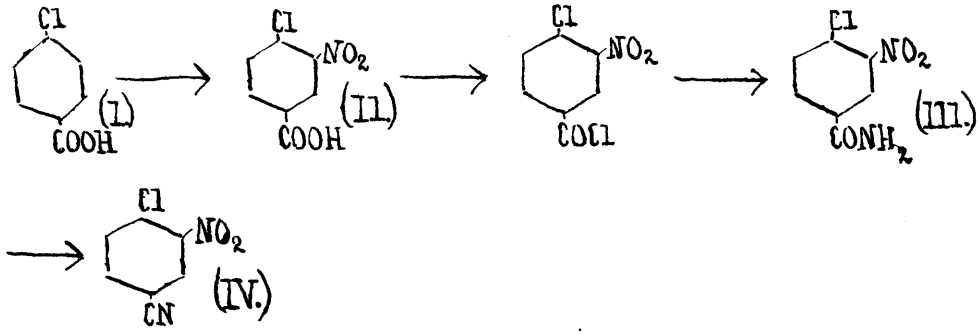
Experimental.

Synthesis of 1-chloro-2-nitro-4-cyanobenzene.

The method given by Claus and Stiebel [1] for the preparation was found unsatisfactory. This method consists of the nitration of the sulphate of p-chloraniline, or reduction of 1-chloro-2:4-dinitrobenzene, to give 1-chloro-2-nitro-4-aminobenzene and the subsequent replacement of the amino group with cyano by Sandmeyer's method. This gave exceedingly poor yields of 1-chloro-2-nitro-4-cyanobenzene, principally because the Sandmeyer gave only a 10% yield.

1.) Ber. 20. 1379.

A method starting with p-chlorbenzoic acid has proved much better. Nitration gave 1-chloro-2-nitro-4-carboxybenzene which with phosphorus pontachloride gave the acid chloride, which with ammonia gave the acid amide, and it finally gave the cyano compound by treatment with phosphorus pentoxide.



1-chloro-2-nitro-4-carboxybenzene (II).

Finely powdered p-chlorbenzoic acid (25g.) was added to fuming nitric acid (42 cc.) and then the beaker was heated until all the p-chlorobenzoic acid dissolved. The whole was now poured into water and the 1-chloro-2-nitro-4-carboxybenzene separated. Yield 86%). This was crystallised from alcohol m.p. 177°-178°.

3-nitro-4-chlorobenzamide (III).

This compound was prepared by adding the corresponding acid chloride to ammonia.

An attempt was made to prepare the acid chloride by heating the acid with a slight excess of thionyl chloride. No acid chloride resulted because when ammonia was added no precipitate was obtained. On evaporation of the ammoniacal solution/

/solution a water soluble compound separated which contained sulphur. A possible explanation of this is that the thionyl chloride reduced the nitro group, and then condensed with it.

3-nitro-4-chlorobenzamide was finally prepared by the following method.... 1-chloro-2-nitro-4-carboxybenzene (50g.) was added to a flask containing phosphorus pentachloride (58g.) On shaking a vigorous reaction set in, and much hydrochloric acid was evolved. The whole of the contents of the flask went into solution in the phosphorus oxychloride formed in the reaction. When the reaction had subsided, the flask was heated on the water bath for an hour and then the phosphorus oxychloride was distilled off on the water bath under reduced pressure. Dry carbon tetrachloride (80c.c.) was added, and then the whole of the contents of the flask were poured into a large beaker containing ·88 ammonia (300 c.c.). The acid amide which separated was filtered off, well washed with water, and then dried and freed from carbon tetrachloride by heating in an open basin on a water bath. (Yield 43g.:87%).

The filtrate was made acid with hydrochloric acid and some unchanged 1-chloro-2-nitro-4-carboxybenzene was recovered.

The acid amide was crystallised from water, 100 parts water dissolving 2 parts of the compound. It crystallises from water in small colourless needles, m.p. 151°-152°.

This compound is easily soluble in alcohol and benzene, very sparingly soluble in carbon tetrachloride, and more soluble in chloroform. (Found:N 14.1. $C_7H_5O_3N_2Cl$ requires N. 14.0%).

1-chloro-2-nitro-4-cyanobenzene.(IV)

3-nitro-4-chlorobenzamide (50g.) was heated on an oil bath to 170° and to the molten amide phosphorus pentoxide (75g.) was added, and the mass kept stirred with a rod. After heating for one hour the flask was removed from the oil bath and allowed to cool. When cold, water was added to destroy the excess phosphorus pentoxide. Sodium carbonate was now added to take up the phosphoric acid, and after standing for 12 hours the nitrile was filtered off. It was crystallised from rectified spirits, m.p. 101°. (Yield 37g.:85%).

Condensation of 2:2'-diaminodiphenyl with 1-chloro-2-nitro-4-cyanobenzene.

2:2'-diaminodiphenyl (9g.), 1-chloro-2-nitro-4-cyanobenzene (30g.), dry potassium carbonate (15g.) and xylene (150 c.c.) were heated together for eighteen hours on an oil bath under a reflux condenser at 140° with vigorous stirring. Carbon dioxide was evolved showing that a condensation was taking place.

The xylene was then distilled off, the residue washed with water to remove potassium salts, and alcohol (200 c.c.) was added and then the flask was heated on the water bath. The alcoholic solution/

/solution was now filtered off hot and on cooling, a bright red compound crystallised out (A). (Yield 1.5g).

The solid now remaining was extracted three times with benzene (100 cc), and from the extracts there crystallised out yellow needles (B). (Yield 14g). The residue was now extracted three times with glacial acetic acid (200 cc) and from the extracts a brick red compound crystallised out (C). (Yield 1.8g).

(A) N-(2-nitro-4-cyanophenyl)-2:2'-diaminodiphenyl - The compound crystallising from the above alcoholic extract was purified by boiling with animal charcoal in alcohol. It crystallises in large brick red plates from alcohol m.p. 141°. This compound is easily soluble in benzene, acetone, alcohol, chloroform but only sparingly soluble in ether. It is soluble in dilute acids. Heated with 1-chloro-2-nitro-4-cyanobenzene it gives compounds B and C. (Found:N 17.9. $C_{19}H_{14}O_2N_4$ requires N.17.0%).

(B) N-(2-nitro-4-cyanophenyl)carbazole. - The compound crystallising from the above benzene extracts was recrystallised from benzene from which it crystallises in fine yellow needles m.p. 229°. This compound is soluble in benzene, acetic acid and acetone, but only sparingly soluble in alcohol. It is insoluble in dilute acids. (Found:N 13.6. $C_{19}H_{11}O_2N_3$ requires N 13.4%).

(C) NN'-Di-(2-nitro-4-cyanophenyl)-2:2'-diaminodiphenyl - The compound from the acetic acid extract was recrystallised several times from glacial acetic acid in which it is soluble only with/

/with difficulty, and from which it crystallises in brick red plates m.p. 242°. It is soluble in acetone and acetic acid but only sparingly soluble in other solvents. (Found:C. 65.8: H. 3.4: N 17.9. $C_{26}H_{16}O_4N_6$ requires C.65.6: H.3.4: N.17.6%). In the above condensation the yields of the three compounds vary very much with the conditions of experiment. The above described method gives the maximum yield of (C) which was the desired product. Heating to a higher temperature gives more of (B) and in the absence of potassium carbonate the product is almost all B. with some A. With inefficient stirring the yield of C falls and with no stirring an exceedingly poor yield of C results.

NN'-di-(2-amino-4-cyanophenyl)-2:2'-diaminodiphenyl.

The above compound (C) (15g.) was added to acetic acid (50c.c.) and iron fillings (4g.) were then added in small portions, the mixture being heated on the water bath. The acetic acid solution became colourless. Heating was continued for half-an-hour after the reaction appeared to stop. Water was added, drop by drop, to the acetic acid solution, until a very slight permanent white precipitate remained, and then the solution was extracted with ether. The ether extract was washed with water until free from acetic acid, and dried over sodium sulphate. The ether solution was then heated gently and petroleum ether (60°-80°) was added until a slight precipitate was obtained and on allowing to cool the amino compound came out in pale yellow crystals. It was recrystallised from a mixture of ether and petroleum/

/petroleum ether, m.p. 175°-190° (with decomposition). This compound is soluble in alcohol, ether, benzene; but only slightly soluble in petroleum ether. (Yield 2.8g= 65%). (Found: N, 20.7. $C_{26}H_{20}N_6$ requires N.20.2%).

Di-(5-cyano-1:2:3-benztriazolyl)-2:2'-diphenyl.

The above compound (2g.) was dissolved in dilute hydrochloric acid (100 cc. 10% HCl) and finely ground sodium nitrite (1g.) was gradually added and the solution was boiled. After boiling for ten minutes, and allowing to cool, the solid was filtered off. (1.3g.) This was crystallised from aqueous acetic acid from which it comes out in small pale yellow crystals.m.p. 263°. This compound is soluble in acetone and acetic acid but only slightly soluble in alcohol, benzene and ether. (Found: N,25.9. $C_{26}H_{14}N_8$ requires N.25.6%).

DI-(5-carboxy-1:2:3-benztriazolyl)-2:2'-diphenyl.

The above compound (1g.) was heated at 160°. in a sealed tube, for three hours, with alcoholic sodium hydroxide (2g.NaOH in 10cc alcohol). To the products of reaction, hydrochloric acid was added until acid, and the so-obtained white solid was filtered off. This was purified by making the sodium salt again and reprecipitating with acid. (The sodium salt is only very sparingly soluble in water). This compound is almost insoluble in the common solvents, and so cannot be crystallised from any solvent. As obtained it is a pure white powder, unmelted/

/unmelted at 330°. (Found: N.17.4. $C_{26}H_{16}O_4N_6$ requires N.17.6%).

Attempts to convert Di-(5-carboxy-1:2:3-benztriazolyl)-2:2'-diphenyl into 1:1'-dicarbazyl - The above compound (0.5g.) was heated in a small dry flask with a small flame, and the flask was kept shaken. The solid in the flask blackened but did not melt. After some heating the solid exploded leaving a cake of carbon, from which nothing could be extracted by benzene or other solvents. When heating was stopped before the compound exploded it was found that no action had taken place.

The above compound (0.2g) was mixed with soda-lime (1g.) and the mixture was heated as before. No apparent explosion occurred but on washing with water, drying, and extracting the residue with solvents, nothing was obtained.

The above compound was then heated in wax to 400°. The compound charred a little. On removing the wax with petroleum ether, a residue was obtained which was found to consist of the unchanged compound.

The above methods were repeated several times but no product of reaction could be isolated.

Decomposition of Di-(5-cyano-1:2:3-benztriazolyl)-2:2'-diphenyl.

Di-(5-cyano-1:2:3-benztriazolyl)-2:2'-diphenyl (1g.) was heated for half-an-hour in a small tube immersed in wax at 320°. Slight charring occurred. The product was extracted with acetic acid and boiled with animal charcoal. From the acetic acid solution, a pure white compound came out, on cooling. This compound was recrystallised/

/recrystallised from acetic acid in which it was much less soluble than Di-(5-cyano-1:2:3-benztriazolyl)-2:2'-diphenyl, m.p. 298-300°. on adding water to the mother liquor unchanged starting material was obtained. There was not enough product for analysis.

Hydrolysis of NN'-di-(2-nitro-4-cyanophenyl)-2:2'-diamino-diphenyl - NN'-di-(2-nitro-4-cyanophenyl)-2:2'-diamino-diphenyl (1g.) was heated for two hours with 80% sulphuric acid (50 c.c.). The product obtained was very soluble in water and was found to contain sulphur, which was not removed by boiling with dilute hydrochloric acid. It is apparent that the compound had been sulphonated.

Using concentrated hydrochloric acid and boiling for two hours it was found that NN'di-(2-nitro-4-cyanophenyl)-2:2'-diamino-diphenyl was not hydrolised. Heating to 150° in a sealed tube with dilute alcohol saturated with hydrochloric acid this compound was returned unaltered. On heating to 250° however, hydrolysis occurred and a very deep red compound resulted. This compound could not be satisfactorily purified as it would not crystallise from any of the recognised solvents. It was soluble in benzene, acetic acid, nitrobenzene and acetone. This compound heated in a wax bath gave off a gas but the residue, when extracted, with acetic acid, gave an extract from which no definite compound could be isolated.

NN'-di-(2-nitro-4-cyanophenyl)-2:2'-diaminodiphenyl (1g.) was heated to 160° with sodium hydroxide (2g.) in alcohol (10 cc) in a sealed tube. The product of reaction was added to dilute hydrochloric acid and the white solid which separated was filtered off. The solid was dissolved in hot alcohol and on cooling a grey solid came out of solution. This was filtered off and the mother liquor was concentrated. On cooling a definitely crystalline compound crystallised out, which was recrystallised from alcohol twice, m.p. 198°. This compound is evidently 1-hydroxy-2-amino-4 carboxybenzene, which would be obtained by fissure of the diphenylamine grouping by the alcoholic sodium hydroxide. (This type of fissure has been previously noted on similar compounds.) The reduction of the nitro group is also to be expected. The first obtained product was probably NN'di-(2-amino-4-carboxyphenyl)-2:2'-diaminodiphenyl. It was purified by boiling with charcoal in alcohol, and was obtained fairly pure, as a yellowish microcrystalline compound. Unmelted at 300°. Heated in paraffin it appeared to decompose as it charred slightly, but no definite product of reaction was isolated.

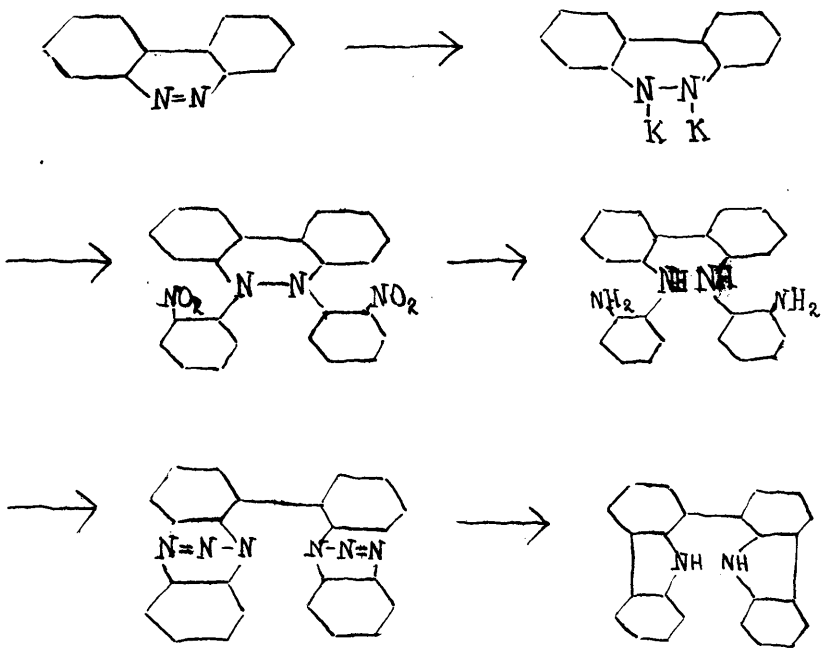
Method C.

Azobenzene gives when dissolved in ether and treated with atomised potassium a compound which has a formula approximating to $C_6H_5NK-NKC_6H_5$, $C_6H_5N=NC_6H_5$.[1].

It/

1.) Ber. 1914, 47, 485.

It was thought that phenazone should give a similar compound which would condense with o-chloronitrobenzene, and make the following synthesis possible:-



A purple compound was got when phenazone was treated with atomised potassium. No condensation product with this and o-chloronitrobenzene could be isolated.

Experimental.

Phenazone (Diphenylenazone).

Azobenzene can be obtained by the electrolytic reduction of nitrobenzene, in good yields. Similar reduction of 2:2'-dinitrodiphenyl/

dinitrodiphenyl was tried to produce phenazone. No reduction product was obtained.

Phenazone was prepared in the way given by Dobbie, Fox, and Gudge.[1])

Phenazone (2g.) was dissolved in ether (50 c.c.) and potassium (1g.) was atomised in toluene (25 c.c.). These were mixed and shaken together, and a purple solid separated which was probably di-potassium phenazone. o-chlornitrobenzene (3g.) was now added and the flask heated under a reflux condenser on a water bath for half-an-hour. The ether was now distilled off and a very little alcohol added to destroy the remaining potassium. More alcohol was added in order to extract the unchanged phenazone and o-chlornitrobenzene. No residue resulted, and so it appeared that the condensation had not taken place. The alcoholic solution was taken down but the first thing to crystallise out was the phenazone, thus showing there to be no condensation product in the alcohol.

1.) J.C.S., 1911, 1617.

The Action of Methyl Magnesium iodide on Methyldiphenylene Acetate. - Acting on methyldiphenylene acetate with an excess of methyl magnesium iodide (4 mols.) ought, according to the general reactions of Grignard reagents, to give 9-fluorenyldimethyl carbinol. None of this latter compound could be isolated from the product of reaction, and no evidence of its formation in the reaction was obtained. Three compounds were isolated from the product of reaction m.p. 141°, 47° and 37° respectively, and evidence of the presence of a fourth compound was obtained as there was obtained small quantities of an oxime, by treating the product of reaction with hydroxylamine.

The compound m.p. 141° was obtained only in very small quantities and its constitution could not be ascertained. It gave no oxime with hydroxylamine. The compound m.p. 47° was found to be 9-methylfluorene, and it formed the bulk of the product of reaction. The compound m.p. 37° proved to be a hydrocarbon, and is either 9-ethylfluorene or 9-n-propylfluorene. As the difference in analysis for these compounds is small, and the results obtained lay between those required for 9-ethylfluorene and 9-n-propylfluorene, the exact constitution of the compound could not be ascertained.

By varying the amount of methyl magnesium iodide used in the reaction different results were obtained. When two mols. of methyl magnesium iodide were used no compound m.p. 141° could be isolated, although the yields were decidedly lower: the remainder of the product of reaction was chiefly unchanged

starting/

/starting material. When eight Mols. of methyl magnesium iodide were used the product of reaction was almost identical with that obtained using four Mols.

Distilling off the excess methyl iodide after the formation of the Grignard reagent, had no apparent effect on the result of the reaction, and 9-methylfluorene was still found to be the principal product. The explanation of the reactions involved in this process is difficult. The unexpected results appear to be due to the active hydrogen atom in the starting compound. The reducing action of methyl magnesium iodide is of course well established, which is sufficient to explain the formation of compound m.p. 37°. The formation of 9-methylfluorene is more difficult to explain. Diphenylene-methyl acetate gives 9-methylfluorene by loss of carbon dioxide, but this only takes place at temperatures much above those used in the reaction, and in the subsequent vacuum distillations. The ketone formed, whose presence could only be shown by formation of its oxime, was probably 9-fluorene-methyl ketone. The formation of the compound m.p. 141° cannot be explained since the identity of the compound has not been established.

Experimental.

Methyldiphenylene Acetate - Fluorene-9-carboxylic acid (20g.) was boiled for two hours on the water bath, with methyl alcohol (80 c.c.) containing five per cent hydrochloric acid. Methyl alcohol (30 c.c.) was now added, and on allowing to cool the ester crystallised/

/crystallised out. It was recrystallised from methyl alcohol m.p. 67°. (Yield, 18g: 84%).

The Action of Methyl Magnesium Iodide on Methyldiphenylene Acetate.

Magnesium (7g.) was covered with sodium dried ether (45 c.c.) and methyl iodide (45g.) was gradually added, the flask being immersed in cold water. When the reaction had subsided methyldiphenylene acetate (15g.) was added in small portions, the addition taking half-an-hour. During the addition much gas was evolved, and a solid came out of solution. The flask was finally heated for an hour on the water bath to complete the reaction.

When cold the magnesium compounds were decomposed with dilute sulphuric acid, and then the oil formed was extracted with ether. The ether solution was dried over sodium sulphate, the ether distilled off, and the remaining oil distilled under sixteen millimetres pressure and was collected between 175° and 185°. The distillate was put in ice but could not be induced to crystallise. A little petroleum ether was added and on scratching the sides of the vessel a small quantity of a compound crystallised out. On purification by crystallisation from alcohol this compound was obtained in fine pale yellow needles m.p. 141°. When treated with hydroxylamine it was returned unchanged.

The above mother liquor could not be induced to yield any more crystals, and the petroleum ether was therefore distilled off. The oil was now boiled with hydroxylamine in the usual manner for the formation of an oxime, but the bulk of the oil remained unchanged, and only a very little of an oxime was obtained.

This/

/This oxime was very easily oxidised by atmospheric oxygen and a good melting point could not be obtained.

The oil from the above was now dried by dissolving in ether and drying over sodium sulphate. It was then distilled in vacue and fractionated. The first fraction (175-179° /16mm.) was redistilled, and the second fraction (179-185 /16mm.) was similarly treated. The fraction distilling at the lower temperature was dissolved in alcohol and on keeping in a refrigerator overnight yielded crystals of 9-methylfluorene (3.7g.)

The other fraction was similarly treated, and after two days in the refrigerator a very little of a compound crystallised out. By adding a little water and placing again in the refrigerator more of the compound was obtained. This compound was recrystallised from aqueous alcohol by dissolving in alcohol and adding water to the alcoholic solution, which was kept at 5° until a very slight milkiness remained. The solution was now heated gently until the milkiness disappeared, and then the solution was put in a refrigerator overnight, when the compound crystallised in fine colourless needles, m.p. 37°. (Yield 1.2g.) (Found: C, 92.5: H.7.5. $C_{15}H_{14}$, (9-ethylfluorene) requires C92.8: H.7.2%. $C_{16}H_{16}$ (9-n-propylfluorene) requires C 92.3: H 7.7%).

2. The Dehydrogenating enzymes of milk.

Schardinger's discovery[1] that fresh cows' milk has the property of decolorising methylene blue in the presence of formaldehyde gave rise to many researches, principally to examine the possibility of the use of this decolorisation in the determination of the bacterial content of milk. It was finally proved by Trommsdorff[2] that this property of milk is independent of bacterial action, but that it is due to the presence of an extracellular enzyme in the milk.

Milk was later found to bring about the aerobic oxidation of aldehydes to acids, and the dismutation of aldehydes to acids and alcohols.[3] H. Wieland attributed all three actions - 1. the aerobic oxidation of aldehydes to acids, 2. the anaerobic reduction of methylene blue and 3. the anaerobic dismutation of aldehydes to acids and alcohols, to one enzyme - a dehydrase.

The Schardinger enzyme has been isolated from milk in varying states of purity by Hopkins and his school in Cambridge[4], by Bach in Moscow[5] and later in Munich by Wieland and Rosenfeld[6].

1) F. Schardinger, Zs. z. Untersuchung.der Nahrungs-u. Genussmittel, 5, 1113 (1902).

2) Zbl.f.Bakt.u.Paras., 1. 49, 291 (1909).

3) Ber. 46, 3327 (1913); 47, 2085 (1914).

4) M. Dixon and S. Thurlow, Bioch.Jl., 18, 971 (1924).

5) B. Sbarsky u. D.Michlin, Biochem. Zs., 155, 485 (1925).

6) Ann., 477, 32 (1929).

The further important biological discovery by Hopkins[1], that the Schardinger enzyme brings about the oxidation of the purine bases, xanthine and hypoxanthine, gave rise to further researches on this enzyme. The Cambridge and the Moscow schools have produced a large amount of work on the nature of these enzyme actions, with the result that there has been a big increase in the knowledge of these actions.

The recent work of Wieland and Rosenfeld[2] has definitely proved the mechanism of the oxidation of xanthine and hypoxanthine to uric acid. The process is essentially a dehydrogenation, the function of the enzyme being to activate the hydrogen of the hydrated forms of the bases, to render them capable of removal by an oxidising agent, the function of which therefore is only that of hydrogen acceptor. The formation of hydrogen peroxide, as the dehydrogenation thoery[3] demands, was definitely proved.

A study has been made of the corresponding dehydrogenation of aldehydes in order to establish the true nature of the mechanism of the process. In this work ample use has been/

1) E.G.Morgan, G.P.Stewart and F.G.Hopkins, Proc. Roy. Soc.Ldn., 94, 109 (1922).

2) loc. cit.

3) H. Wieland, Ber., 55, 3639 (1922); Ergebnis. d. Physiol., 20, 477, (1922).

made of the methods used in the corresponding experiments on the purine bases.

Work has been completed to establish the relationship between the anaerobic dismutation of aldehydes to acids and alcohols, and the aerobic dehydrogenation to acids, which Wieland[1] first showed to be brought about by fresh cows' milk with salicylaldehyde, and which Bach[2] found was also brought about by his enzyme preparation from butter-milk.

Experimental evidence has been gathered bearing on the question of the distinct nature or identity of Aldehydrase and Xanthine dehydrase, which subject is not yet quite clear. In addition, further experiments have been carried out, which, it is hoped, add to the previous knowledge of the dehydrogenating enzymes of milk.

1) loc. cit.

2) A. Bach, u.K. Nikolajeff, Biochem. Ze., 169, 105 (1925).

The General Properties of the Dehydrases of milk and the Question of the Identity or Distinct Nature of Aldehydrase and Xanthine Dehydrase.

Method of Measurement.

The methylene blue method first used by Thunberg[1] and later by Dixon and Thurlow[2] in their studies on the kinetics of the dehydrogenation of xanthine and Hypoxanthine, and still later by Wieland and Rosenfeld[3], has been used. This method of measurement is dependent on the fact that the time of decolorisation of a given quantity of methylene blue is dependent on the quantity of enzyme present.

The technique of the last-mentioned authors has been used, and only one slight modification has been introduced. It was found advantageous in the estimation of xanthine dehydrase to add the substrate with the methylene blue, and not at the beginning. This precaution prevents the aerobic dehydrogenation of xanthine which is accompanied by destruction of enzyme.

The convenient standards of enzyme content - Aldehydrase and Xanthine Dehydrase, adopted by Wieland and Rosenfeld, have also been used. 0.4 cc. of $\frac{m}{50}$ salicylaldehyde has, however, been used in the measurement of aldehydrase. The unit of quantity/

1) T. Thunberg, Scand. Arch. Phys., 35, 163 (1917).

2) loc. cit.

3) Loc. cit.

/quantity of salicylaldehyde dehydrase therefore, is the amount of enzyme, that in the presence of 0.4 cc. $\frac{m}{50}$ salicylaldehyde, at p H = 8.0, in a total volume of 5 c.c., at 37°, decolorises 1 c.c. of $\frac{n}{1000}$ methylene blue in 5.0 minutes.

Enzyme Preparation.

The Cream preparation of Wieland and Rosenfeld[1] has been used in the course of this work.

The yields of enzymes obtained were similar to those obtained by the original authors, and the stability of the enzymes of this preparation, attributed to the preparation, has been verified. One preparation when kept 6 months still contained 65% of its original enzyme content.

Further Purification of Cream Preparation.

When a solution of cream preparation was brought to p H = 5.5, it was noticed that a slight precipitation of protein occurred, which increased in quantity as the acidity increased. The precipitate it was found contained the greater part of the enzyme, and a method for the further purification of the enzymes has been devised, making use of this precipitation.

150 mg. of cream preparation was dissolved in 12 c.c. of water, at 37°. To the solution 3.6 c./

1) Loc. cit.

/3.C.C. of $\frac{m}{4}$ acetate buffer, p H= 4.5, was added and the solution shaken for two minutes at 37°. The precipitated protein was centrifuged off, and was washed once with 10 c.c. of distilled water to which several drops of the above mentioned buffer had been added. The solid was again centrifuged off. The, thus obtained, paste was dissolved in 10 c.c. of $\frac{m}{10}$ phosphate buffer, p H= 8.0, in which it was easily soluble.

The results of three typical purifications are given.

Table 1.

		A.	B.	C.
Dry weight of cream preparation used.		150 mg.	150 mg.	150 mg.
Total dehydrase content.	X.	81.0	81.0	86.2
	Sa.	28.7	28.7	30.5
Dry weight of product.		59.0 mg.	55.5 mg.	57.0 mg.
Total dehydrase content.	X.	82.9	67.2	74.5
	Sa.	23.7	24.0	25.5

A preparation is thus obtained which is twice as active as the cream preparation. In the process about 20% of the enzymes are lost. This preparation contains very little buffering materials, compared with cream preparation, and considerable use has been found for it on account of this.

When dried this preparation loses 30% to 35% of its enzyme content, and the so-obtained powder dissolves only partially in phosphate buffer p H = 8.0. This dry preparation is therefore unsatisfactory/

The adsorption of the whey enzymes by A. Kaolin and B. Alumina.

Dixon and Kadoma[1] obtained a purification of the enzymes of whey by dissolving the precipitate obtained by half saturating whey with ammonium sulphate in water, and adsorbing the enzymes with kaolin at p H= 5.0. The enzymes were eluted from the kaolin with sodium carbonate solution. Those authors were only interested in the xanthine dehydrase. Wieland and Rosenfeld[2] also studied the adsorption of the cream enzymes by Kaolin and Alumina.

A study of the adsorption of xanthine dehydrase as compared with that of aldehydrase by the above adsorbents was carried out, to find if it was possible to definitely prove the distinct nature of these two enzymes.

A. Kaolin.

To 200 c.c. of skim-milk at 37°, 0.5 g. of Birk's Junket Powder, dissolved in 10 c.c. of water, was added. The milk was shaken to ensure complete mixing of the rennet. The milk was kept 10 minutes at 37°, then removed from the thermostat and on cooling the curd set. The curd was broken up with a glass rod and the casein was centrifuged off. The whey thus obtained contained nearly all the dehydrases contained in the skim-milk. (2 c.c. skim-milk contained 2.44X. and 0.89 Sa.: 2 c.c. whey contained 2.38X. and 0.86 Sa.)

1) Loc. cit.

2) Loc. Cit.

To 40 c.c. whey 0.5 $\frac{m}{1}$ acetate buffer, p H=4.5 was added giving a final p H=5.0. 400 c.c. of a Kaolin suspension (1.0 c.c.=50 mg. kaolin) made up to 24 c.c. with water, was added and the mixture was shaken for 3 minutes. The kaolin was now centrifuged off, and the solution, after being brought back to p H=8.0, with $\frac{m}{10}$ ammonia, was measured for xanthine dehydrase and aldehydrase. This was repeated using increasing quantities of adsorbent. All adsorptions were carried out in equal solution volumes, by decreasing the water as the volume of the kaolin suspension increased.

Table 2.

mg. Kaolin used in adsorption.	0	200	400	800	1000	1200
X. in total adsorbed solution.	53. 3.	52.0	37.2	27.9	9.8	2.5
X. adsorbed by kaolin.	0	1.3	16.1	25.4	43.5	50.8
% X. adsorbed.	0	2.4	30.1	47.6	81.6	95.3
Sa. in total adsorbed solution.	17. 8	16.1	14.3	8.5	2.7	0.8
Sa. adsorbed by kaolin.	0	1.7	3.5	9.3	15.1	17.0
% Sa. adsorbed.	0	9.5	19.7	52.3	85.0	95.5

Xanthine dehydrase and aldehydrase are therefore adsorbed by kaolin in the same proportions, for the above noted differences are within the bounds of the experimental error.

The adsorption with 200 mg. of kaolin brought about very little adsorption of enzyme, but a distinct purification of the whey resulted, for it became much clearer. This adsorption appears to remove principally the heavier protein bodies, with which the dehydrases are not associated. For experiments with whey it is therefore advisable to run this preliminary adsorption.

the kaolin centrifuged from the experiment where 1000 mg. of kaolin was used, was treated with 10 c.c. $\frac{n}{50}$ ammonia, and allowed to stand for 10 minutes. The kaolin was again centrifuged off and again eluted with the same amount of ammonia solution. The two elutions were measured for the dehydrases after being brought to p H.=8.0 with $\frac{m}{10}$ acetic acid.

Table 3.

	1st Elution.	2nd Elution
X. Eluted.	5.2	2.7
% X. in original whey eluted.	9.8	5.1
Sa. Eluted.	2.2	1.2
% Sa. in original whey eluted.	12.3	6.7

A very incomplete elution of the dehydrases from the kaolin is therefore obtained. The elutions are moreover, the same with both the dehydrases, for the difference found is within the bounds of the experimental error.

B. Alumina.

To 10 c.c. of whey, at p.H.=5.0, which had previously been adsorbed with kaolin (200 mg.kaolin to 40 c.c. whey) 15 mg. of alumina contained in 5 c.c. of water was added. The solution was shaken for 3 minutes and then the alumina was centrefuged off. The solution after being brought to p H.=8.0 with $\frac{n}{10}$ ammonia, was measured for the dehydrases. This was repeated with increasing quantities of alumina.

Table 4.

mg. alumina used in adsorption	0	15	30	45
X. in total adsorbed solution	11.9	8.5	5.0	4.0
X. adsorbed by alumina	0	3.4	6.9	7.9
% X adsorbed	0	28.6	58.0	66.5
Sa. in total adsorbed solution	4.2	3.1	2.3	1.8
Sa. adsorbed by alumina	0	1.1	1.9	2.4
% Sa adsorbed	0	26.2	45.3	57.0

As with kaolin no distinct preferative adsorption of either xanthine dehydrase or aldehydrase is noticable, although a slightly stronger adsorption of xanthine dehydrase was perhaps obtained. The difference recorded however, is not great enough to denote a definite difference in the adsorptions.

Attempts to elute the dehydrases from the alumina with $\frac{n}{50}$ ammonia were unsuccessful.

The above adsorption experiments with kaolin and alumina must be regarded as evidence in favour of the identity of xanthine dehydrase and aldehydrase, for no distinct differences in the adsorptions by either kaolin or alumina were obtained, and further the elutions of the two dehydrases from kaolin were similar.

The increase in Enzyme Activity of Milk.

In the course of this work it was noted that milk obtained fresh from the cow had a very low dehydrase content, measured with methylene blue, but that the activity increased greatly as the milk stood, and it was only after several days that the milk reached its maximum dehydrase content. Provided the milk was kept fresh it retained this maximum activity for several weeks.

Table 5. Milk No.1. (6. XI.1929.)

Hours after milking.	2	8	30
X. contained in 1 c.c.	0.76	0.94	2.22
Sa. contained in 1 cc.	1.07	-	1.10

Table 6. Milk No.11.(21.1.1930).

Hours after milking	4	28	52
X.contained in 1.cc.	0.99	3.45	3.51
Sa. contained in 1 c.c.	1.14	1.41	1.41

Table 7. Milk No.111.(30.IV.1930).

Hours after milking	4	7	10	26	51
X. contained in 1 c.c.	0.85	1.01	1.16	1.60	2.70
Sa. contained in 1 c.c.	0.75	0.79	0.86	1.08	1.08

Table 8. Milk No.1V.(1.V. 1930).

Hours after milking	4	26	51	100
X. contained in 1 c.c.	0.76	1.14	2.50	2.66
Sa. contained in 1 c.c.	0.74	-	1.04	1.03

Table 9. Milk No.V. (5.V.1930).

Hours after milking	4	9	29	50	73	98	170
X. contained in 1 c.c.	0.42	0.73	0.93	1.15	1.60	2.18	2.18
Sa. contained in 1 c.c.	0.64	0.73	0.78	-	-	0.87	-

Table 10. Milk No.V1. (17.V1.1930).

Hours after milking	4	11	29
X.contained in 2 c.c.	1.37	2.14	4.16
Sa. contained in 2 c.c.	1.29	1.66	1.75

Table 11. Milk No.V11 (20.V1.1930).

Hours after milking	3	7	11	28
X. contained in 2 c.c.	1.29	1.62	1.92	4.55

The milks 1 - V1 were obtained from the Veterinary College attached to Munich University. The milks 1 and 11 were from one cow. The milks 111, 1V, V, and V1 were also obtained from one cow, but a different cow than milks 1 and 11. The milk V11 was from one cow and was obtained from an entirely different source (Nederling). In all the cases the milks were treated immediately after milking with thymol, and in milk V toluene was also added, so that the increase/

/increase in activity cannot be attributed to bacterial action. None of the above milks were artificially cooled. Before each measurement the milks were shaken until homogeneous, so that true samples were obtained.

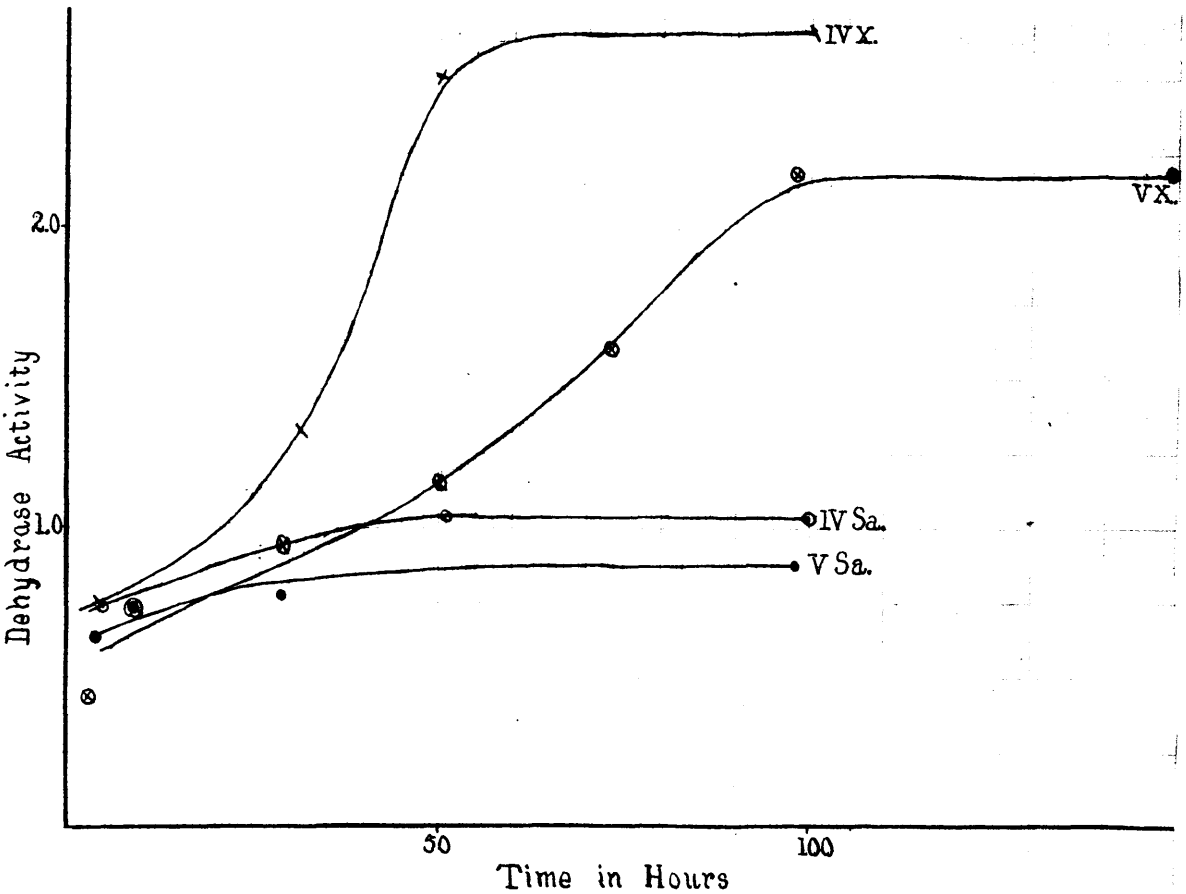


Fig.1. The increase in enzyme activity of milk on standing. IVX. and IVSa., increase in xanthine dehydrase and aldehydrase in milk No.IV. (Table 8.) VX. and V sa., increase in xanthine dehydrase and aldehydrase in milk No. V. (Table 9.).

The milks examined vary considerably, both in the amount of the increase and in the time the milks take to reach the maximum activity. All the milks however, show a very big increase in xanthine dehydrase, and a smaller yet apparent increase in aldehydrase. The increase in xanthine dehydrase activity goes slowly at the start and increases in velocity after some hours. This increase in enzyme activity is a property of all milks, for milks obtained from different cows, from different quarters, and at different seasons of the year, all showed this phenomenon.

Milks from various sources got at different times, have been found to have very similar maximum dehydrase activities. Nine milks out of ten measured have had xanthine dehydrase contents between 2.18X and 2.70X in 1 c.c. The tenth milk had an abnormally high dehydrase content of 3.51X in 1 c.c. The abnormally low xanthine dehydrase contents recorded by Wieland and Rosenfeld[1] of 1.45X, 1.20X, and 1.22X in 2 c.c. are undoubtedly due to the milks having been measured before they had reached their maximum activities. A similar constancy of the aldehydrase contents of various milks has been noted. From eight milks measured, seven had aldehydrase contents between 0.86 Sa. and 1.10 Sa. in 1 c.c. The eighth milk had 1.41 Sa. in 1 c.c., and it is of interest that it was the same milk which showed the abnormally high xanthine dehydrase content.

1) Loc. cit.

Skim-milks obtained by centrifuging off the cream from full milk within two hours of the time of milking were examined.

Table 12. Skim-milk from Milk 1.(6.XI.1929).

Hours after milking	4	48	96
X.contained in 2 c.c.	1.11	1.37	1.72
Sa. contained in 2 c.c.	0.68	0.69	-

Table 13. Skim-milk from Milk 11.(21.1.1930).

Hours after milking	4	28	52
X. contained in 2 c.c.	1.73	2.46	2.50
Sa. contained in 2 c.c.	0.83	0.94	0.94

A definite increase in the dehydrase activity is therefore also shown by skim-milk.

2 g. of cream which had been centrefuged from Milk 111 within 2 hours of milking was made up to 20 c.c. with distilled water containing thymol. This suspension when measured immediately and measured the next day showed a big increase in enzyme activity, the xanthine dehydrase content rising from 2.50 X. to 5.56 X. in 1 c.c. and the aldehydrase rising from 1.51 Sa. to 1.71 Sa. in 1 c.c.

The cream from milk VI was similarly treated to the above cream. The increase in dehydrase content was carefully followed.

Table 14. Cream from Milk v1. (17.V1.1930).

Hours after centrifuging	1	2	2½	5½	6	8¼
X. contained in 1 c.c.	0.49	-	0.85	1.53	-	2.30
Sa. contained in 1 c.c.	-	1.01	-	-	1.57	

A large increase in the activities of both dehydrases is therefore obtained, in such cream suspensions. The velocity of the increase is much greater than in milk.

Experiments to determine the cause of this increase in enzyme activity were carried out on Milk Vll, which was an average milk. Like all the recorded milks, this milk was obtained from one cow, and was not artificially cooled. Thymol was added shortly after milking.

Exp.1. Through 10 c.c. of this milk, at room temperature, pure nitrogen was slowly led for 3 hours. The Xanthine Dehydrase before and after this treatment was measured. A similar experiment passing air slowly instead of nitrogen was also completed.

X. in 2.0 c.c. milk before treatment with N2 and air- 1.29.
X. in 2.0 c.c. milk after treatment with N2 - 1.35.
X. in 2.0 c.c. milk after treatment with air - 1.30

The passing of the above gases appears to have inhibited the normal increase in dehydrase activity, for the milk by standing would have increased in activity to 1.55 X. in 2 c.c. (This figure is derived from the graph of dehydrase activity plotted against the time after milking of the examined milk).

Exp.2. Through 10 c.c. of milk, at room temperature, air was led very quickly for 2 hours. The strong agitation caused butter to be formed. The xanthine dehydrase was measured before and after.

X. in 2.0 c.c. original milk - 1.90
X. in 2.0 c.c. after passing air quickly - 4.54

A strong increase in activity is thus brought about. The butter formation is undoubtedly the cause.

Exp.3. Milk was shaken in an atmosphere of air, and also in a nitrogen atmosphere, obtained by passing nitrogen through the milk for 10 minutes. The milks were shaken simultaneously for 20 minutes.

X. in 2.0 cac. unshaken milk	- 1.90.
X. in 2.0 c.c. shaken with air	- 4.54
X. in 2.0 c.c. shaken with N2	- 4.65

No definite butter formation was apparent to the naked eye, but under the microscope the shaken milks were observed to have much bigger fat particles than the unshaken milk, and therefore shaking produced a coagulation of the fat particles.

Exp. 4. 2.0 c.c. of milk was treated with nitrogen for 5 minutes. The milk was now boiled in vacuum at 37° for 2 minutes. The vacuum was released with nitrogen and re-evacuated, and again released with nitrogen. The, thus-obtained, oxygen-free milk was shaken vigorously on a machine for 60 minutes.

X. in 2.0 c.c. original milk	- 2.06
X. in 2.0 c.c. after evacuation	- 2.04
X. in 2.0 c.c. after shaking	- 5.70

The experiment definitely proves that oxygen plays no part in enzyme activity. Evacuation as above has no effect.

Only one explanation can be given to explain the phenomenon. The enzymes contained in milk immediately after milking, appear to be adsorbed on the fat particles, or are in some way associated with them, in such a way that their activities are inhibited. As the milk stands there is a coagulation of the fat particles, accompanied by a reduction in the surface of the fat, with the result that the dehydrases are forced from the fat, and are thus capable of exercising their normal activities. Brisk agitation causes coagulation of the fat particles, and therefore accelerates the normal increase in activity.

Milks obtained from three different dairies and measured within 6 hours of milking, contained 4.85 X, 5.15 X. and 5.00 X. in 2 c.c. respectively. All the milks when kept 2 days showed no tendency to increase in activity. The activities shown compare with the maximum activities of the other milks examined.

The law demands that milk for sale be artificially cooled. The milk when obtained from the cow is immediately brought to the temperature of tap water (10°) by allowing it to flow slowly through coolers. A milk obtained from the Veterinary College which had been cooled in this way measured within 3 hours showed 5.00 X. in 2 c.c. and even after 2 days was found to have remained unchanged.

This artificial cooling of milk causes the increase in dehydrase activity to go very much more quickly than it normally does. In 3 hours or less the change is complete, whilst the uncooled milk requires days to reach its maximum activity. The artificial cooling of milk is known to change the creaming properties of the milk. In this cooling therefore some change in the physical state of the fat particles occurs.

The Effect of Various Factors on the Dehydrases.

A. The effect of the concentration of substrate on the reaction velocity of the dehydrogenation of xanthine and salicylaldehyde. The reaction velocity in the presence of both substrates.

A. Xanthine.

Dixon and Thurlow [1], and Wieland and Rosenfeld [2] studied the effect of the concentration of xanthine on the speed of dehydrogenation. This work has been repeated and verified.

1) Bioch. Jl., 18, 976 (1924).
2) Loc. cit.

Xanthine dehydrase has such a strong affinity for its substrate that the maximum activity is reached at very small substrate concentrations. The addition of more substrate reduces the velocity, probably due to the adsorptive blocking of the enzyme.surface by xanthine molecules. At a certain concentration an activity is reached, which is unaltered by the addition of more xanthine; at this concentration it is possible that the complete surface of the enzyme is covered with xanthine molecules.

The effect of substrate concentration on the reaction velocity has been studied with three different amounts of cream preparation. The experiments were done by the methylene blue decolorisation method, at p H. = 8.0, 37°, in a total volume of 5 c.c.

Table 15.

Moles Xanthine	$1X10^{-6}$	$2X10^{-6}$	$4X10^{-6}$	$8X10^{-6}$	$1.6X10^{-5}$	$3.2X10^{-5}$
X.in 10 mg.cream preparation.	4.55	4.00	3.37	3.03	4.92	1.87
X. in 7.5 mg.cream preparation	3.18	2.82	2.15	1.65	1.24	1.20
X. in 5 mg. cream preparation	2.22	2.02	1.62	1.02	0.79	2.74

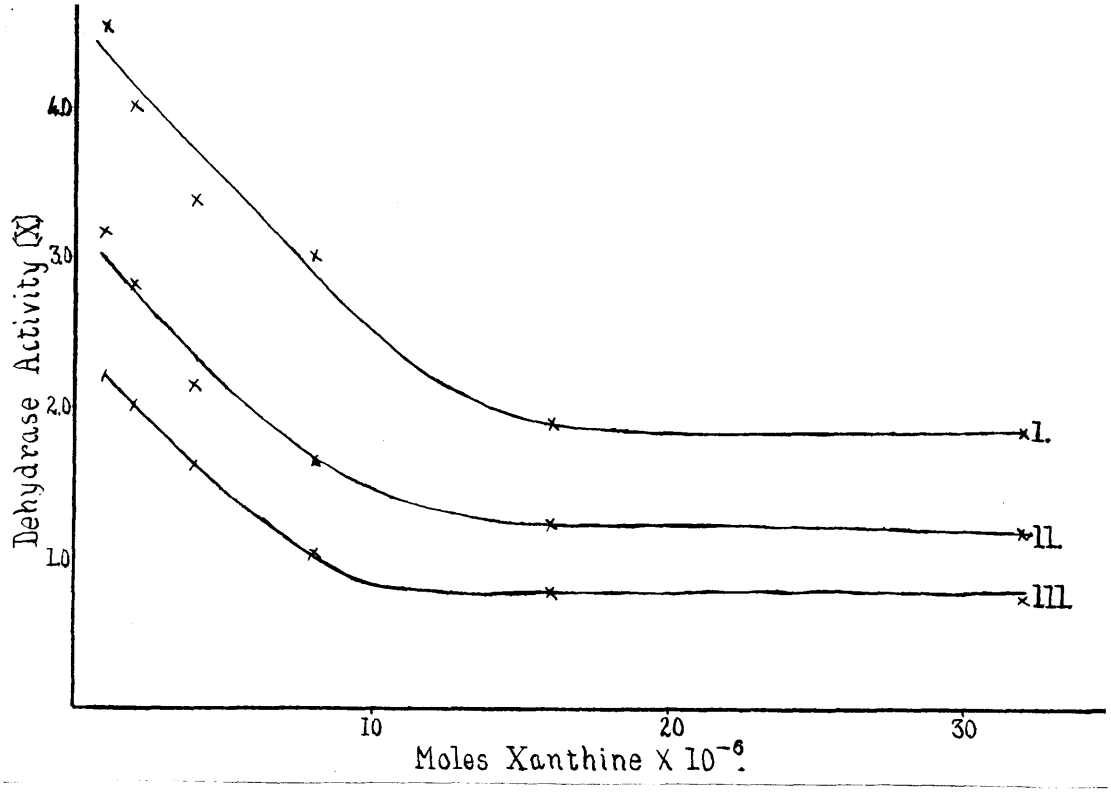


Fig.2. The effect of substrate concentration on the activity of xanthine dehydrase. I. 10 mg. cream preparation, II. 7.5 mg. cream preparation, III. 5 mg. cream preparation (Table 15.)

B. Salicylaldehyde and p.anisaldehyde.

The effect of concentration of Salicylaldehyde on activity of aldehydrase in cream preparation has formerly been made[1]. This was repeated using skim-milk as enzyme material. pH. = 8.0, 37°, volume = 5 c.c.

Table 16.

Moles salicylaldehyde	$2X10^{-6}$	$4X10^{-6}$	$8X10^{-6}$	$1.6X10^{-5}$	$3.2X10^{-5}$
Sa. in c.c. skim-milk	0.28	0.61	0.80	0.76	0.50

A similar series of experiments were carried out with p.anisaldehyde. The skim-milk used was slightly more active than that used for the salicylaldehyde experiments as 2 c.c. contained 0.94 Sa. as compared with 0.80 Sa. in the former milk. p H.=8.0, 37°, total volume = 5 c.c.

Table 17.

Moles p. anisaldehyde	$4X10^{-6}$	$8X10^{-6}$	$1.6x10^{-5}$	$3.2x10^{-5}$
An.A. in 2 c.c. skim-milk	0.25	0.40	0.48	0.46

1) H.Wieland and B.Rosenfeld, Loc.cit.

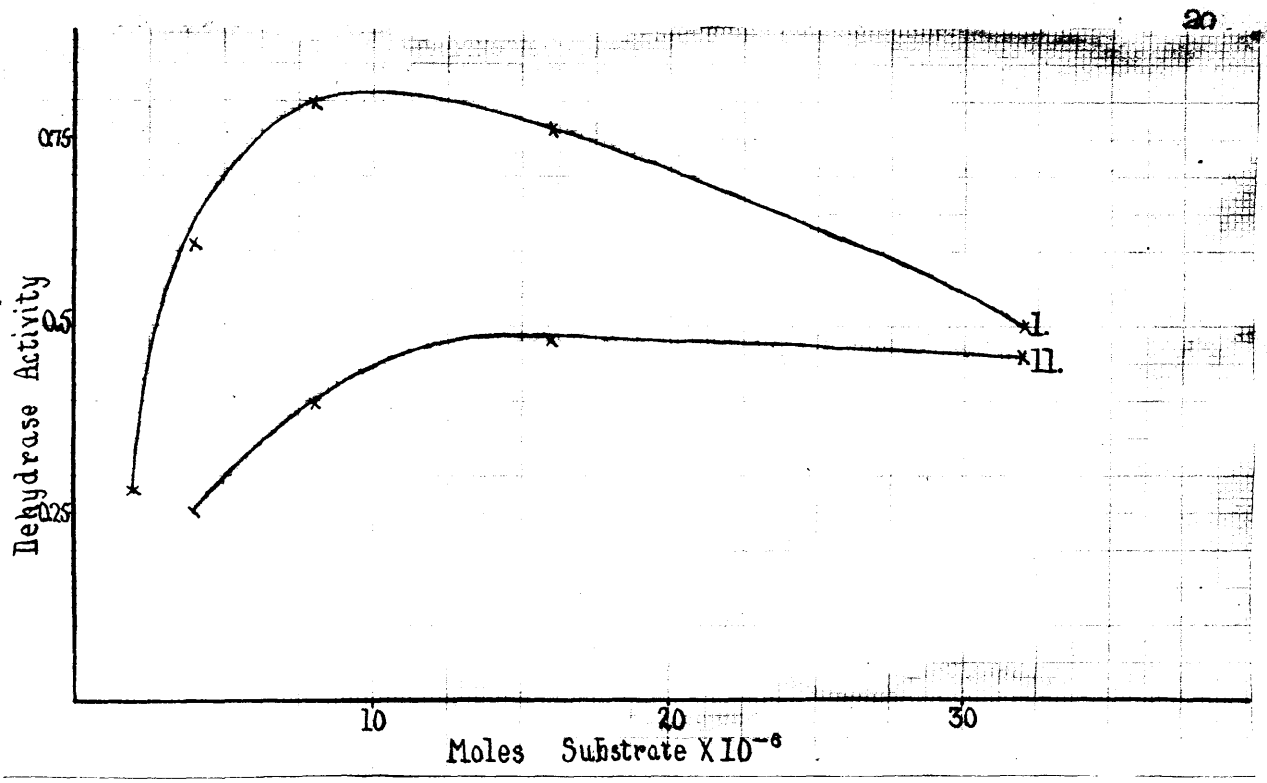


Fig. 3. The effect of substrate concentration on the activity of old hydrase. I. Salicylaldehyde (Table 16). II. p Anisaldehyde (Table 17).

c. Xanthine and Salicylaldehyde.

In the hope of showing that Xanthine dehydrase and Aldehydrase are distinct enzymes, experiments were carried out with mixed substrates for should the enzymes be distinct then in the presence of both substrates both enzymes should function and thus show increased activities with methylene blue.

The following experiments were carried out with equal quantities of cream preparation (10 mg.) at p H.=8.0, 37°, in a total volume of 5 c.c. The substrates were added with the methylene blue.

Table 18.

Moles Xanthine	$2X10^{-6}$	$2X10^{-6}$	$2X10^{-6}$	$2X10^{-6}$	$2X10^{-6}$	$2X10^{-6}$
Moles Salicylaldehyde	0	$2X10^{-6}$	$4X10^{-6}$	$8X10^{-6}$	$16X10^{-6}$	$3.2X10^{-5}$
Dehydrase activity	5.88	5.00	4.54	3.92	3.57	3.23

Table 19.

Moles Xanthine	$5X10^{-7}$	$5x10^{-7}$	$5x10^{-7}$	$5X10^{-7}$	$5x10^{-7}$
Moles Salicylaldehyde	$1x10^{-6}$	$2X10^{-6}$	$4X10^{-6}$	$8X10^{-6}$	$1.6X10^{-5}$
Dehydrase activity	5.72	6.25	5.16	4.17	3.70

Table 20.

Moles Xanthine	0	$1X10^{-6}$	$2X10^{-6}$	$4X10^{-6}$	$8X10^{-6}$	$1.6X10^{-5}$
Moles Salicylaldehyde	$4x10^{-6}$	$4X10^{-6}$	$4X10^{-6}$	$4X10^{-6}$	$4X10^{-6}$	$4X10^{-6}$
Dehydrase Activity	1.92	5.40	4.54	3.50	2.67	2.27

No distinct increase in dehydrase activity is noted by adding two substrates. It is evident however, that the addition of salicylaldehyde strongly inhibits the action of xanthine dehydrase, and that xanthine inhibits the action of aldehydrase. In the experiments it is possible that two partially inhibited reactions proceeded, namely, the xanthine dehydrogenation inhibited by salicylaldehyde and salicylaldehyde dehydrogenation inhibited by xanthine, the combined effect of which was to give times of decolorisation of methylene blue, comparable with those obtained using only one substrate.

d. Xanthine and Acetaldehyde.

The following experiment was carried out using xanthine and acetaldehyde together. The substrates were added with the methylene blue. p H.=8.0, at 37°, in a total volume of 5 cc.

Table 21.

Moles xanthine	$2X10^{-6}$	0	$2X10^{-6}$
Moles Acetaldehyde	0	$1X10^{-4}$	$1X10^{-4}$
Dehydrase activity	5.72	5.56	7.70

An apparent increase in dehydrase activity is thus obtained. The activity is not however the sum of the single activities, which must be due to the inhibitory action of acetaldehyde on xanthine dehydrase and of xanthine on aldehydrase.

This experiment is good evidence for the distinct nature of xanthine

/xanthine dehydrase and aldehydrase. Should these enzymes be identical it is difficult to explain how the above obtained result is possible.

B. The effect of sodium bicarbonate on the dehydrases contained in whey and cream preparation.

Wieland and Rosenfeld[1] found that the action of sodium bicarbonate on whey was to destroy the action of aldehydrase, but to have little effect on the xanthine dehydrase. These results have also been obtained.

To 60 c.c. of whey, 1.5 g. $NaHCO_3$ was added and allowed to stand at 37° for 10 minutes. For measurement the solution was brought to p H.=8.0 with $\frac{m}{5}$ primary phosphate.

Table 22.

	X.	%X.	Sa.	%Sa.	X.	%X.	Sa.	%Sa.
2.0 c.c. whey before $NaHCO_3$	2.65	100	0.89	100	2.05	100	0.86	100
2.0 c.c. whey after $NaHCO_3$	2.70	101.8	0.40	44.9	1.97	96.5	0.38	44.2

50 mg. of cream preparation was dissolved in 5 c.c. of thymol water and 0.5.g. $NaHCO_3$ added. The solution was kept at 37° for 15 minutes, and was then brought to p H.=8.0 with $\frac{m}{5}$ KH_2PO_4 for measurement.

Table 23.

	X.	%X.	Sa.	%Sa.
0.5 c.c. (5 mg) before $NaHCO_3$	2.65	100	1.05	100
0.5 c.c. (5 mg) after $NaHCO_3$	2.56	97.2	1.02	97.1

A very great difference between the enzymes in whey and cream preparation is apparent, for with cream preparation no destruction of the aldehydrase was obtained.

In order to find if the aldehydrase of whey is actually destroyed by $NaHCO_3$ or its action merely inhibited, a sample of whey which had been treated with the above quantity of $NaHCO_3$ was added to three/

1) Loc. cit.

/three times its volume of pure acetone. The precipitated enzyme bearing protein was redissolved in $\frac{m}{10}$ phosphate buffer p H=8.0. The solution obtained contained 14.6% of the original xanthine dehydrase and only 1% of the original aldehydrase. The yields of the dehydrases are so poor that this experiment has little value, but since the yield of xanthine dehydrase was much greater than aldehydrase it would appear that actual destruction of the aldehydrase is produced by $NaH\ CO_3$.

The dehydrases of whey are less stable than those in cream preparation in other ways. On standing for 24 hours at room temperature 25-35% of the enzymes in whey are destroyed, whilst with enzyme preparation less than 10% are destroyed. The whey enzymes are also more sensitive to destruction by evacuation.

C. The Effect of Hydrogen Peroxide on the Dehydrases of Cream Preparation.

The effect of hydrogen peroxide on xanthine dehydrase was measured by Wieland and Rosenfeld[1] and separately by Bernheim and Dixon[2]. The Method used in both cases was similar. To the enzyme solution, buffer, hydrogen peroxide and xanthine were added. After passing nitrogen for some time the solutions were evacuated and methylene blue added. The time of decolorisation was noted and allowing for the re-oxidation of the leuco methylene blue by hydrogen peroxide, it was possible to estimate the inhibition caused by the hydrogen peroxide. Both workers found that complete inhibition was brought about by concentrations of hydrogen peroxide of 5×10^{-4} m.

This work was repeated and similar results obtained. It was found however, that when the substrate was added with the methylene

1) Loc. cit.

2) F. Bernheim a. M. Dixon, Bioch. Jl., 22, 113(1928).

/methylene blue, instead of at the beginning of the experiment, that concentrations of hydrogen peroxide of $5X10^{-4}$ m. had practically no effect on the time of decolorisation, and even concentrations of $1X10^{-3}$ m. had little effect. The cause of this great difference produced by adding the xanthine with the methylene blue, instead of at the beginning, is at present inexplicable.

To enzyme solution $\frac{m}{5}$ phosphate buffer, p H.=8.0, and hydrogen peroxide were added. After nitrogen had been passed for 3 minutes, the solution was evacuated for 1 minute, released with nitrogen and re-evacuated. The methylene blue-xanthine mixture was now added, and the time of decolorisation noted.

Table 24.

Substrate = 0.2 c.c. $\frac{m}{10}$ xanthine; total volume = 5 c.c.; Temp.= 37°.

Concentration of H_2O_2	0	$5X10^{-4}$ m.	$1X10^{-3}$ m
X.	6.94	5.88	5.4
% Inhibition by H_2O_2	-	15.3	22.0

Similar experiments were carried out with acetaldehyde as substrate.

Table 25.

Substrate = 0.4 c.c. $\frac{m}{1}$ acetaldehyde; p H.=8.0; total volume 5 c.c. Temp.= 37°.

Concentration of H_2O_2	0	$5X10^{-4}$ m	$1X10^{-3}$ m
Aa.	12.5	11.7	11.1
% inhibition by H_2O_2	-	6.0	11.3

It is apparent that concentrations of even $1X10^{-3}$ m. H_2O_2 have little effect on either xanthine dehydrase or aldehydrase, for in the above experiments the larger times of decolorisation in the presence of hydrogen peroxide, must have at least been partially due to re-oxidation of the leuco methylene blue by hydrogen peroxide.

A more careful study of the effect of hydrogen peroxide on the dehydrases has been completed. To prevent the measurements being disturbed by hydrogen peroxide, catalase was added in the following experiments, just before the solutions were measured for the dehydrases. It was thus possible to determine accurately the actual destruction of the dehydrases by hydrogen peroxide.

To 25 mg. cream preparation dissolved in 2.5 c.c. $\frac{m}{10}$ phosphate buffer, p H.=8.0, and 2.5 c.c. $\frac{m}{5}$ phosphate buffer p.H.= 8.0, 5 c.c. $\frac{m}{5}$ H_2O_2 was added. The solution was kept 100 minutes at 37°. 1 c.c. of a solution of catalase (100 mg.catalase in 50 c.c.) was now added. The solution was now left 10 minutes at 37° after which time it gave no yellow coloration with titanium sulphate. The dehydrases were now estimated with methylene blue in the usual way. For each estimation 2.2 c.c. of the solution was used which contained 5 mg. of cream preparation.

This experiment was repeated with diminishing concentrations of hydrogen peroxide.

Table 26.

Concentration H_2O_2	10^{-1} m.	10^{-2} m.	10^{-3} m.	10^{-4} m.	10^{-5} m.	0
X.in 2.2 c.c. solution	0.10	1.39	2.26	2.57	2.57	2.58
Percentage present	3.9	54.1	88.0	100	100	-
Xa.in 2.2 c.c. solution	0.092	0.72	0.96	0.98	1.02	1.02
Percentage present	9.0	70.6	94.1	96.1	100	-

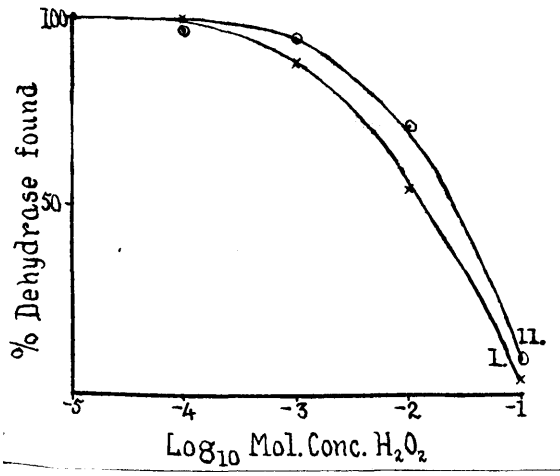


Fig.4. The effect of hydrogen peroxide on the dehydrases of cream preparation. 1. Effect on xanthine dehydrase, 11. effect on aldehydrase (Table 26.)

It is only when the concentration of hydrogen peroxide reaches 10^{-3}m. that destruction of enzyme occurs. At 10^{-1}m. the destruction is almost complete. Xanthine dehydrase is destroyed somewhat more quickly than aldehydrase, but the difference is not great.

The effect of evacuation on the enzymes of cream preparation, skim-milk, full milk and whey.

Cream Preparation.

In the estimation of the enzymes by the methylene blue method, the solutions are evacuated for 1 minute in order to remove the last traces of oxygen from the solution. It was noticed that when the solutions of cream preparations were kept in vacuum for longer periods, that the time of decolorisation of the methylene blue became greater. It appeared that during evacuation under these conditions that there occurred either a destruction of enzymes or an inhibition of their actions.

The destruction of aldehydrase obtained was always similar to the destruction of xanthine dehydrase and it was therefore regarded as sufficient to make a full study of the destruction of xanthine dehydrase.

The following experiments were carried out in order to ascertain the cause of the change in the enzymes. In all experiments the same amounts of enzyme solution were used.

Exp. a. Through 10 mg. of cream preparation dissolved in 1 c.c. $\frac{m}{10}$ phosphate buffer, p H=8.0, and 2.8 c.c. $\frac{m}{5}$ phosphate buffer, p H.= 8.0, in a Thunberg tube, nitrogen was passed for 3 minutes. A microburette, containing a mixture of five parts of $\frac{m}{1000}$ methylene blue to one part of $\frac{m}{100}$ xanthine, was introduced into the rubber stopper. The solution was evacuated for 1 minute, the vacuum released with nitrogen, and re-evacuated. A steady stream of nitrogen was now allowed to enter and 1.2.c.c. of the methylene blue-xanthine mixture was allowed to run in. The time of decolorisation of the methylene blue was noted. This is the normal method for the estimation of xanthine dehydrase, and no inhibition or destruction of enzyme occurs using this method.

Exp.b. This experiment was carried out exactly as experiment a. except that, after the second evacuation, the solution was allowed to stand 10 minutes in vacuum, before the addition of the methylene blue-xanthine mixture. whilst standing this methylene blue-xanthine mixture below the tap boiled and 1-2 drops of this mixture entered the solution.

Exp. C. Nitrogen was passed through the enzyme and buffer mixture for 3 minutes, and then a glass rod was introduced into the rubber stopper instead of the burette. The solution was evacuated for 1 minute, released with nitrogen, re-evacuated and allowed to stand 10 minutes. The vacuum was now released with nitrogen, the glass rod removed and the burette inserted. The tube was now evacuated and the methylene blue-xanthine mixture immediately added. This differs from Exp.b. in that during the long period of evacuation no methylene blue-xanthine mixture could enter the enzyme solution.

Exp.d. To the enzyme solution 2 drops of the methylene blue-xanthine mixture was added, and the experiment was then carried out in the same way as Exp.c.

Exp.e. To the enzyme solution 2 drops of methylene blue was added but no xanthine. The experiment was now carried out in exactly the same way as Exp.c.

Exp.f. To the enzyme solution 1 drop of $\frac{m}{100}$ xanthine was added, and the experiment carried out in the same way as Exp. c.

Exp. g. To the enzyme and buffer solution 0.2 c.c. $\frac{m}{100}$ xanthine was added, and nitrogen passed for 3 minutes. The solution was evacuated for 10 minutes, the vacuum released with nitrogen, and then the burette containing $\frac{n}{1000}$ methylene blue inserted. The solution was re-evacuated and then 1 c.c. of methylene blue run in immediately.

Exp.h. Exp. g. was repeated but instead of 2.8 c.c. buffer solution only 1.8 c.c. was added and 1 c.c. of a catalase solution ($\frac{4 \text{ mg.}}{\text{c.c.}}$)

Exp. i. The usual enzyme and buffer solution was evacuated for 10 minutes and then 0.2 c.c. $\frac{m}{100}$ xanthine was added. Nitrogen was passed for 3 minutes, the burette containing $\frac{n}{1000}$ methylene blue inserted, the solution evacuated and then 1 c.c. of methylene blue run in.

Exp. k. To the enzyme and buffer solution 1 drop of $\frac{m}{50}$ salicyl-aldehyde was added. Nitrogen was passed for 3 minutes and the solution was evacuated for 10 minutes. The vacuum was released with nitrogen, a burette containing the methylene blue-xanthine mixture inserted, the tube re-evacuated and 1.2 c.c. of the methylene blue-xanthine mixture added.

Exp.l. Exactly as Exp.k. except that 3 drops of $\frac{m}{50}$ salicylaldehyde was added instead of 1 drop.

Exp.m. Exp.l. was repeated but instead of 2.8 c.c. buffer solution only 1.8 c.c. was added and 1.c.c. of a catalase solution (4.mg./c.c.).

Table 27.

Exp.	a	b	c	d	e	f	g	h	i	j	k	m
X.	5.90	3.28	5.33	2.37	5.12	3.22	0.34	6.05	1.02	1.79	0.76	2.94
A.	100	55.7	90.6	40.2	87.0	54.8	5.8	102	17.3	30.3	12.9	49.8

In Exp.b., where 1-2 drops of the mixture of methylene blue and xanthine entered the tube during the long period of evacuation, the enzyme activity was reduced to almost half of the original activity.

Exp.c. shows that the small quantity of the methylene blue-xanthine mixture which entered the tube in Exp.b. played a part in the destruction of enzyme, for when this was avoided no appreciable destruction occurred. Exp.d. shows the effect of the deliberate addition of this quantity of methylene blue-xanthine mixture.

Exp.e. and Exp.f. show that xanthine is the destroying factor of the methylene blue-xanthine mixture.

Exp.g. shows that the destruction is increased by increasing the quantity of xanthine.

The protective action of catalase is shown in Exp.h. Exp.k. and Exp.l. show that salicylaldehyde has a similar effect to xanthine, but Exp.m. shows that catalase in this case produces only partial protection of the enzyme.

Exp.i. is important for it shows that ten minutes evacuation produces some change in the enzyme that renders it very sensitive to later inhibition by xanthine.

The probable cause of the foregoing results is the production of traces of hydrogen peroxide, by the dehydrogenation of the small traces of xanthine with the traces of oxygen, which are unavoidably present, as hydrogen acceptor. The concentration of hydrogen peroxide thus formed cannot be more than 10^{-4}m., which concentration has been shown to be harmless to the enzymes under normal conditions. It therefore appears that evacuation produces some change in the enzyme that makes it very sensitive to poisoning by hydrogen peroxide.

Wieland and Haussmann[1] showed that evacuation of a catalase solution destroyed the catalase, probably because during evacuation a coagulation of the enzyme particles occurs. It is possible that some similar change in the physical state of the enzyme occurs with cream preparation. The preserving action of catalase may be due to its power of decomposing hydrogen peroxide, but it is also possible that catalase prevents some change in the physical state of the enzyme, perhaps by functioning as a protecting colloid.

Skim-milk. To 2 c.c. of skim-milk 1.8 c.c. $\frac{m}{5}$ phosphate buffer, p H.=8.0 was added. Nitrogen was passed for 3 minutes and then a burette containing five parts of $\frac{n}{1000}$ methylene blue to one part of $\frac{m}{100}$ xanthine was inserted into the stopper. The solution was evacuated for 1 minute, the vacuum released with nitrogen, re-evacuated and allowed to stand 10 minutes, as before 1-2 drops of the methylene blue-xanthine mixture dropped into the solution. The enzyme content was now estimated by adding 1.2 c.c. of the methylene blue-xanthine mixture.

1) Ann. 445, 181 (1925).

Table 28.

	X.	% X.	Sa.	% Sa.
2.0 c.c. skim-milk without standing in vacuum	1.98	100	0.69	100
2.0 c.c. skim-milk after 10 minutes in vacuum	2.05	103.2	0.69	100

No destruction of the enzymes of skim-milk is therefore produced by evacuation under these conditions. The enzymes are apparently protected by something contained in skim-milk. This may be catalase or some protecting proteins.

Full Milk.

2.0 c.c. of full milk was treated in exactly the same way as the skim-milk.

Table 29.

	X.	% X.
2.0 c.c. full milk without standing in vacuum	1.49	100
2.0 c.c. full milk after standing 30 mins.in vacuum	1.37	92.0

Full milk apparehtly resembles skim-milk in its behaviour.

Whey.

2.0 c.c. of whey, obtained from skim-milk by coagulation of the casein with rennin, was treated in the same way as the skim-milk.

Table 20.

	X.	% X.
2.0 c.c. whey measured without standing in vacuum	2.04	100
2.0 c.c. whey measured after standing 10 mins.in vacuum	0	0

The whey enzymes appear to be even more sensitive to evacuation than even those in cream preparation.

The Effect of Calcium Chloride and Ammonium Carbonate on the Enzymes of Whey.

It was thought possible to obtain a purification of the enzymes of whey by adsorbing them on calcium carbonate, dissolving the adsorbent in acetate buffer and precipitating the enzymes from this solution with acetone. A good enzyme preparation was not obtained by this method, but a more important result was forthcoming. It was found that the addition of the ammonium carbonate and calcium chloride to whey produces complete inhibition of the xanthine dehydrase, but has little effect on the aldehydrase. When, however, the protein from this xanthine dehydrase inert solution are precipitated by acetone and redissolved the xanthine dehydrase regains its activity.

To 40 c.c. of whey, which had previously been treated with 200 mg. of kaolin to remove the heavier proteins (Soln. A), 8 c.c. of a freshly prepared saturated solution of ammonium carbonate was added. 2 c.c. of a calcium chloride solution (1 c.c.=118 mg. CaO) was added gradually, the solution being shaken meanwhile. A heavy precipitate of calcium carbonate separated. The flask was shaken 3 minutes and then the calcium carbonate was centrifuged off.

The enzymes in the remaining solution (Soln. B) were measured. In the measurement some calcium phosphate was precipitated due to the presence of a little calcium in the solution.

The calcium carbonate was dissolved in 16 c.c. $\frac{m}{1}$ acetate buffer, p H.=4.5 and this solution (Soln. C) was brought to p H.=8.0 with (a) $\frac{n}{20}$ $Na_2B_4O_7$ and (b) $\frac{n}{10}$ ammonia, for measurement.

Table 31.

	X.	% X.	Sa.	% Sa.
2.0 c.c. of whey (Solution A.)	2.38	100	0.68	100
25 c.c. of CaCO3 adsorbed solution (Solution B.)	0	0	0.215	35.8
1.0 c.c. of CaCO3 solution in acetate buffer (Solution C.) brought to p H.=8.0 with m/20 sodium borate	0.07	2.3	0.19	17.3
1.0 c.c. of CaCO3 solution in acetate buffer (Solution C.) brought to p H.=8.0 with n/10 ammonia.	0.055	1.8	0.18	16.5

The solution B. was also found to be inactive against hypoxanthine.

To 50 c.c. of solution B. 150 c.c. of acetone, previously purified by boiling with permanganate, was added. The precipitated protein was centrifuged off and washed with a mixture of 5 c.c. water and 15 c.c. acetone. After drying in a vacuum desiccator the precipitate weighed 560 mg.

250 mg. of this dry preparation was treated with 10 c.c. m/10 phosphate buffer, p H.=8.0 for 15 minutes at 37° and was then centrifuged. The solution was measured for enzymes.

Table 32.

	X.	% X.	Sa.	% Sa.
2.0 c.c. of above solution.	1.05	24.2	0.345	21.2

Solution C. was treated with acetone in the same way as Solution B. but the solid obtained was found inactive. Electro-dialysis of Solution C. to remove calcium salts also brought about destruction of the enzymes.

The Solution B. therefore contains xanthine dehydrase the action of/

/of which is in some way, inhibited. It was proved that ammonium carbonate is not the inhibiting factor, for the addition of ammonium carbonate to whey produces no inhibition.

2 c.c. of ammonium carbonate solution was added to 10 c.c. of whey and after heating for 10 minutes at 37° and allowing to stand 2 hours at room temperature no inhibition of the xanthine dehydrase was produced.

The precipitation of calcium phosphate in the measurement of Solution B. could possibly have caused the absence of the action of xanthine dehydrase, by its completely adsorbing the enzyme. The following experiments, where the calcium in Solution B. was removed by the addition of phosphate, before the precipitation of the proteins with acetone, prove that the precipitation of calcium phosphate in the measurement of Solution B. adsorbs very little xanthine dehydrase.

40 c.c. of whey was treated with 8.0 c.c. of ammonium carbonate solution and 2 c.c. calcium chloride solution. After removal of the calcium carbonate, 5 c.c. $\frac{m}{5}$ phosphate buffer, p H.=8.0 was added, and after being kept at 37° for 5 minutes the calcium phosphate was centrifuged off. The solution was treated with three times its volume of acetone as before, washed once and dried (dry weight= 570 mg.) 250 mg. of this dry preparation was dissolved in 10 c.c. $\frac{m}{10}$ phosphate buffer, p H.=8.0, centrifuged and measured.

Table 33.

	X.	% X.	Sa.	% Sa.
2.0 c.c. whey	2.10	100	0.87	100
2.5 c.c. solution after treatment with CaCO3	0	0	0.505	58.0
2.75 c.c. solution after treatment with phosphate buffer	0	0	0.42	48.3
2.0 c.c. solution of dry preparation	0.89	23.3	0.318	20.2

The cause of this peculiar effect of ammonium carbonate and calcium chloride cannot be determined. Some change in the physical state of the xanthine dehydrase, such as coagulation of the enzyme particles, may occur, accompanied by a loss of enzyme activity. This physical change could be reversed by redissolving the protein precipitated by acetone, in buffer solution, which would explain the reactivation of the xanthine dehydrase. A simpler explanation is that in the Solution B. there is something which is adsorbed by the xanthine dehydrase producing a blocking of the enzyme surface, and a consequent loss in activity. When the precipitated protein is redissolved in phosphate buffer the substance would no longer be present. It is impossible however, to say what could produce the adsorptive blocking.

The Question of the identity or distinct nature of aldehydrase and Xanthine dehydrase.

Whether the dehydrogenations of xanthine and aldehydes are brought about by the same enzyme, or whether the enzymes are distinct in character has not yet been definitely proved. Wieland and Rosenfeld[1] advanced experimental evidence favouring the distinct nature of the enzymes. They found that the proportions of alde-

1) Loc.cit.

/aldehydrase to xanthine dehydrase varied with the different samples of milk, cream, skim-milk, whey, or enzyme preparation measured. By adsorption on various adsorbents of these enzymes, these authors also found that the proportions of aldehydrase to xanthine dehydrase adsorbed, varied, which, of course, is only possible if aldehydrase and xanthine dehydrase are distinct enzymes.

It appears necessary to summarise the experimental evidence recorded on the foregoing pages, bearing on this subject.

The experiments on the adsorption of the enzymes of whey with kaolin and alumina (p.7) are definitely in favour of the view that the enzymes are identical, for the adsorption of xanthine dehydrase was always similar to that of aldehydrase, the differences recorded being almost within the range of the experimental error. The destructive effect of hydrogen peroxide (p.23) must also be regarded as evidence in favour of this view, for although a slightly stronger destructive effect on xanthine dehydrase was obtained, the difference was not great.

In favour of the view that the enzymes are distinct more experimental evidence has been gathered. The increase in the enzyme activity of milk (p.10) is perhaps the most important. It was found that the xanthine dehydrase content increased very much more than the aldehydrase. This appears to be possible only if xanthine dehydrase and aldehydrase are distinct. The experiments with mixed substrates (p.20) also favour the view that the enzymes are distinct. The complete inhibition of the action of xanthine dehydrase, and not of aldehydrase, by the addition of ammonium carbonate and calcium chloride to whey (p.31) is inexplicable if aldehydrase and xanthine dehydrase are identical.

A general survey of all the experimental evidence which has been gathered on this subject points definitely to the view that the enzymes are distinct in character. It appears, however, that they are associated with the same, or similar, proteins, for which reason they are exceedingly difficult to separate by selective adsorption or by other methods.

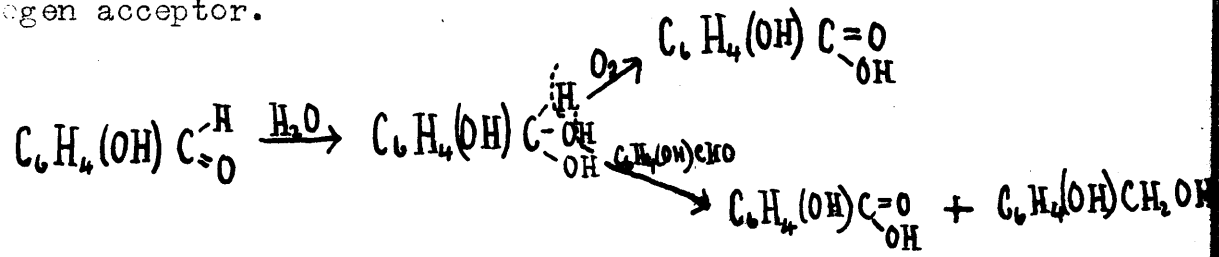
Part 2.

The Mutase of Cream Preparation and the Question of its Identity with Aldehydrase.

As was first discovered by Wieland [1], cows' milk has the property of bringing about the dismutation of salicylaldehyde into salicylic acid and salicyl alcohol. Under aerobic conditions the direct dehydrogenation of aldehyde to acid is the principal reaction, but the dismutation also proceeds to some extent. Under anaerobic conditions the dismutation alone proceeds.

Bach [2] found that his enzyme preparation from butter-milk could also bring about the Cannizzaro reaction with salicylaldehyde.

The dehydrogenation and dismutation were attributed by Wieland to the action of the same ferment - aldehydrase. Both reactions were regarded as dehydrogenations of the hydrated form of the aldehyde. Under aerobic conditions oxygen is the hydrogen acceptor, whilst under anaerobic conditions the aldehyde itself plays the part of hydrogen acceptor.



The dismutation of salicylaldehyde, and to a greater extent of acetaldehyde, by cream preparation, have been studied. Mutase and aldehydrase have been shown to be identical as is demanded by the above mentioned theory.

1) Loc. cit.

2) Loc. cit.

The Dismutation of Salicylaldehyde.

Using cream preparation, purified by precipitation from an aqueous solution, by acetate buffer p H.=4.5, and redissolving the precipitate in water, an enzyme solution was obtained, which contained so little buffering materials that it was possible to titrate minute quantities of salicylic acid therein, in the presence of salicylaldehyde, with baryta.

The addition of baryta to a solution of salicylaldehyde produces a solution the p H of which is from 8.0 to 8.4, due to the formation of the barium salt of salicylaldehyde. It is only in the presence of an excess of baryta that a strongly alkaline solution is obtained.

The procedure adopted for this work was to add a known quantity of baryta to an anaerobic solution of enzyme and salicylaldehyde, and after a given time to titrate the excess baryta with salicylic acid solution. In the dismutation salicylic acid was formed which was immediately neutralised by the barium salt of salicylaldehyde, so that no appreciable change in the p H occurred in the course of the reaction. The difference between the salicylic acid required to give methyl red a certain colour, and the salicylic acid required to give methyl red the same colour in a control experiment, carried out exactly in the same way except that a boiled enzyme solution was used, gave the quantity of salicylic acid formed in the dismutation.

To 500 mg. of cream preparation dissolved in 50 c.c. of water 2 c.c. $\frac{m}{1}$ acetate buffer, p H.=4.5 was added. After 2 minutes at 37° the precipitated protein was centrifuged off. This was washed twice with 10 c.c. of water. The precipitate was now dissolved in 12 c.c. water and a few drops of $\frac{n}{10}$ ammonia was added until it was just alkaline to methyl red. The solution thus obtained contained 6.1 Sa. in 1 c.c.

Through 3 c.c. of this solution (18.3 sa.) in a Thunberg tube, placed in a thermostat at 37°, nitrogen was led for 3 minutes. The solution was then evacuated to boiling for 1 minute, the vacuum released with nitrogen, re-evacuated and again released with nitrogen. In a strong stream of nitrogen 10 c.c. $\frac{m}{50}$ salicylaldehyde was added and then 1 c.c. $\frac{n}{20}$ baryta. The tube was kept at 37° for 1 hour.

Simultaneously with the above experiment a control experiment was run using 3 c.c. of the same enzyme solution, which had previously been boiled for 10 minutes over a Bunsen to ensure complete destruction of the enzymes. This control experiment was carried out exactly as the above experiment. To the control experiment 2 drops of methyl red solution was added and then the solution was titrated with $\frac{m}{100}$ salicylic acid until a slight redness was obtained. The solution from the experiment with unboiled enzyme solution was now titrated with $\frac{m}{100}$ salicylic acid until the same coloration was obtained. The difference in the titrations gave the amount of $\frac{m}{100}$ salicylic acid formed in the dismutation of salicylaldehyde.

Further experiments were carried out allowing 2 and 4 hours for the reactions to proceed. A further series of experiments was carried out at 50°.

Table 34.

Experiments carried out at 37°, using 18.3 Sa and 10 c.c. $\frac{m}{50}$ salicylaldehyde in a total volume of 14 c.c.

Time allowed at 37°	60 minutes	120 minutes	240 minutes.
c.c. $\frac{m}{100}$ Salicylic acid required for control	5.60	5.60	5.60
c.c. $\frac{m}{100}$ Salicylic acid required for experiment	5.20	5.10	4.85
c.c. $\frac{m}{100}$ Salicylic acid formed	0.40	0.50	0.75

Table 35.

Experiments carried out at 50° using 13.3 Sa. and 10 $\frac{m}{50}$ c.c. Salicylaldehyde in a total volume of 14 c.c.

Time allowed at 50°	30 minutes	30 minutes	120 minutes
c.c.m Salicylic acid required for control / 100	5.70	5.70	5.60
c.c.m Salicylic acid required for experiment / 100	5.15	4.85	4.70
c.c.m Salicylic acid formed / 100	0.55	0.85	0.90

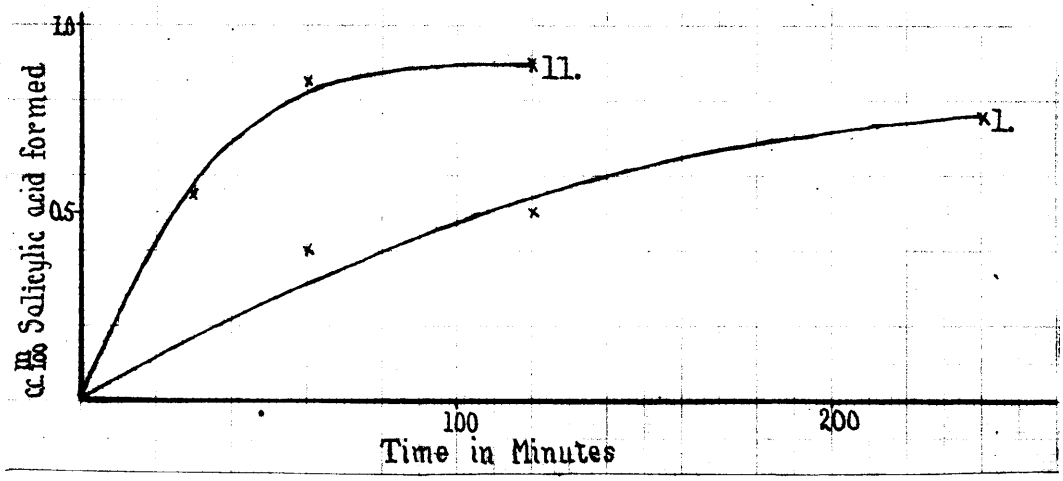


Fig. 5. Salicylic acid formed by the dismutation of Salicylaldehyde using the enzymes of cream preparation 1. at 37° (Table 34). 11. at 50° (Table 35).

The dismutation of salicylaldehyde is therefore brought about by the enzymes of cream preparation and cream preparation therefore contains the mutase contained in milk and in the preparation of Bach.

The dismutation proceeds more quickly at 50° than at 37°.

The Dismutation of Acetaldehyde.

In the enzyme solution used in the dismutation experiments with salicylaldehyde it was found that small quantities of acetic acid could be estimated in the presence of acetaldehyde, by titration with baryta using phenolphthalein as indicator. A much sharper end-point was obtained than in the corresponding experiments with salicylic acid and salicylaldehyde. A method was therefore/

/therefore obtained for the study of the dismutation of acetaldehyde by the cream preparation enzymes.

To 5 c.c. of enzyme solution containing 18.0 Sa., in a Thunberg tube, 4 c.c. of water was added and 2 drops of a 1% alcoholic solution of phenolphthalein. Through this solution pure nitrogen was passed for 3 minutes, the solution boiled by evacuation for 1 minute and the vacuum released with nitrogen. The solution was re-evacuated and the vacuum again released with nitrogen. In a strong stream of nitrogen 1 c.c. $\frac{m}{1}$ acetaldehyde was added and a micro burette containing $\frac{n}{20}$ baryta then introduced into the stopper. Baryta was added until a very slight coloration occurred (This required about 0.28.c. and was due to the unavoidable presence of acetic acid in the acetaldehyde.) 0.05 c.c. of baryta was now added and a distinct redness in the solution was obtained which gradually disappeared as acetic acid was formed by the dismutation of the acetaldehyde. On decolorisation more baryta was added. This was continued until no further decolorisation of the phenolphthalein occurred, which only was the case after 3 days.

A control experiment was run as above using boiled enzyme solution. At the beginning of the experiment some slight decolorisation of the phenolphthalein occurred but after 30 minutes no further decolorisation was noticeable. The baryta added in the control experiment amounted to only about 10% of the baryta required in the dismutation experiments. In calculating the amount of the dismutation this was allowed for.

Exp.1 a. Carried out at 37° using 18 Sa. in a concentration of m/10 acetaldehyde and a total volume of 10 c.c.

Table 36.

Time in minutes.	25	52	127	257	387	520	1260	1470	1760	2700
c.c.m/100 acetic acid found.	0.57	0.85	1.14	1.42	1.71	2.00	3.36	3.65	3.93	4.39
Controlled amount of acetic acid.	0.27	0.50	0.74	1.02	1.31	1.60	2.96	3.25	3.53	3.99

Exp.1.b. Repeat of Exp.1 a.

Table 37.

Time in minutes	35	145	295	1140	4050
c.c.m/100 acetic acid found.	0.57	0.85	1.14	3.12	3.88
Controlled amount of acetic acid.	0.27	0.50	0.74	2.72	3.48

Exp.11. Carried out at 37° using 18 Sa. in a concentration of m/5 acetaldehyde and a total volume of 10 c.c.

Table 38.

Time in minutes	40	97	155	1200	2700
c.c.m/100 acetic acid found.	0.57	0.85	1.14	4.18	4.46
Controlled amount of acetic acid.	0.27	0.50	0.74	3.78	4.06

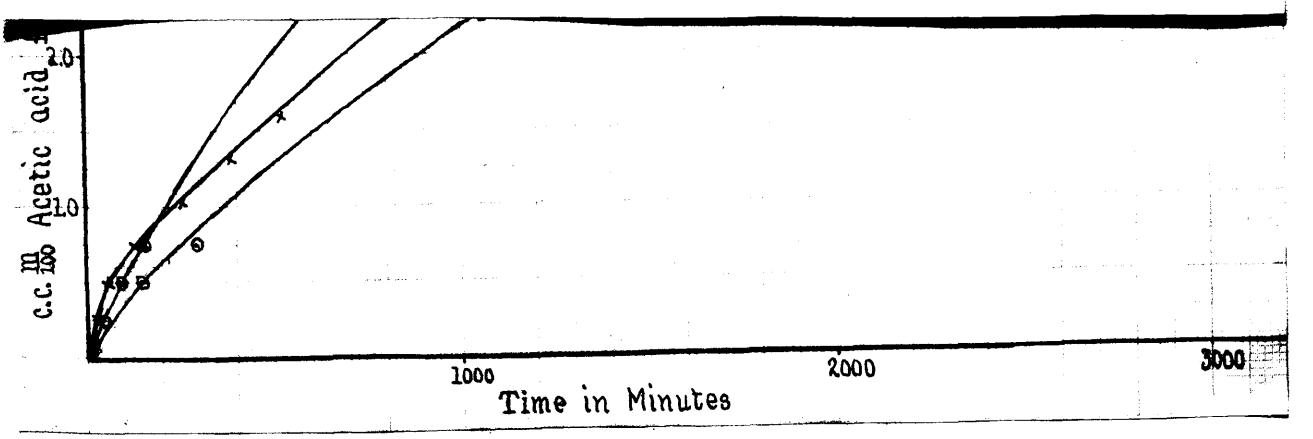


Fig. 6. The formation of acetic acid by the dismutation of acetaldehyde using cream preparation. 1. a. With m/10 acetaldehyde (Table 36) 1.b. With m/10 acetaldehyde (Table 37.) 11. With m/5 acetaldehyde (Table 38).

Exp.1 a. Carried out at 37° using 18 Sa. in a concentration of m/10 acetaldehyde and a total volume of 10 c.c.

Table 36.

Time in minutes.	25	52	127	257	387	520	1260	1470	1760	2700
c.c.m/100 acetic acid found.	0.57	0.85	1.14	1.42	1.71	2.00	3.36	3.65	3.93	4.39
Controlled amount of acetic acid.	0.27	0.50	0.74	1.02	1.31	1.60	2.96	3.25	3.53	3.99

Exp.1.b. Repeat of Exp.1 a.

Table 37.

Time in minutes	35	145	295	1140	4050
c.c.m/100 acetic acid found.	0.57	0.85	1.14	3.12	3.88
Controlled amount of acetic acid.	0.27	0.50	0.74	2.72	3.48

Exp.11. Carried out at 37° using 18 Sa. in a concentration of m/5 acetaldehyde and a total volume of 10 c.c.

Table 38.

Time in minutes	40	97	155	1200	2700

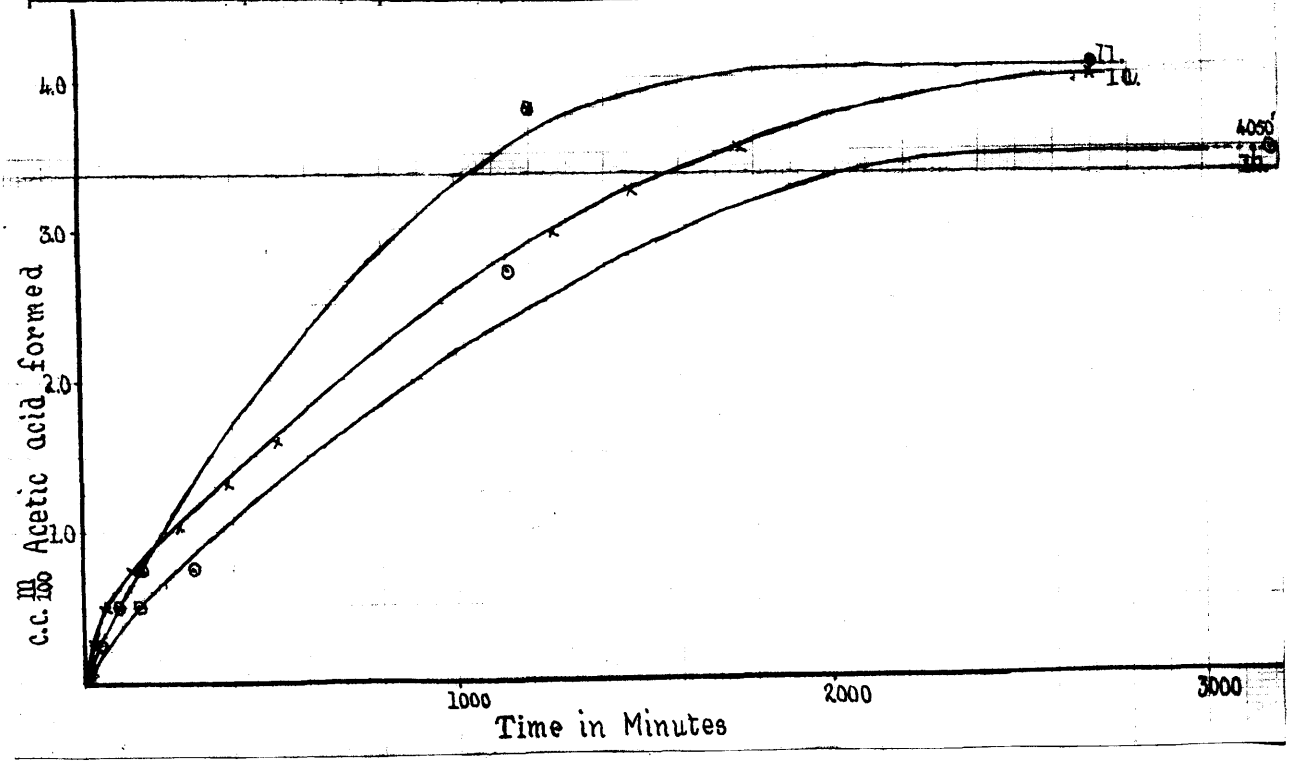


Fig. 6. The formation of acetic acid by the dismutation of acetaldehyde using cream preparation. 1. a. With m/10 acetaldehyde (Table 36) 1.b. With m/10 acetaldehyde (Table 37.) 11. With m/5 acetaldehyde (Table 38).

From the above experiments it is apparent that the dismutation of acetaldehyde proceeded with a diminishing velocity, due to the destruction of mutase which destruction was completed after 4000 minutes. In the experiments 1.and 11. the solutions were measured by adding 1 c.c. $\frac{m}{5}$ phosphate buffer, p H.= 8.0 and 1 c.c. $\frac{n}{1000}$ methylene blue, and measuring the time of decolorisation of the methylene blue. In experiment 1.a. 0.80% of the original aldehydrase was found and in 11, 0.48%. This shows that the destruction of mutase was accompanied by a destruction of aldehydrase.

The amounts of the dismutations in the experiments 1.a, 1.b. and 11. were similar, but the speed of dismutation in 11 was greatest - here the aldehyde concentration was twice that in 1 a. and 1 b. This increase in reaction velocity with increase in concentration agrees with the requirements of the dehydrogenation theory.

Other experiments exactly the same as experiments 1 a. and 1 b. were carried out. After varying times during which the dismutation had been allowed to proceed, the amounts of aldehydrase present were estimated, by adding 1 c.c. $\frac{m}{5}$ phosphate buffer p H.=8.0, 2.c.c. $\frac{n}{1000}$ methylene blue, and measuring the time of decolorisation. For each determination a complete new dismutation experiment was necessary.

Table 39.

Time of Dismutation reaction	120'	300'	1080'	2700'
% of original aldehydrase found present	54.8	39.8	15.2	0.80

Careful comparison of the results with the destruction of mutase as shown in the graphs 1 a. and 1 b., (Fig. 6) shows definitely that the destruction of aldehydrase and mutase proceed at very similar speeds.

At the beginning of the reactions there appear to be big destructions of enzymes, due, apparently, to the presence of traces of oxygen, for the graphs show that the rate of formation of acetic acid during the first 60 minutes is abnormally high. The only reasonable explanation that can be tendered, is that traces of oxygen cause the dehydrogenation of acetaldehyde, which type of dehydrogenation proceeds very quickly and which is accompanied by a big destruction of enzyme.[1] After a short time all the oxygen appears to be used up and the dismutations then proceed normally. A destruction of enzyme amounting to 30-35% of the total is apparently brought about in the early stages of the experiments by these traces of oxygen.

The destruction of aldehydrase subsequently is directly proportional to the acetic acid formed, which in turn is proportional to the amount of mutase destruction, so that the destruction of aldehydrase and mutase proceeds at similar speeds, which seems only possible if mutase and dehydrase are the same enzyme.

The Effect of Cyanide on (a) the Anaerobic Dehydrogenation of Acetaldehyde with Methylene blue, (b) the dismutation of Acetaldehyde.

(a) The anaerobic dehydrogenation with methylene blue.

Dixon[2] measured the effect of the addition of HCN on the dehydrogenation of acetaldehyde using methylene blue as hydrogen acceptor. Results similar to those obtained by Dixon have been obtained using cream preparation.

To 1 c.c. cream preparation containing 10 mg. dry preparation, so much $\frac{m}{5}$ phosphate buffer, p H.= 8.0 was added in order to give a final solution of 5 c.c. Nitrogen was passed for 3 minutes and then/

1) See p.49. 2) Bioch, Jl. 21, 940 (1927).

/then the solution was evacuated for 1 minute, released with nitrogen, re-evacuated and then a mixture of methylene blue, acetaldehyde, and HCN which had previously been allowed to stand 15 minutes to allow time for the formation of cyanhydrin, and which contained 1 c.c. $\frac{n}{1000}$ methylene blue, 0.5 c.c. $\frac{m}{1}$ acetaldehyde and the quantity $\frac{m}{10}$ HCN required to give the desired cyanide concentration, was run in. The time of decolorisation of the methylene blue was taken. The temperature was 37°.

Table 40.

Cyanide concentration	0	$\frac{m}{1000}$	$\frac{m}{500}$	$\frac{m}{250}$	$\frac{m}{100}$
Aa.	4.08	4.00	3.62	2.98	2.74
% Aa.	100	98.1	88.8	73.3	67.0

$\frac{m}{1000}$ Cyanide has therefore no inhibiting action on the anaerobic dehydrogenation of acetaldehyde whilst $\frac{m}{100}$ has only a very small inhibiting action.

(b) The Dismutation of Acetaldehyde.

Neuberg and Windisch [1] found that HCN has no effect on the dismutation of acetaldehyde by the acetic acid bacteria.

The effect of HCN on the dismutation of acetaldehyde by cream preparation has been studied. It has been found that $\frac{m}{100}$ HCN has a considerable inhibiting effect but $\frac{m}{500}$ HCN has little effect.

The experiments were carried out in exactly the same way as in the previous dismutation experiments. $\frac{m}{1}$ acetaldehyde and $\frac{m}{10}$ HCN solutions were mixed in the proportions required to give the desired acetaldehyde and cyanide concentrations in the final solutions. This mixture was allowed to stand for 15 minutes before being added to the anaerobic solution of enzyme. The further proceedure was exactly as in the foregoing experiments.

1) Biochem. Zs., 166, 454 (1925).

Experiment 1. Control experiment. Carried out at 37° with 18 Sa. Concentration of acetaldehyde = $\frac{m}{10}$. Total volume=10 c.c. No HCN.

Table 41.

Time in minutes	35	145	295	1140	4050
c.c. $\frac{m}{100}$ acetic acid found	0.57	0.85	1.14	3.12	3.88
Controlled amount of acetic acid.	0.27	0.50	0.74	2.72	3.48

Exp.11.a. Carried out at 37° with 18 Sa. Concentration of acetaldehyde = $\frac{m}{10}$ Concentration of HCN = $\frac{m}{500}$. Total volume = 10 c.c.

Table 42.

Time in minutes	45	130	300	1200	2700
c.c. $\frac{m}{100}$ acetic acid found	0.57	0.85	1.14	2.74	3.42
Controlled amount of acetic acid.	0.27	0.50	0.74	2.54	3.02

Exp.11.b. Repeat of 11.a.

Table 43.

Time in minutes	35	120	1140	4050
c.c. $\frac{m}{100}$ acetic acid found	0.57	0.85	2.85	3.67
Controlled amount of acetic acid.	0.27	0.50	2.45	3.27

Exp.111.a. Carried out at 37° with 18 Sa. Concentration of acetaldehyde = $\frac{m}{10}$. Concentration of HCN = $\frac{m}{100}$. Total volume = 10 c.c.

Table 44.

Time in minutes	30	75	900	1500	4350
c.c. $\frac{m}{100}$ acetic acid found	0.57	0.85	1.14	1.42	1.82
Controlled amount of acetic acid.	0.27	0.50	0.74	1.02	1.42

Exp.111.b. Repeat of 111.a.

Table 45.

Time in minutes	35	90	1000	2000	4350
c.c. $\frac{m}{100}$ acetic acid found	0.57	0.85	1.14	1.43	1.76
Controlled amount of acetic acid.	0.27	0.50	0.74	1.03	1.36

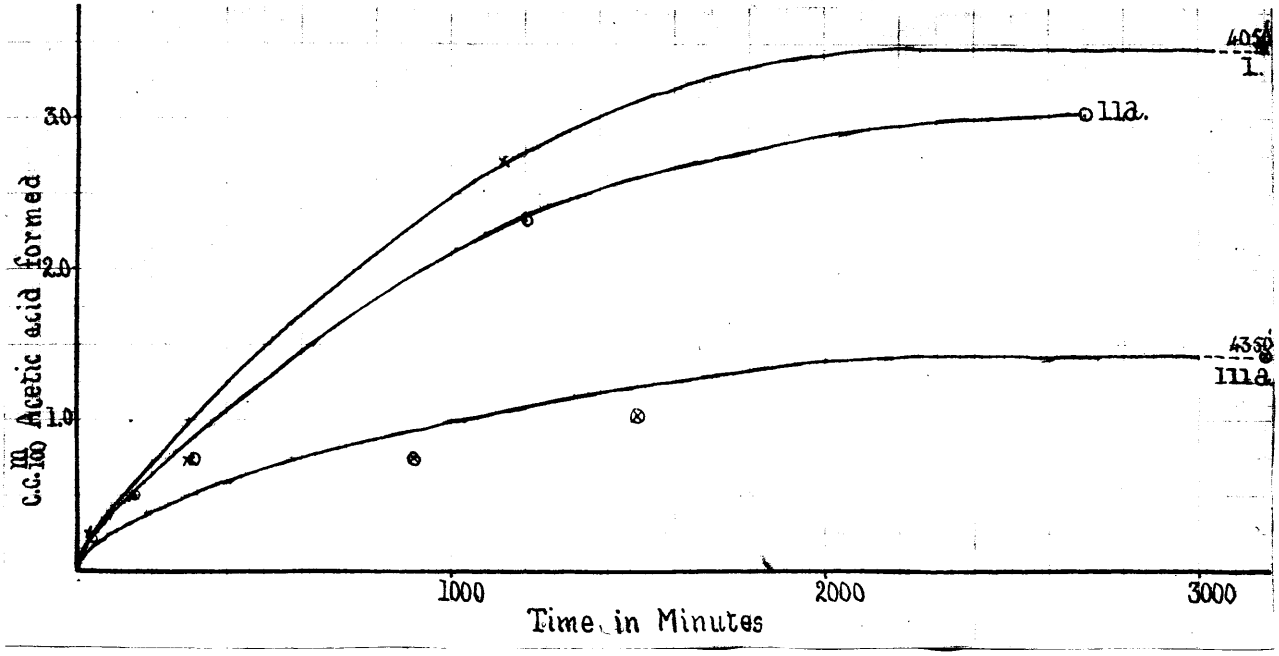


Fig. 7. The effect of cyanide on the dismutation of acetaldehyde. I. with no Cyanide (Table 41.) II.a. with $\frac{n}{500}$ cyanide (Table 42.) III.a. with $\frac{m}{100}$ Cyanide (Table 44.).

A comparison of the effect of cyanide on the anaerobic dehydrogenation with methylene blue, and the dismutation of acetaldehyde shows that the effects are very similar. $\frac{m}{500}$ cyanide has very little effect in both reactions, but $\frac{m}{100}$ has, in both reactions, an apparent inhibiting action. The inhibition of the dismutation is somewhat stronger, which is most probably due to the much longer time that the enzyme is under the influence of the cyanide.

The aerobic dehydrogenation of Acetaldehyde.

The aerobic dehydrogenation of acetaldehyde was followed, in the same way as the dismutation, by titrating the acetic acid formed.

To the enzyme solution containing 18 Sa. 2 drops of phenolphthalein was added. Nitrogen was passed for 3 minutes and the solution evacuated for 1 minute, the vacuum released with nitrogen, re-evacuated and then again released with nitrogen. 1 c.c. $\frac{m}{1}$ acetaldehyde was added in a strong stream of nitrogen and the solution was brought to a pale pink with baryta. A strong stream of CO_2-free air was then passed through the solution, and $\frac{n}{20}$ baryta was added as the phenolphthalein was decolorised.

Exp.1. Carried out at 37° with 18 Sa. Concentration of acetaldehyde= $\frac{m}{10}$. Total volume = 10 c.c.

Table 46.

Time in minutes	9	18	32	62	159	410
c.c.$\frac{m}{100}$ acetic acid found.	0.57	0.85	1.14	1.42	1.71	1.80.
Controlled amount of acetic acid.	0.27	0.50	0.74	1.02	1.31	1.40.

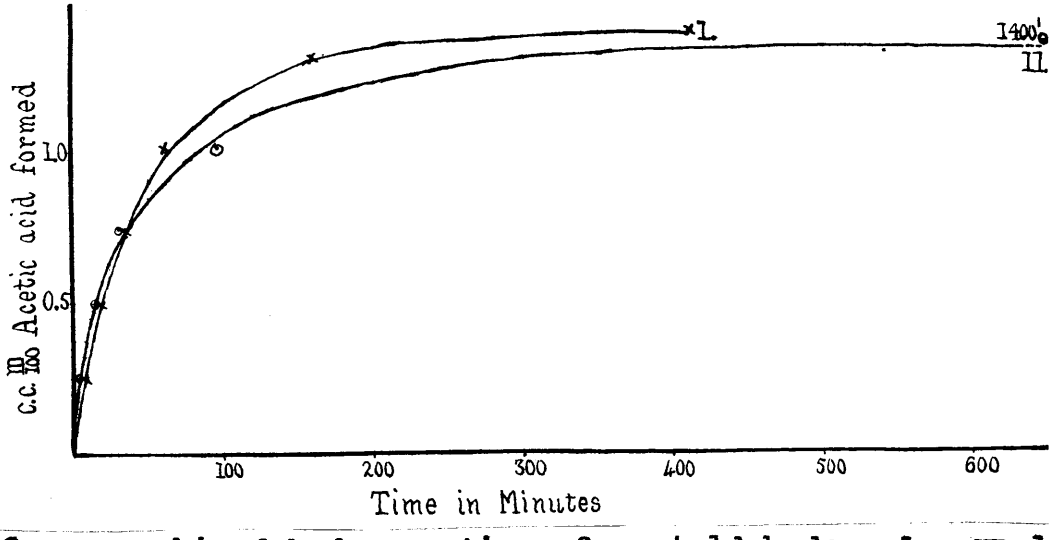


Fig.8. Aerobic dehydrogenation of acetaldehyde. I. Exp.1.(Table 46.) II. Exp. 11.(Table 47.)

Exp.11. Repeat experiment. As Exp.1.

Table 47.

Time in minutes	7½	14	32	97	300	1400
c.c.m acid found 100	0.57	0.85	1.14	1.42	1.71	1.77
Controlled amount of acetic acid.	0.27	0.50	0.74	1.02	1.31	1.37

The above enzyme solutions after the aerobic dehydrogenation would proceed no further were made oxygen free, by the usual method. More acetaldehyde was added and the solutions were kept at 37° for 18 hours. No further formation of acetic acid could be detected and the solutions no longer contained mutase.

A comparison of the aerobic dehydrogenation of acetaldehyde and the dismutation shows that the aerobic dehydrogenation goes very much more quickly and is complete in several hours, whilst the dismutation goes very much more slowly and requires several days to reach completion. The acetic acid formed by the dismutation is almost three times that produced in the aerobic dehydrogenation.

Bach[1] found that more salicylic acid resulted from the anaerobic dismutation of salicylaldehyde than in the aerobic reaction, using an enzyme preparation from butter-milk. Wieland[2] found, working with raw milk, that the aerobic reaction produced the more salicylic acid. The production of the greater quantity of salicylic acid in the aerobic dehydrogenation of salicylaldehyde with milk, is apparently due to the known protective actions of catalase and casein on the enzyme, allowing the enzyme to function for a greater time.

1) Loc. cit.

2) Loc. cit.

The Question of the Identity of Aldehydrase and Mutase.

The foregoing experiments must be regarded as definite proof of the identity of aldehydrase and mutase. The three following pieces of experimental evidence appear to be conclusive.

1. In the dismutation of acetaldehyde it has been shown that there is a gradual decrease in mutase and an equal gradual decrease in aldehydrase content. Further when the dismutation would proceed no further, it was found that no aldehydrase, measured against methylene blue, could be found. That is, that as mutase functions there is a gradual destruction of not only mutase, but also aldehydrase.
2. In the aerobic dehydrogenation of acetaldehyde it was found that when aldehydrase had been destroyed, that the enzyme solution no longer contained mutase. Therefore the functioning of aldehydrase not only brings about the destruction of aldehydrase, but also mutase.
3. The effects of cyanide on aldehydrase, measured against methylene blue and the effect on the dismutation of acetaldehyde are very similar.

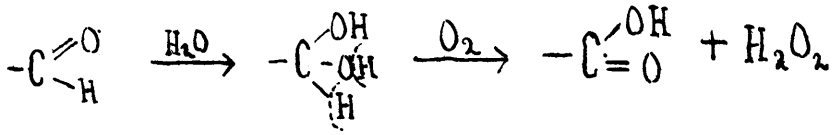
The mechanisms of the dismutation and aerobic dehydrogenation of aldehydes are therefore most probably the same, both being dehydrogenations of the hydrated form of the aldehyde; in the dismutation another molecule of aldehyde is hydrogen acceptor whilst in the aerobic dehydrogenation oxygen plays the part of hydrogen acceptor.

Part 3.

The Reaction Mechanism of the Aerobic Dehydrogenation of Aldehydes.

In the aerobic dehydrogenation of xanthine and hypoxanthine, it was shown by Wieland and Rosenfeld[1] that the reaction is a true dehydrogenation. One molecule of oxygen gives with one molecule of xanthine, one molecule of uric acid and one molecule of hydrogen peroxide.

It has been shown that the aerobic dehydrogenation of aldehydes proceeds in the same way, being true dehydrogenations.



The formation of hydrogen peroxide has been proved both directly and indirectly.

The destruction of enzymes in the aerobic dehydrogenation of xanthine, salicylaldehyde and acetaldehyde, and the protective action of Catalase thereon.

It has been noted by several observers that in the aerobic dehydrogenation of xanthine and salicylaldehyde that a destruction of enzyme occurs. Catalase has been shown to protect the enzymes in the aerobic dehydrogenation of xanthine, the function of catalase being to decompose the destructive hydrogen peroxide formed in the reaction as it is formed.

The actual destructions of aldehydrase and xanthine dehydrase brought about by the aerobic dehydrogenation of the above mentioned substrates have been measured. For the first time the effect of catalase on the aerobic dehydrogenation of aldehydes has been studied.

1) Loc.cit.

Exp.1. To 4 c.c. cream preparation solution containing 5.13 X and 2.14 Sa. per c.c., 3 c.c. $\frac{m}{5}$ phosphate buffer, p H.=8.0 were added and 1 c.c. $\frac{m}{100}$ xanthine. The solution was shaken for 15 minutes in air at 37°. 1 c.c. of a catalase solution (1 c.c.= 2 mg. catalase preparation) was added and the solution kept at 37° for 5 minutes. The xanthine dehydrase and aldehydrase were now measured with methylene blue in the usual way. Catalase was added to destroy the hydrogen peroxide formed, which, because of its power of oxidising leuco methylene blue, would interfere with the estimations.

Exp.2. Carried out as Exp.1, only 0.5 c.c. $\frac{m}{100}$ xanthine added, and 3.5 c.c. $\frac{m}{5}$ phosphate buffer p H.=8.0, in order to keep solution volumes the same.

Exp.3. To the same quantity of enzyme 3.5 c.c. $\frac{m}{5}$ phosphate buffer, p H.= 8.0, and 0.5 c.c. $\frac{m}{50}$ salicylaldehyde were added. The solution was shaken in air for 15 minutes at 37°. 1 c.c. of catalase solution was added and after 5 minutes at 37° the enzymes were measured.

Exp.4. Carried out as Exp.3, only 0.5 c.c. $\frac{m}{25}$ acetaldehyde added instead of 0.5 c.c. $\frac{m}{50}$ salicylaldehyde.

Exp.5. To 4 c.c. of the same enzyme solution 3.c.c. $\frac{m}{5}$ phosphate buffer, p H.= 8.0 and 1 c.c. of catalase were added. 1 c.c. $\frac{m}{100}$ xanthine was now added and the solution shaken under air for 20 minutes at 37°. The enzymes were then measured.

Exp.6. Carried out as Exp.5. Only 0.5 c.c. $\frac{m}{50}$ salicylaldehyde added instead of the xanthine and a correspondingly greater quantity of phosphate buffer.

Exp.7. Carried out as Exp.5, only with 9.5 c.c. $\frac{m}{5}$ acetaldehyde and no xanthine.

Table 48.

Exp.	1.	2.	3.	4.	5.	6.	7.
X.in 2.25 c.c.	1.80	3.44	0.77	2.38	4.87	2.53	4.87
% original X.	35.2	67.0	15.0	46.5	95.0	49.2	95.0
Sa.in 2.25 c.c.	0.85	1.45	0.52	1.56	2.34	1.50	2.40
% original Sa.	35.3	60.1	21.6	64.8	97.0	62.3	99.6

A very strong destruction of enzyme is therefore produced by the aerobic dehydrogenation of all the above substrates. The destruction of xanthine dehydrase and aldehydrase are in all cases similar. Catalase produces complete protection of the enzymes with xanthine and acetaldehyde, whilst with salicylaldehyde the protection is only partial.

Those experiments are strong evidence in favour of the theory that hydrogen peroxide is formed in the aerobic dehydrogenation of dehydrases, for the protective action of catalase must be due to its destroying hydrogen peroxide as it is formed. With acetaldehyde it appears that hydrogen peroxide is alone responsible for the destruction of enzymes. With salicylaldehyde it appears that two factors are responsible, for catalase only partially protects the enzymes. It is very probable that salicylaldehyde itself has a destructive action on the enzymes because of its phenolic nature, and if that is so then catalase plays the same part in the dehydrogenation as it does with acetaldehyde, namely, it decomposes hydrogen peroxide as it is formed.

The Effect of Methylene blue on the aerobic dehydrogenation of Salicylaldehyde and Anisaldehyde.

Leuco methylene blue has the property of reducing peroxides, and therefore because of this property should have a similar/

/similar protective action to catalase on the enzymes, in the aerobic dehydrogenation of aldehydes.

In the Barcroft apparatus enzyme was shaken with salicylaldehyde and anisaldehyde with air. The effect of the addition of methylene blue to this aerobic dehydrogenation was studied. In all the following experiments the enzyme was added from side vessels so that the reaction was followed from the beginning. The temperature was 37°.

Table 49.

	I.A.	I.B.	II.A.	II.B.	III.A.	III.B.	IV.A.	IV.B.
c.c.cream preparation.	3.0 (3Sa)	3.0 (3Sa)	3.0 (3Sa)	3.0 (3Sa)	3.0 (4Sa)	3.0 (4Sa)	3.0 (4Sa)	3.0 (4Sa)
c.c.m salicylaldehyde $\frac{M}{50}$	1.0	1.0	1.0	1.0	-	-	-	-
c.c.m phosphate buffer $\frac{M}{50}$ p H.=8.0	3.5	3.5	4.0	4.0	1.5	1.5	2.0	2.0
c.c.n methylene blue $\frac{N}{100}$	0.5	0.5	-	-	0.5	0.5	-	-
Oxygen absorbed $\times 10^{-5}$ M.	0.76	0.80	0.35	0.38	1.47	1.47	0.73	0.80

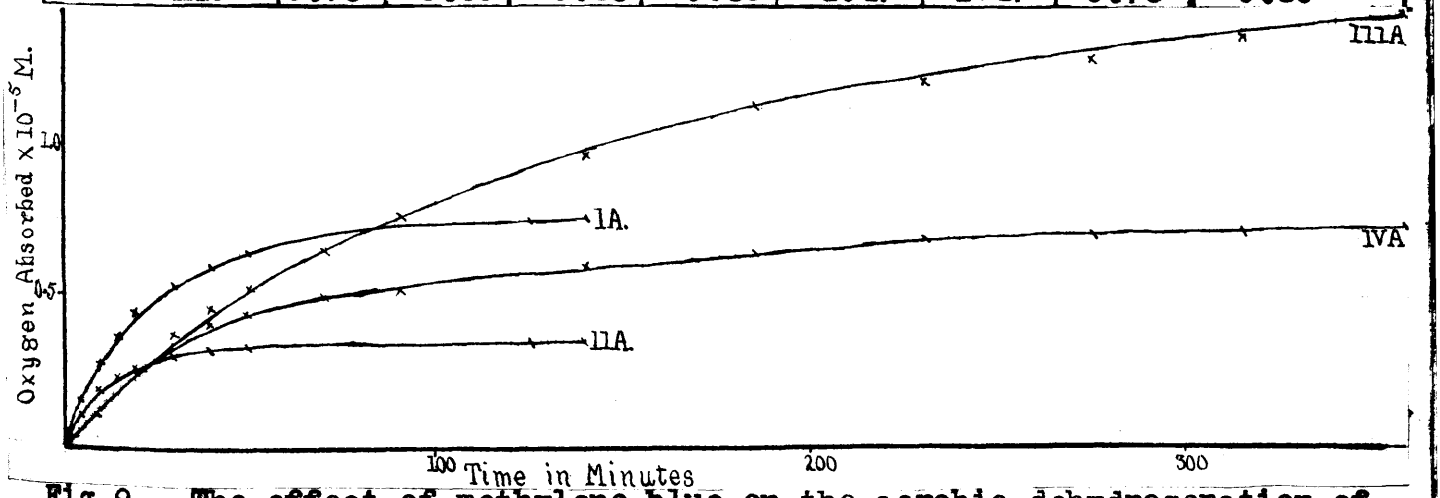


Fig.9. The effect of methylene blue on the aerobic dehydrogenation of salicylaldehyde and anisaldehyde. I.A. Salicylaldehyde with methylene blue. II.A. Salicylaldehyde without methylene blue. III.A. Anisaldehyde with methylene blue. IV.A. Anisaldehyde without methylene blue (Table 49.)

The protective action of methylene blue in the above reactions is very strong. This protective action is most probably due to the action of methylene on the formed peroxides.

The Aerobic Dehydrogenation of aldehydes and the effects of catalase and cerous hydroxide on the Dehydrogenations.

The estimation of H_2O_2 using $Ce(OH)_3$.

Wieland and Rosenfeld[1] first used cerous hydroxide for the estimation of the hydrogen peroxide formed in enzymatic reactions. The method of estimation of the ceric peroxide formed, used by the above authors has been improved. The method formerly used was to add potassium iodide and sulphuric acid to the ceric peroxide and titrate the liberated iodine with thiosulphate. Only about 60% of the expected quantity of hydrogen peroxide was found by this method. Using pure ceric peroxide a similar deficit has been obtained, the cause of which has been traced to the interaction of ceric salts and hydrogen peroxide giving oxygen. Even in the presence of potassium iodide this reaction can proceed.

$$2\,Ce(OH)_3OOH \xrightarrow{acid} 2\,Ce^{\cdots\cdot} + 2\,H_2O_2 + --$$

$$2\,Ce^{\cdots\cdot} + H_2O_2 \longrightarrow 2\,Ce^{\cdots} + O_2 + ---$$

Because of this secondary reaction no accurate estimation of ceric/

1) Loc. cit.

/ceric peroxide is possible by this method.

Two methods have been found to give theoretical results in the estimation of pure ceric peroxide. The addition of permanganate and sulphuric acid [1] to the ceric peroxide in the Barcroft apparatus and estimating the oxygen evolved, gave theoretical results. The second method used was to decompose the ceric peroxide with acetate buffer p H.=4.0, in the presence of catalase. The catalase destroyed the hydrogen peroxide as it was formed and the reaction proceeded thus:-

$$2\,Ce(OH)_3OOH \longrightarrow 2\,Ce^{\cdots\cdot} + O_2 + \cdots$$

The ceric salt was now estimated by making strongly acid with sulphuric acid, adding potassium iodide and titrating the liberated iodine with thiosulphate. The resulting titration represented only one third of the original hydrogen peroxide, for two thirds was lost as oxygen.

The method using permanganate and sulphuric acid was found unpractical for the estimation of ceric peroxide in the presence of enzyme, due to a strong evolution of gas resulting from the action of permanganate and sulphuric acid on enzyme preparation itself.

The catalase method has given good results in the presence of cream preparation.

From the enzyme solution the ceric peroxide and the adsorbed enzyme were centrifuged off. This was washed once with water.

1) R. Schwarz. u.H.Giese, Z.a.CH., 176, 217 (1928).

To the precipitate 3 c.c. of a catalase solution containing 4 mg. catalase per c.c. and 3 c.c. $\frac{m}{1}$ acetate buffer, p H.=4.0 were added. The mixture was kept for 1 hour at 37° and the brown colour of the ceric peroxide gradually changed to the yellow colour of ceric salts - oxygen was evolved. A few drops of potassium iodide solution was added and then sulphuric acid until the solution was strongly acid. After allowing to stand 1 minute at room temperature the liberated iodine was titrated with $\frac{n}{100}$ thiosulphate solution.

Using this method it was found possible to estimate 1×10^{-5} M H_2O_2, the experimental error being less than 10%.

The Aerobic Dehydrogenation of Acetaldehyde.

The absorption of oxygen in the aerobic dehydrogenation of acetaldehyde was measured. The influence of catalase on this reaction and also that of $Ce(OH)_3$ were studied. The experiments were carried out with cream preparation at p H.= 8.0 and 10.5, at 0° which temperature was maintained by running a strong stream of tap water through the thermostat. The enzyme solution was in all cases run in from a side vessel, so that the absorptions were followed from the time of mixing.

Table 50.

Exp.I.a, II.a. and III.a. carried out with one enzyme solution. Exp.I.b, II.b. and III.b. with a different solution. Temperature= 10.5°.

	I.		II.		III.	
	a.	b.	a.	b.	a.	b.
c.c.cream preparation	1.0 (7.2Sa)	1.0 (6.5Sa)	1.0 (7.2Sa)	1.0 (6.5Sa)	1.0 (7.2Sa)	1.0 (6.5Sa)
c.c.m $\frac{1}{1}$ acetaldehyde	0.5	0.5	0.5	0.5	0.5	0.5
c.c.catalase solution (4 mg/c.c.)	-	-	2.0	2.0	-	-
c.c.m $\frac{1}{50}$ $Ce_2(SO_4)_3$	-	-	-	-	2.0	2.0
c.c.m $\frac{1}{20}$ $Na_2B_4O_7$	-	-	-	-	2.0	2.0
c.c.m $\frac{1}{5}$ phosphate buffer p H.=8.0	2.0	2.0	2.0	2.0	-	-
c.c. water	4.5	4.5	2.5	2.5	2.5	2.5
Oxygen absorbed $\times 10^{-5}$M.	3.20	2.93	3.92	2.61	2.71	1.85
H_2O_2 found by estimation $\times 10^{-5}$M	-	-	-	-	2.33	1.68
% theory	-	-	-	-	86	91
% original aldehydrase found at end of Exp.	-	5	-	100	-	-

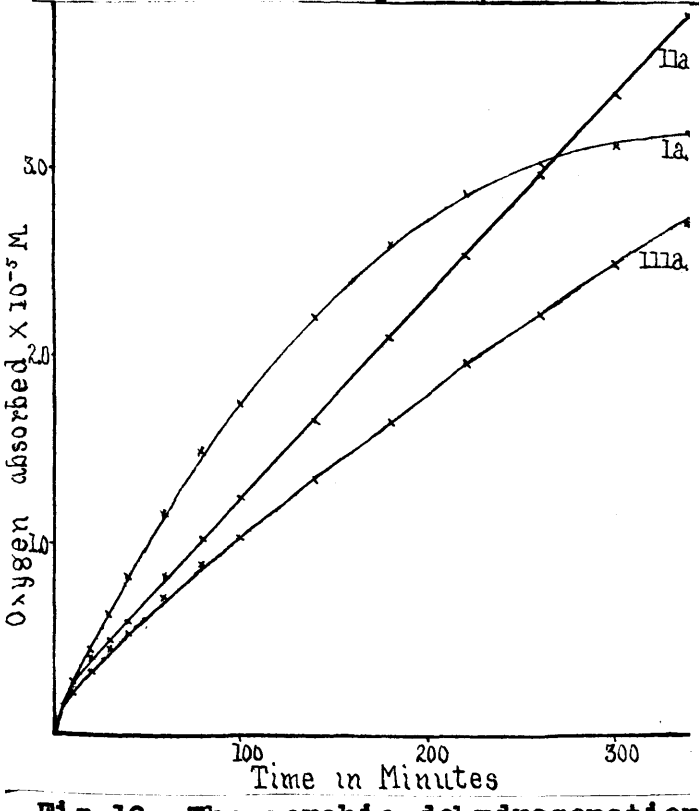


Fig.10. The aerobic dehydrogenation of acetaldehyde. I.a.with phosphate buffer alone (Table 50.) II.a.with catalase. III.a.with $Ce(OH)_3$(Table 50.)

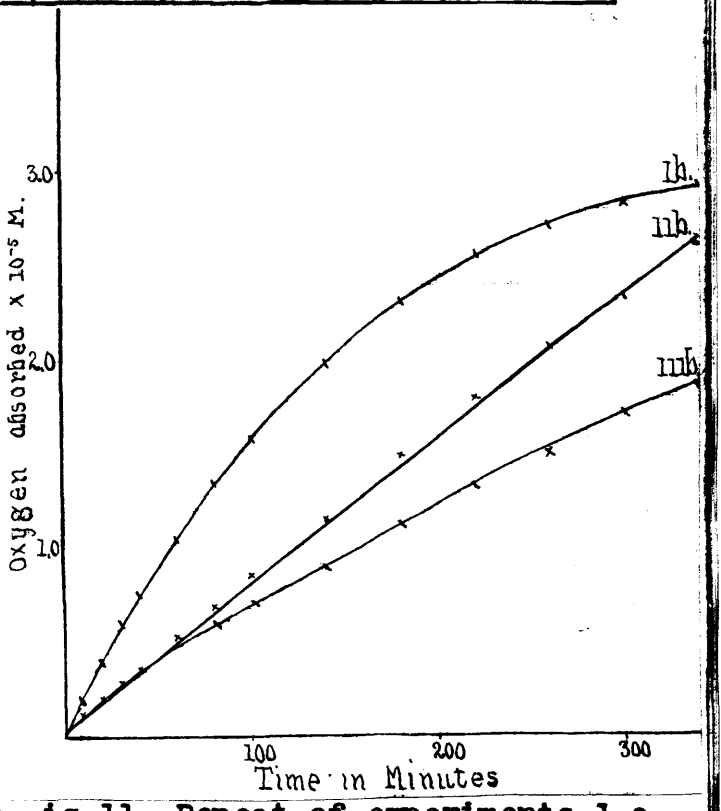


Fig.11. Repeat of experiments I.a. II.a.& III.a.using less ferment. I.b. with phosphate alone. II.b. with catalase. III.a.with $Ce(OH)_3$

In the dehydrogenation of acetaldehyde in the absence of cerous hydroxide and catalase (Exp.1.a. and 1.b.) there is a destruction of enzyme as is shown by the diminishing speed with which the reaction proceeds, and also by measurement of the enzyme at the end of the experiment, for only 5% of the original enzyme was found in the solution by estimation with methylene blue.

In the presence of catalase (Exp.11.a. and 11.b.) the oxygen absorption proceeds more slowly, but it continues with a uniform speed right to the end of the experiment. At the end of the experiment, estimation by the methylene blue method showed that no destruction of enzyme had taken place. The cause of the difference between the experiments with and without catalase must be due to the formation of hydrogen peroxide in the dehydrogenation, which in the absence of catalase brings about the destruction of enzyme, but which in the presence of catalase is immediately decomposed before it can affect the enzyme. The greater velocity of oxygen uptake at the start shown in the absence of catalase is not due to the dehydrogenation speed being greater, but is due to half of the absorbed oxygen in the experiment with catalase, being regenerated by decomposition of the hydrogen peroxide.

The experiments with cerous hydroxide (ExpIIIa. and ExpIIIb.) prove definitely that hydrogen peroxide is formed in the aerobic dehydrogenation of acetaldehyde. As the experiments proceeded the white cerous hydroxide suspension gradually changed to the yellow-brown ceric peroxide, due to the interaction of cerous hydroxide and the hydrogen peroxide formed. The estimation of the hydrogen peroxide formed was done by centrifuging off the cerium precipitate, washing it once with water and estimating the ceric peroxide present by the/

/the catalase method previously described. The hydrogen peroxide found was nearly the theoretical quantity required by the dehydrogenation theory. The deficiency of about 10% is most probably due to some hydrogen peroxide reacting with acetaldehyde before cerous hydroxide.

The speed of the reaction in the presence of cerous hydroxide is slow compared with the speeds with catalase and with neither catalase nor cerous hydroxide. This is undoubtedly caused by the adsorption of the enzyme on the cerous hydroxide, for a similar diminution of enzyme activity was obtained by adding the same quantity of cerous hydroxide to the anaerobic dehydrogenation of acetaldehyde using methylene blue as hydrogen acceptor. In this anaerobic reaction the addition of cerous hydroxide reduced the velocity of the reaction to 35.0% of its velocity in the absence of cerous hydroxide.

The aerobic dehydrogenation in the presence of cerous hydroxide proceeds at a practically uniform speed showing that the cerous hydroxide has a protective action on the enzyme. This is evidently due to its combining with hydrogen peroxide and thus removing it from the solution.

<u>The Aerobic Dehydrogenation of formaldehyde.</u>

The following experiments were carried out in exactly the same way as the corresponding experiments with acetaldehyde. Three times the enzyme quantity used in experiments with acetaldehyde was used in these experiments. The enzyme solution was again added from the side vessels.

Table 51.

Exp. I.a, II.a. and III.a. carried out with the same enzyme solution. Exp. I.b. II.b. and III.b. with a different solution. Temperature = 10.6°.

	I.		II.		III.	
	a.	b.	a.	b.	a.	b.
c.c. enzyme solution m/50	3.0 (21.6.Sa)	3.0 (19.5Sa)	3.0 (21.6Sa)	3.0 (19.5Sa)	3.0 (21.6Sa)	3.0 (19.5Sa)
c.c. m/10 formaldehyde	0.5	0.5	0.5	0.5	0.5	0.5
c.c. Catalase solution (4 mg./c.c.)	-	-	2.0	2.0	-	-
c.c. m/50 $Ce_2(SO_4)_3$	-	-	-	-	2.0	2.0
c.c. m/10 $Na_2B_4O_7$	-	-	-	-	2.0	2.0
c.c. m/5 phosphate buffer p H.=8.0	2.0	2.0	2.0	2.0	-	-
c.c. water	2.5	2.5	0.5	0.5	0.5	0.5
Oxygen absorbed X 10^{-5}M	2.77	2.51	3.35	3.16	1.91	1.54
H_2O_2 found by estimation X10^{-5}M.	-	-	-	-	1.38	1.25
% theory	-	-	-	-	73	81
% aldehydrase found at end of experiment.	-	3	-	26	-	-

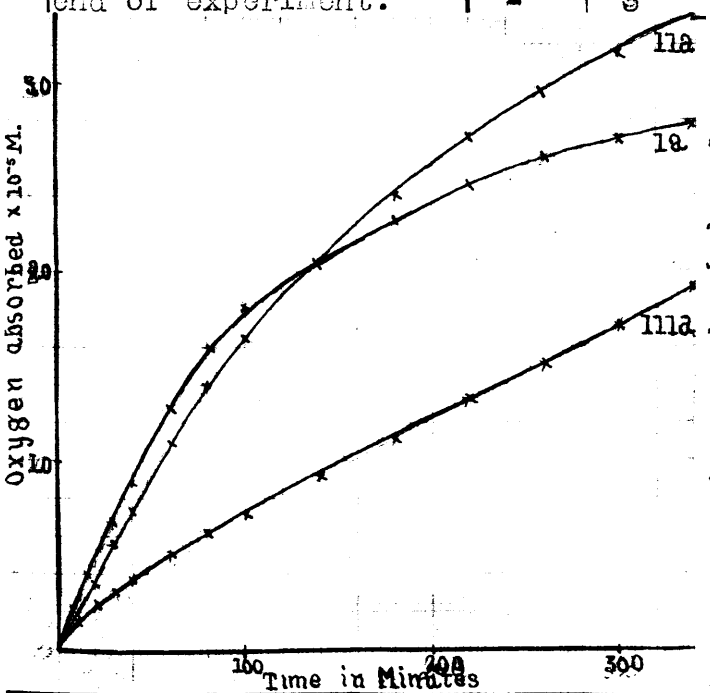


Fig.12. The aerobic dehydrogenation of formaldehyde. I.a. phosphate buffer alone (Table 51) II.a. with catalase (Table 51) III.a. with Ce $(OH)_3$.

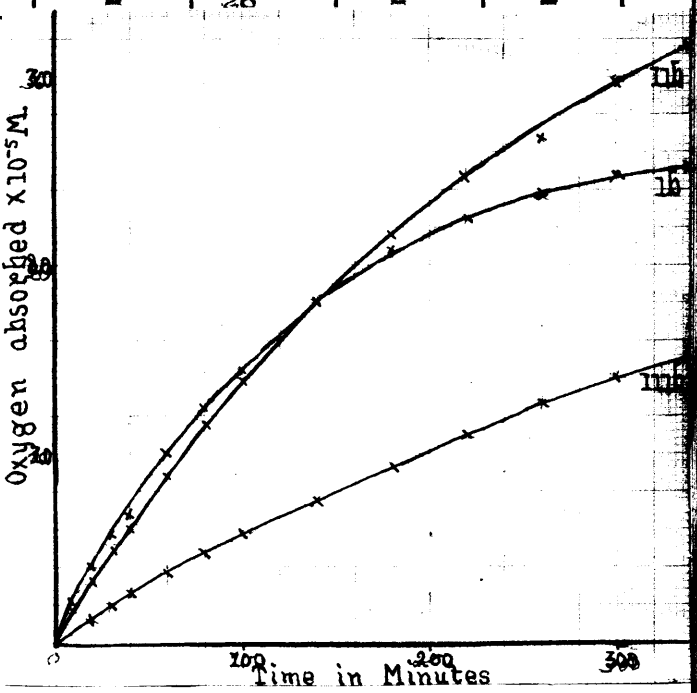


Fig.13. Repeat of experiments I.a. II.a. and III.a. using less ferment. I.b. with phosphate alone (Table 51), II.b. with catalase (Table 51) III.b. with Ce $(OH)_3$.

In the absence of both catalase and cerous hydroxide (Exp.1.a. and 1.b.) there is again a very quick destruction of enzyme. Only 3% of the original aldehydrase, estimated by the methylene blue method, was found at the end of the experiment showing the destruction of enzyme to be almost complete.

With catalase (Exp.11.a. and 11.b.) the absorption again starts more slowly than in the absence of catalase due to the regeneration of oxygen from hydrogen peroxide. In these experiments however, there is a very considerable diminution in the speed of absorption as the reaction proceeds which was not so in the corresponding experiments with acetaldehyde. This is due to a destruction of enzyme in the dehydrogenation of formaldehyde even in the presence of catalase for only 26% of the original enzyme was found at the end of the reaction. It is apparent however that catalase had a decided protective action even with formaldehyde, as is shown by the graphs and also by estimation of the enzymes at the end of the reactions. This destruction of enzyme in the presence of catalase is probably caused by a poisoning action of formaldehyde itself, on either the catalase or more probably on the aldehydrase.

The experiments with cerous hydroxide (Exp.111.a. and 111.b.) show a very strong resemblance to the corresponding experiments with acetaldehyde. The formation of a yellow-brown coloration proceeded with the absorption of oxygen, showing the formation of ceric peroxide. At the end of the experiment the ceric peroxide was estimated by the catalase method, and the amount of hydrogen peroxide this represented was again found to be nearly the theoretical quantity. Again the deficiency may have been due to the interaction of hydrogen peroxide and formaldehyde. The inhibition of the reaction by cerous hydroxide

is again probably due to the adsorption of the enzyme on the cerous hydroxide, for the anaerobic dehydrogenation of formaldehyde with methylene blue as hydrogen acceptor was inhibited to 53% of its activity by the presence of cerous hydroxide. That cerous hydroxide has a protecting action on the aldehydrase is shown by the uniform speed with which the reaction proceeds.

The Aerobic Dehydrogenation of benzaldehyde.

The following experiments with benzaldehyde were carried out in the same way as those with acetaldehyde and formaldehyde. The quantity of enzyme used in the experiments with formaldehyde was used. As aldehydrase has a much greater affinity for benzaldehyde than for acetaldehyde and formaldehyde it was possible to work with much smaller concentrations of benzaldehyde. The enzyme solution was again added from the side vessels.

Table 52.

Exp.I.a., II.a. and III.a. carried out with the same enzyme solution.
Exp.I.b., II.b. and III.b. carried out with a different solution.
Temperature = 10.5°.

	I.		II.		III.	
	a.	b.	a.	b.	a.	b.
c.c. enzyme solution	3.0 (21.6Sa)	3.0 (19.5Sa)	3.0 (21.6Sa)	3.0 (19.5Sa)	3.0 (21.6Sa)	3.0 (19.5Sa)
c.c. $\frac{m}{50}$ benzaldehyde	1.0	1.0	1.0	1.0	1.0	1.0
c.c. catalase solution (4mg./c.c.)	-	-	2.0	2.0	-	-
c.c. $\frac{m}{50}$ $Ce_2(SO_4)_3$	-	-	-	-	2.0	2.0
c.c. $\frac{m}{20}$ $Na_2B_4O_7$	-	-	-	-	2.0	2.0
c.c. $\frac{m}{5}$ phosphate buffer p H.= 8.0	2.0	2.0	2.0	2.0	-	-
c.c. water	2.0	2.0	-	-	-	-
Oxygen absorbed $\times 10^{-5}$M.	0.101	0.83	0.80	0.63	1.83	1.41
H_2O_2 found by estimation $\times 10^{-5}$M.	-	-	-	-	1.35	1.25
% theory	-	-	-	-	74	89
% aldehydrase found at end of experiment	-	32	-	78	-	-

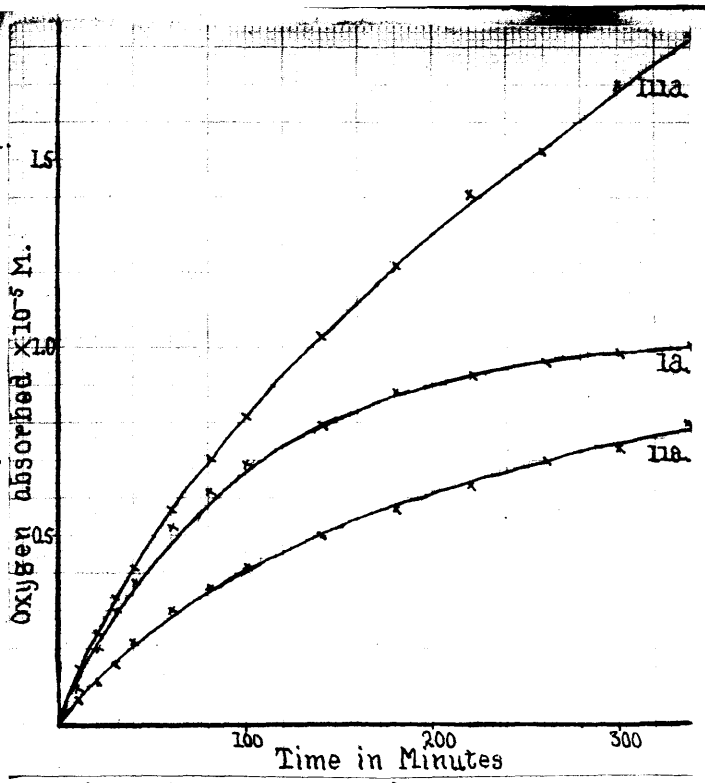


Fig.14. The aerobic dehydrogenation of benzaldehyde. 1.a. with phosphate buffer alone (Table 52), 11. a. with catalase (Table 52), 111.a. with $Ce(OH)_3$ (Table 52.).

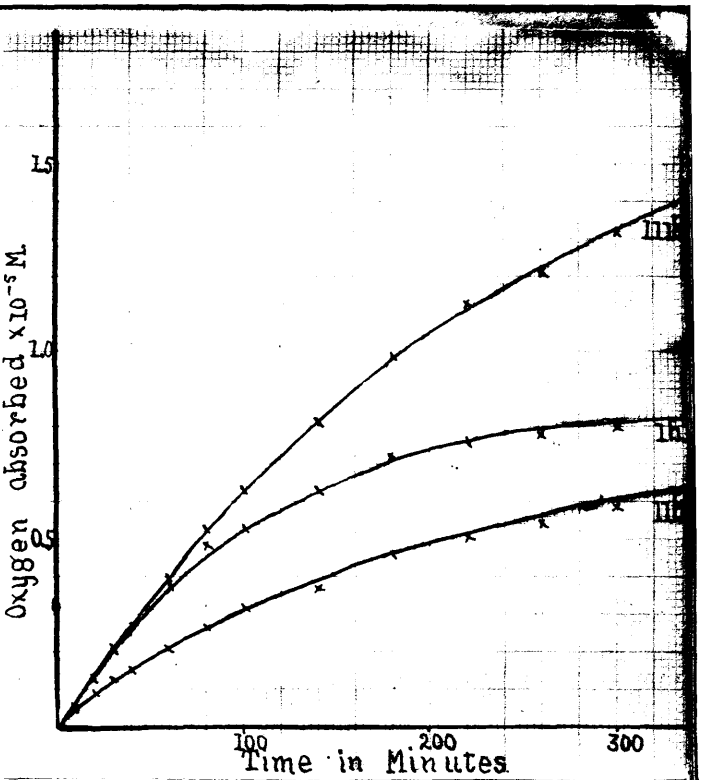


Fig.15. Repeat of experiments 1.a. 11.a. and 111.a. with less ferment. 1.b. with phosphate (Table 52) 11.b. with catalase (Table 52), 111.b. with $Ce(OH)_3$ (Table 52.).

In the absence of both catalase and $Ce(OH)_3$ (Exp.1.a. and 1.b.) the oxygen absorption starts with a considerable speed but the speed diminished very quickly. This is due to two factors; first to the destruction of enzyme and also to the decrease in substrate concentration. With this experiment the principal of these factors is the destruction of enzyme for at the end of the experiment only 32% of the original enzyme was found.

In the presence of catalase (Exp.11.a. and 11.b.) the absorption again begins more slowly than in the experiments without catalase due again to the regeneration of oxygen from hydrogen peroxide. Again as the reaction proceeds a diminution in speed is noted, but in this case the principal factor is the decrease in substrate concentration. In Exp.11.a. practically all the added aldehyde was used up, for 80% of the theoretical quantity was absorbed and about/

10% of the aldehyde must have undergone the Cannizzaro action, so that the aldehyde concentration could not have been more than 10% of the original concentration. The anaerobic dehydrogenation of benzaldehyde with methylene blue as hydrogen acceptor goes much more slowly in such concentration of benzaldehyde. The finding of 78% of the original enzyme at the end of experiment 11.b. definitely shows that catalase protects the aldehydrase.

With cerous hydroxide (Exp. 111.a. and 111.b.) the formation of hydrogen peroxide in the dehydrogenation was again definitely found. The yellow-brownish coloration of ceric peroxide was obtained, and the subsequent estimation of the peroxide gave results which agree with the requirements of the dehydrogenation theory. The deficiency again recorded is probably due to the oxidation of benzaldehyde with hydrogen peroxide. With benzaldehyde no inhibition of the dehydrase is produced by cerous hydroxide, which is perhaps due to the dehydrase having a higher affinity for benzaldehyde than for acetaldehyde and formaldehyde.

Cerous hydroxide has a strong protecting action on the enzyme for the reaction proceeded almost to completion and the diminution in reaction velocity can be fully explained by the diminution in the substrate concentration.

The Aerobic Dehydrogenation of Salicylaldehyde.

The following experiments were carried out at 37° in the Barcroft apparatus. The enzyme solutions were added from the side vessels.

Table 53.

Exp.I.a. II.a. and III a. carried out with the same enzyme solution. Exp.I.b. II.b. and III.b. carried out with onethird the quantity of enzyme used in Exp.I.a. II.a. and III.a. Temperature = 37°.

	I.		II.		III.	
	a.	b.	a.	b.	a.	b.
c.c. enzyme preparation m/15Sa	3.0	1.0 (5Sa)	3.0 (15Sa)	1.0 (5Sa)	3.0 (15Sa)	1.0 (5Sa)
c.c. m/50 Salicylaldehyde	1.5	1.5	1.5	1.5	1.5	1.5
c.c. catalase solution (4 mg./c.c.)	-	-	2.0	2.0	-	-
c.c. m/50 $Ce_2(SO_4)_3$	-	-	-	-	2.0	2.0
c.c. m/20 $Na_2B_4O_7$	-	-	-	-	2.0	2.0
c.c. m/5 phosphate buffer p H.=8.0	2.0	2.0	2.0	2.0	-	-
c.c. water	2.0	4.0	-	2.0	-	2.0
Oxygen absorbed X10⁻⁵M.	1.49	0.52	1.12	0.73	2.52	1.29
H202 found by estimation x10⁻⁵M	-	-	-	-	2.19	-
% theory	-	-	-	-	87	-
% aldehydrase found at end of experiment.	0.6	-	34.0	-	-	-

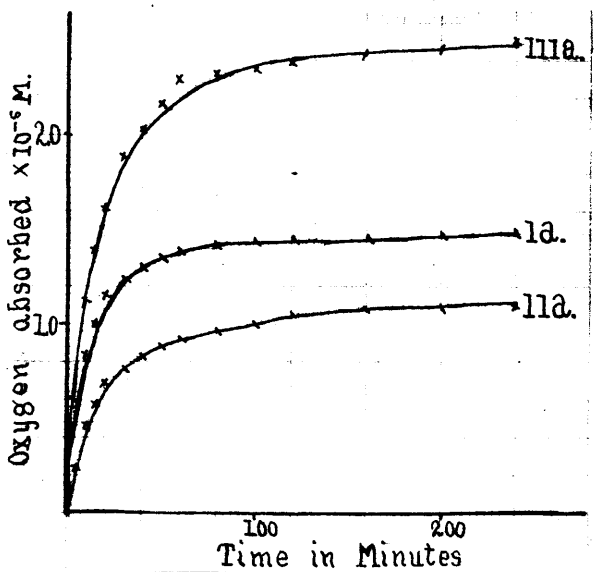


Fig.16. The aerobic dehydrogenation of Salicylaldehyde. I.a. with phosphate buffer alone (Table 53.) II.a. with catalase added (Tab.53) III.a. with $Ce(OH)_3$ added (Table53)

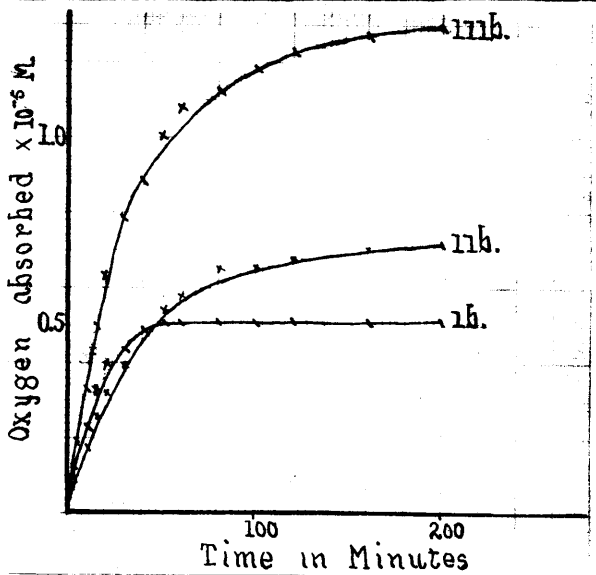


Fig.17. Repeat of experiments I.a. II.a. and III.a. using less ferment. I.b. with phosphate buffer alone (Table 53) II.b. with catalase added (Table 53). III.b. with $Ce(OH)_3$ added (Table 53).

In the absence of both catalase and cerous hydroxide (Exp.1.and 1.b.) the oxygen absorption starts very quickly but very soon stops. A very quick destruction of enzyme results for the decrease in substrate concentration cannot explain the stopping of the reaction. The finding of only 0.6% of the original enzyme, at the end of the experiment, shows that the destruction of enzyme is almost complete.

With catalase present (Exp.11.a. and 11.b.) the absorption of oxygen begins more slowly due again to regeneration of oxygen from hydrogen peroxide. As the reaction proceeds the velocity of reaction becomes less, but not so quickly as in the experiments without catalase. The protective action of catalase is proved by the finding of 34% of the original enzyme at the end of the reaction. As with benzaldehyde and formaldehyde however the protection is not complete. It appears that salicylaldehyde itself has a destructive action on the enzyme. The difference between the experiments 1.a. and 11 a. and 1.b. and 11.b. are due entirely to the amounts of enzyme used. Owing to the smaller amounts of enzyme used in experiments 1.b. and 11.b. the protective action of catalase is more apparent.

With cerous hydroxide (Exp.111.a.and 111.b.) the formation of hydrogen peroxide was again proved. The amount of ceric peroxide formed in the experiment, was very nearly the theoretical amount required for the dehydrogenation theory, for in experiment 111.a. 87% was found and in a similar experiment run simultaneously 89% of the theoretical was found. The protecting action of cerous hydroxide is very apparent for in experiment 111.a. the reaction went almost to completion whereas in experiment 1.a. where no cerous hydroxide was present, the reaction went to only about half way.

The Aerobic Dehydrogenation of Anisaldehyde.

The experiments which follow were carried out in exactly the same way as those with salicylaldehyde.

Table 54.

All experiments carried out with the same enzyme solution.
Temperature 37°.

	I.	II.	III. a.	III. b.
c.c. enzyme solution	3.0 (15Sa)	3.0 (15Sa)	3.0 (15Sa)	3.0 (15Sa)
c.c. $\frac{m}{50}$ Anisaldehyde	2.0	2.0	2.0	2.0
c.c. catalase solution (4 mg./c.c.)	-	2.0	-	-
c.c. $\frac{m}{50}$ $Ce_2(SO_4)_3$	-	-	2.0	2.0
c.c. $\frac{m}{20}$ $Na_2B_4O_7$	2.0	2.0	-	-
c.c. $\frac{m}{5}$ phosphate buffer p H. = 8.0	2.0	2.0	-	-
c.c. water	2.0	-	-	-
oxygen absorbed $\times 10^{-5}$ M	2.62	2.04	3.58	3.86
H_2O_2 found by estimation $\times 10^{-5}$ M	-	-	3.44	3.52
% theory	-	-	93	91
% aldehydrase found at end of experiment.	2.2	36.5	-	-

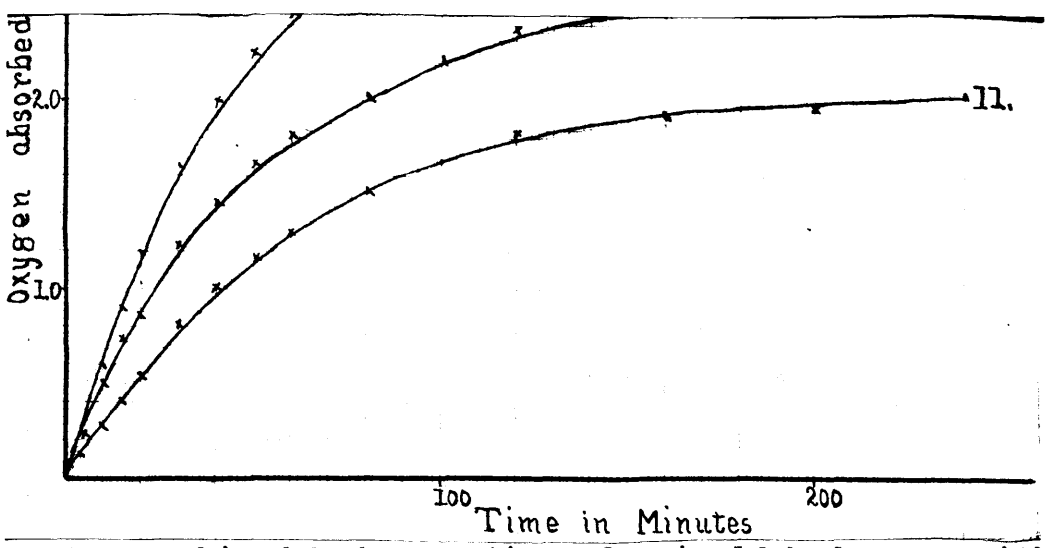


Fig. 18. Aerobic dehydrogenation of anisaldehyde. I. With phosphate buffer alone. II. With Catalase. III a. With $Ce(OH)_3$

The Aerobic Dehydrogenation of Anisaldehyde.

The experiments which follow were carried out in exactly the same way as those with salicylaldehyde.

Table 54.

All experiments carried out with the same enzyme solution.
Temperature 37°.

	I.	II.	III. a.	III. b.
c.c. enzyme solution	3.0 (15Sa)	3.0 (15Sa)	3.0 (15Sa)	3.0 (15Sa)
c.c. $\frac{n}{50}$ Anisaldehyde	2.0	2.0	2.0	2.0
c.c. catalase solution (4 mg./c.c.)	-	2.0	-	-
c.c. $\frac{m}{50}$ $Ce_2(SO_4)_3$	-	-	2.0	2.0
c.c. $\frac{n}{20}$ $Na_2B_4O_7$	2.0	2.0	-	-
c.c. $\frac{m}{5}$ phosphate buffer p H.=8.0	2.0	2.0	-	-
c.c. water	2.0	-	-	-
oxygen absorbed $\times 10^{-5}$ M	2.62	2.04	3.58	3.86
H_2O_2 found by estimation $\times 10^{-5}$ M	-	-	3.44	3.52

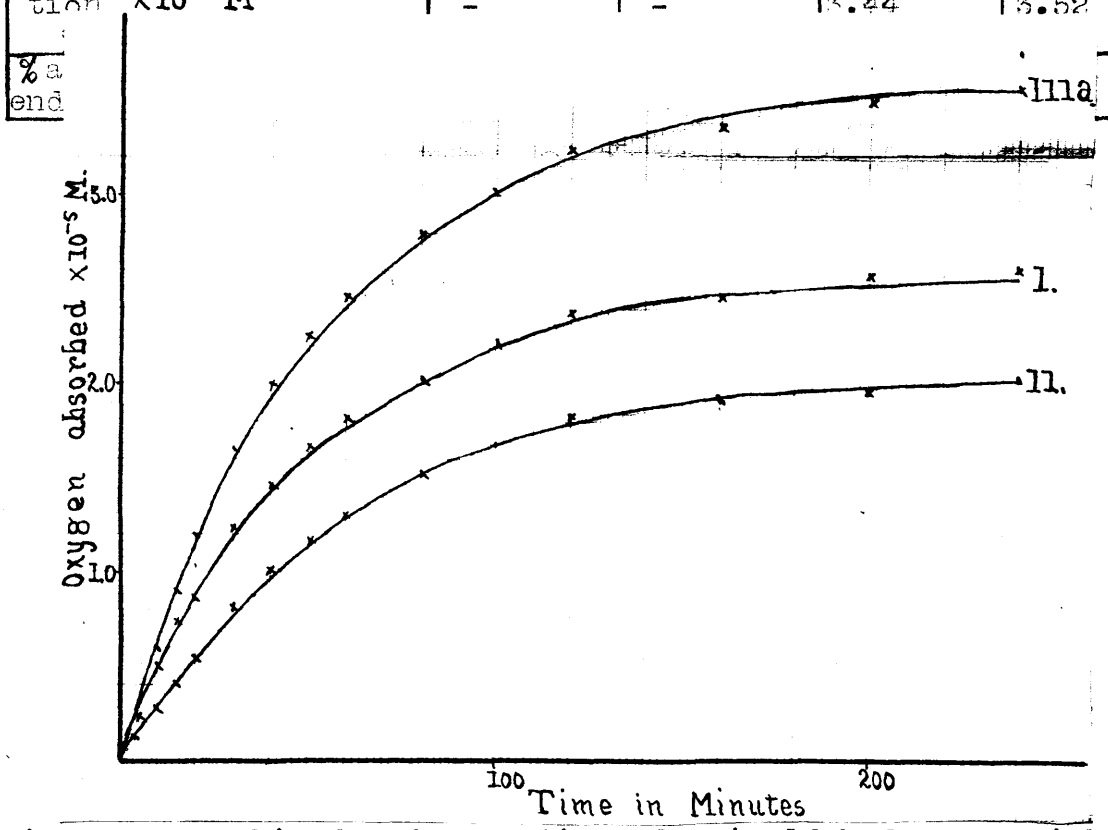


Fig. 18. Aerobic dehydrogenation of anisaldehyde. I. With phosphate buffer alone. II. with Catalase. III a. with $Ce(OH)_3$

The experiments with Anisaldehyde are very similar to those with benzaldehyde and salicylaldehyde. In the absence of catalase and cerous hydroxide (Exp.1.) there is a rapid destruction of enzyme, as only 2.2% of the original aldehydrase was found at the end of the experiment.

With Catalase (Exp.11.) although a considerable destruction of aldehyde took place, the destruction was not so rapid, and at the end 36.5% persisted. The actual speed of the oxygen absorption is again less, because of the regeneration of oxygen from hydrogen peroxide. The oxygen absorbed in Experiment 11. is the theoretical quantity.

With cerous hydroxide (Exp.111.a. and 111.b.) the formation of hydrogen peroxide was again manifested. The estimation of the ceric peroxide again gave theoretical results. The protective action of cerous hydroxide is very apparent by comparison of the experiments 1.and 111a. and 111 b. The oxygen uptakes in 111.a. and 111.b. are nearly the theoretical quantity for the anisaldehyde added.

The formation of hydrogen peroxide in the dehydrogenation of five aldehydes has therefore been proved. It must therefore be taken that hydrogen peroxide is always formed in the enzymatic aerobic dehydrogenation of aldehydes. The reaction must therefore proceed according to Wieland's dehydrogenation theory. The protective actions of catalase and cerous hydroxide in these aerobic dehydrogenations also agree with the theory that it is hydrogen peroxide that is the destroying factor in these dehydrogenations.

The Effect of Cyanide on the aerobic dehydrogenation of aldehydes.

The Warburg theory of biological oxidation [1] postulates that iron plays a part in the oxidation, it acting as an oxygen activator. The principal evidence in favour of this theory is that cyanide inhibits several enzymatic oxidations, which Warburg attributes to the action of cyanide on iron salts. Wieland attributes the inhibiting action of cyanide to its effect not on the dehydrogenating enzymes, but on catalase, which is present in living cells, and which acts as a protective enzyme to the dehydrases by decomposing hydrogen peroxide as it is formed. In the absence of catalase there is an accumulation of hydrogen peroxide, which brings about destruction of the dehydrades.

The effect of the addition of cyanide to the aerobic dehydrogenation of several aldehydes has been studied. The experiments were carried out in the Barcroft apparatus. The aldehydes were previously mixed with the HCN solution, and the mixture was allowed to stand 15 minutes to allow time for the formation of the cyanhydrins. The enzyme solutions were added from side vessels.

Table 55.

All experiments carried out with the same enzyme solution (1 c.c.= 7.0Sa) Temperature = 10.5°.

	I.			II.			III	
	a	b	c	a	b	c	a	b
c.c.cream preparation	1.0	1.0	1.0	3.0	3.0	3.0	3.0	3.0
c.c.macetaldehyde $\frac{M}{1}$	0.5	0.5	0.5	-	-	-	-	-
c.c.mFormaldehyde $\frac{M}{1}$	-	-	-	0.5	0.5	0.5	-	-
c.c.m Benzaldehyde $\frac{M}{50}$	-	-	-	-	-	-	1.0	1.0
c.c.m HCN $\frac{M}{10}$	-	0.16	0.8	-	0.16	0.8	-	0.16
c.c.mphosp.buff.p_H=8.0 $\frac{M}{5}$	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
c.c.water	4.5	4.34	3.7	2.5	2.34	1.7	2.0	1.84
Oxygen absorbed X10^5M.	2.25	2.43	1.99	2.55	1.95	0.78	0.78	0.75

1) Bioch.Zs. $\frac{M}{K}$ 471 (1927).

Table 56.(Repeat of experiments on Table 55.)

All experiments carried out with M/15 acetate buffer solution (1 c.c.= 3.2 mg.) Temperature = 10.5°.

	I.			II			III	
	a	b	c	a	b	c	a	b
c.c. cresyl red solution	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
c.c. acetaldehyde $\frac{M}{1}$	0.5	0.5	0.5	-	-	-	-	-
c.c. formaldehyde $\frac{M}{1}$	-	-	-	0.5	0.5	0.5	-	-
c.c. benzaldehyde $\frac{M}{50}$	-	-	-	-	-	-	1.0	1.0
c.c. $\frac{M}{10}$ HCN	-	0.13	0.3	-	0.13	0.3	-	0.16
c.c. phosp. buffer $\frac{M}{5}$ P.=7.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
c.c. water	4.5	4.37	4.7	4.5	4.37	4.7	4.0	4.84
Oxygen absorbed $\times 10^{-5}$M.	0.05	0.05	0.42	1.08	1.35	0.64	0.76	0.63

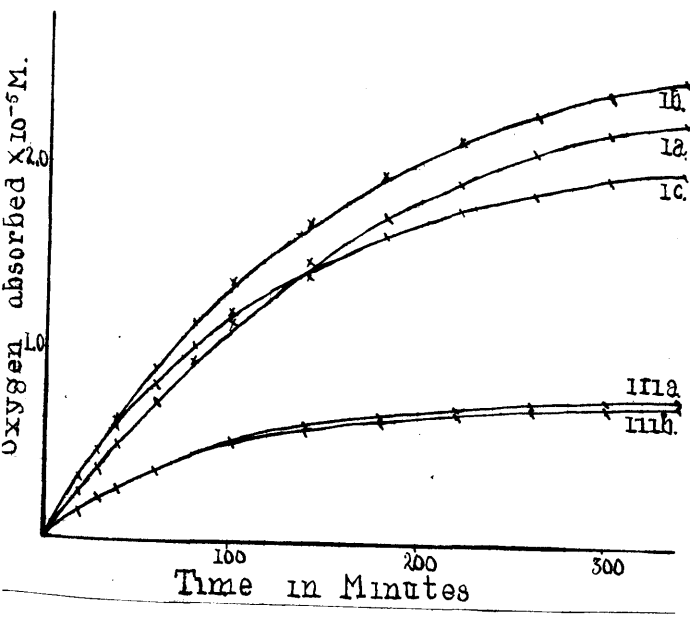


Fig.19. The effect of cyanide on the aerobic dehydrogenation of acetaldehyde and benzaldehyde. I.a. acetaldehyde without HCN, I.b. acetaldehyde with $\frac{M}{500}$ HCN, I.c.- acetaldehyde with $\frac{M}{100}$ HCN III.a. benzaldehyde without HCN. III.b. benzaldehyde with $\frac{M}{500}$ HCN. (Table 55.)

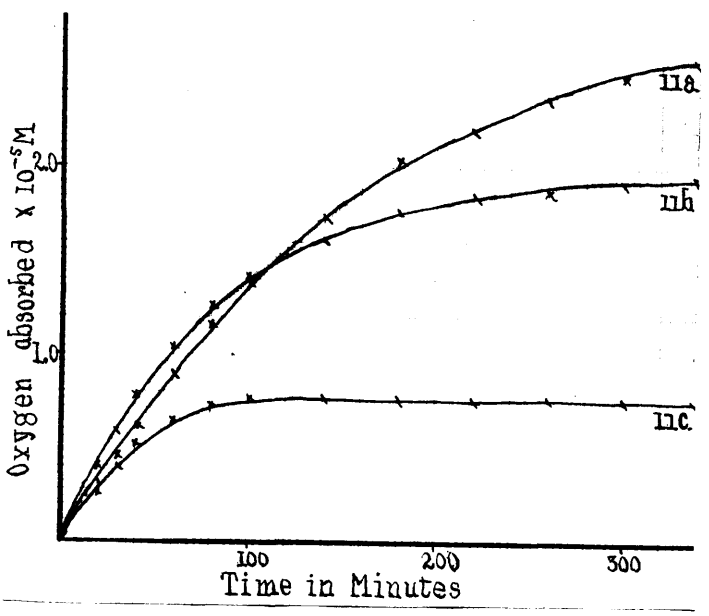


Fig. 20. The effect of cyanide on the aerobic dehydrogenation of formaldehyde. II.a. without HCN, II.b.with $\frac{M}{500}$ HCN, II.c.with $\frac{M}{100}$ HCN. (Table 55.)

The effect of HCN on the aerobic dehydrogenation by methylene blue, of the above substrates was measured. The method used was to add the aldehyde-cyanidemixture, which had previously been allowed to stand, to the methylene blue, and to add this mixture to an anaerobic solution of the enzyme, and note the time of decolorisation. The concentrations of aldehydes and cyanide were exactly the same as those used in the above aerobic experiments.

Table 57. Temperature 37°.

Aldehyde.	Acetaldehyde.			Formaldehyde.			Benzaldehyde.	
Concentration of Cyanide.	0	m/500	m/100	0	m/500	m/100	0	m/500
Aldehydrase found.	10.5	9.1	6.3	6.5	3.5	2.3	2.3	2.2
% inhibition by cyanide.	-	14	40	-	46	64	-	4

The inhibitions produced by cyanide in the anaerobic and aerobic dehydrogenations are very similar. In both types of dehydrogenations, cyanide had a much stronger inhibiting action with formaldehyde as substrate, than with either acetaldehyde or benzaldehyde as substrates. The inhibitions produced even with formaldehyde as substrate are not strong compared with the action of cyanide on other enzymes, and it appears that iron can play no part in the enzyme action.

Why there should be a much stronger inhibiting action in the presence of formaldehyde as compared with the other aldehydes, is difficult to explain. According to Warburg's theory similar inhibitions by cyanide in the dehydrogenation of the aldehydes must be expected, and according to this theory, the cyanide has an effect on the enzyme itself. It is probable that the inhibiting action of cyanide is due to an adsorptive blocking of the enzyme by the cyanide.

The effect of hydrogen peroxide on the aerobic dehydrogenation of aldehydes.

In the aerobic dehydrogenation of aldehydes and in several other aerobic dehydrogenations there is a destruction of enzyme. In the presence of catalase the destruction is not so great and in several dehydrogenations (xanthine, acetaldehyde) there is no destruction of the enzyme.[1] The dehydrogenation theory attributes this destruction of enzyme to the hydrogen peroxide formed in the dehydrogenation.

The effect of hydrogen peroxide on xanthine dehydrase and aldehydrase has already been discussed[2]. It was found that 1×10^{-3} m. H_2O_2 has very little effect on the anaerobic dehydrogenation of both xanthine and acetaldehyde. Actual destruction of the enzymes was only brought about by concentrations of hydrogen peroxide of 10^{-2} m.

The effect of the addition of hydrogen peroxide on the aerobic dehydrogenation of acetaldehyde, formaldehyde and benzaldehyde has been studied. The enzyme solution, buffer and hydrogen peroxide were introduced into the Barcroft vessels and the substrates were added from the side vessels.

1) Loc. cit.
2) See p.23

Table 58. Temperature = 10.5°.

1 c.c. enzyme solution = 7.0 Sa.

	1a.	1b.	1c.	11a.	11b.	11c.	111a.	111b.	111c.
c.c. cream preparation.	1.0	1.0	1.0	3.0	3.0	3.0	3.0	3.0	3.0
c.c. m/5 phosphate buffer p H.=8.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
c.c. n/50 H202	-	0.4	2.0	-	0.4	2.0	-	0.4	2.0
c.c. water	4.5	4.1	3.5	2.5	2.1	0.5	2.0	1.6	-
c.c. m/1 acetaldehyde	0.5	0.5	0.5	-	-	-	-	-	-
c.c. m/1 formaldehyde	-	-	-	0.5	0.5	0.5	-	-	-
c.c. m/50 benzaldehyde	-	-	-	-	-	-	1.0	1.0	1.0
Oxygen absorbed. X 10^{-5} M.	2.68	2.13	0.97	1.68	1.42	0.99	0.77	0.51	0.11.

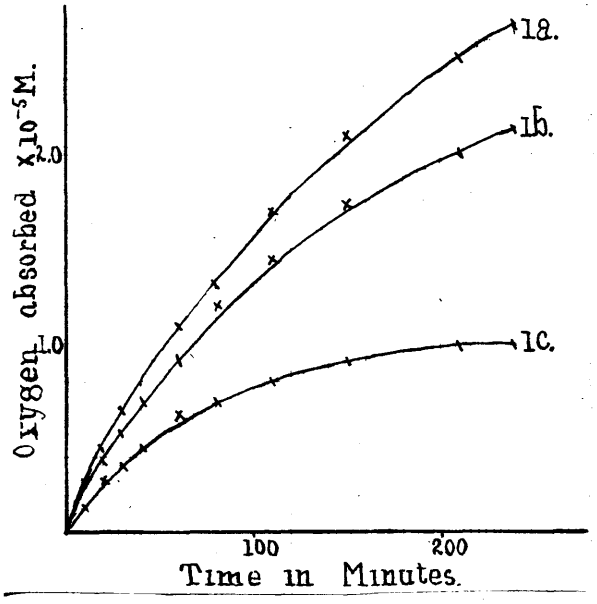


Fig.21. The effect of hydrogen peroxide on the aerobic dehydrogenation of acetaldehyde. 1.a. without hydrogen peroxide. 11.b. with $1X10^{-3}$m H202. 1.c. with $5x10^{-3}$m H202 (Table 58.)

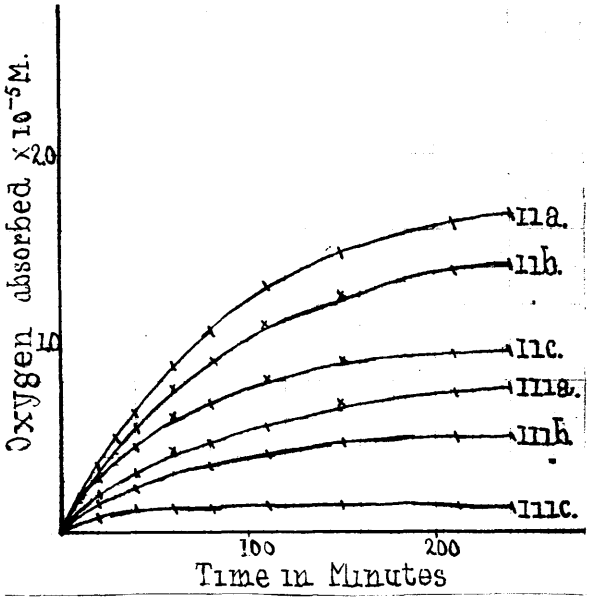


Fig.22. The effect of hydrogen peroxide on the aerobic dehydrogenation of formaldehyde and benzaldehyde. 11.a. formaldehyde without hydrogen peroxide. 11.b. with $1X10^{-3}$m H202. 11.c. with $5X10^{-3}$ m.H202. 111.a. benzaldehyde without hydrogen peroxide. 111.b. with $1X10^{-3}$m.H202. 111.c. with $5X10^{-3}$m H202. (Table 58.).

concentrations of $1X10^{-3}$ m. hydrogen peroxide have therefore very little effect on the above dehydrogenations but a certain amount of inhibition is apparent. With $5X10^{-3}$ m. hydrogen peroxide a very strong inhibition is noticeable. It is noteworthy that with benzaldehyde the inhibition is much stronger than with the aliphatic aldehydes, for it is also the case that in the aerobic dehydrogenation of benzaldehyde that the enzyme is more quickly destroyed. It therefore appears that hydrogen peroxide is the destroying factor in both cases. It is possible that the stronger destruction of enzyme in the aerobic dehydrogenation of benzaldehyde than with acetaldehyde and formaldehyde is due to the latter aldehydes giving peroxides with the hydrogen peroxide and thus destroying its tonic effect.

In the experiments I.c. II.c. and III.c. catalase solution was added at the end of the experiments. After the evolution of oxygen had stopped, the experiments were continued for one hour but no further absorption of oxygen resulted. The added hydrogen peroxide had therefore brought about destruction of the aldehydrase, and not only the temporary inhibition of its action.

The destructions noted by the addition of hydrogen peroxide are not so great as must be expected if hydrogen peroxide is the only factor which produces destruction of enzyme, in the aerobic dehydrogenation of aldehydes. In the aerobic dehydrogenation of benzaldehyde for instance (Exp. III.a.) no greater a concentration of hydrogen than $1X10^{-3}$ m. occurred. The addition of hydrogen peroxide to this molecular concentration (Exp. III.b.) although it had a considerable destroying action, it did not completely destroy the enzyme.

It appears that hydrogen peroxide added differs from hydrogen peroxide formed in a dehydrogenation, in its destructive action. It is possible for this to be so, for when the hydrogen peroxide is formed in a dehydrogenation, it must be formed on the surface of the enzyme, and so an immediate destruction of enzyme can thus occur.

These experiments show that hydrogen peroxide has a strong inhibiting effect on the aerobic dehydrogenation of aldehydes, and it is therefore very probable that hydrogen peroxide is the destroying factor in these dehydrogenations.

Summary.

1. A further purification of the cream preparation of Wieland and Rosenfeld was carried out.

2. It was found that the actions of the enzymes in fresh cows' milk are partially inhibited, and that it is only when the fat particles of the milk coagulate that the maximum activity is attained.

3. The dehydrases of cream preparation were shown to be sensitive to evacuation.

4. Further evidence has been obtained to prove that xanthine dehydrase and aldehydrase are distinct enzymes.

5. Cream preparation has been shown to contain mutase, and to be capable of bringing about the dismutation of salicylaldehyde and acetaldehyde.

6. The aldehydrase and mutase of cream preparation have been proved identical.

7. The aerobic dehydrogenation of aldehydes has been fully studied, and has been shown to proceed according to the dehydrogenation theory of Wieland.