I. A Study of the Optical Activity of some Derivatives of Lactic Acid.

II. The Dehydrogenation of Succinic Acid and Aldehydes by Animal Tissue.

Thesis submitted to Glasgow University
for the degree of Ph.D. in the Faculty of Science.

by

Alexander Lawson.

September 1930.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Part 1. of the following research was carried out in the Organic Chemistry Department of Glasgow University during sessions 1927-29 under the supervision of Professor T. S. Patterson. This work was published in J.C.S., 1929, 2042. The work contained in Part 11. was conducted during 1929-30 at the "Bayerische Akademie der Wissenschaft", Munich, attached to Munich University, Germany, under the supervision of Herr Geheimrat Professor Dr. H. Wieland.

I take this opportunity of expressing my sincere thanks to Professor T. S. Patterson and to Herr Geheimrat Professor Dr. H. Wieland for their helpful advice and continued interest in the carrying out of this research.

I am indebted to the Department of Scientific and Industrial Research, and the Carnegie Trust for the Scottish Universities, for research grants which enabled me to carry out the work described.

Contents. Page.

Part 1:

A Study of the Optical Activity of some derivatives of Lactic Acid ____ 1.
Experimental ____ 16.

Part 11.

The Dehydrogenation of Succinic Acid and Aldehydes by Animal Tissue.

Introduction ____ 1.

Comparison of the effect of Cyanide on the Succinodehydrase and Catalase action ____ 7.

Influence of Cerous Hydroxide on the Succinodehydrase action ____ 12.

Influence of Carbon Monoxide on the Succinodehydrase action ____ 24.

Hydroquinone as Hydrogen Donator ____ 30.

Effect of Cyanide on Hydroquinone Dehydrogenation ____ 32.

Succinic Acid and Hydroquinone acting together as Substrate ____ 34.

Tyrosine and other Phenolic Substances as Hydrogen Donators ____ 38.

Methyl Glyoxal, Acetic Acid, and Pyruvic Acid as Hydrogen Donators ____ 41.

The Press Liquor from Horse Heart ____ 42.

Attempts at Isolation of Succinodehydrase ____ 47.

Dehydrogenation of Aldehydes by Animal Tissue ____ 50.

Preparation of Suitable Enzyme Material ____ 51.

Effect of Cyanide on Dehydrogenation of Acetaldehyde ____ 56.

Concentration of Substrate and Rate of Reaction of Aldehydrase ____ 58.

The Cannizzaro Reaction ____ 60.

Part. 1.

A Study of the Optical Activity of some Derivatives of Lactic Acid.

A very considerable amount of research work has been carried out on the esters of Tartaric acid with a view to arriving at some relation between optical activity and chemical constitution, the presentation of the characteristic diagram being one of the chief results. Lactic acid appeared to offer an interesting line of study, since it contains only one carbon atom, and since many of its derivatives are liquid, requiring no solvent to enable their optical rotations to be studied. On the other hand, of course, their low boiling nature in many cases limits the temperature range over which work can be carried out. It seemed desirable to work with one asymmetric carbon atom, since the influence of several asymmetric units on the rotary powder is as yet little understood.

Patterson and Forsyth[1] studied the optical behaviour of some derivatives of Lactic acid as regards the influence of solvents and temperature change. At that time, however, the work was carried out using light of only one colour (yellow D light), but on account of the interest of derivatives of lactic acid in regard to rotation dispersion, this work was repeated with light of six different colours, and some addition derivatives were included.

1) J. 1913, 103 2264.

Optically active compounds of lactic acid have been prepared in several ways; by fractional hydrolysis of the menthyl esters (McKenzie and Thomson)[1] ; By the standard method of resolution using active bases such as quinine (Jungfleisch)[2], Strychnine (Purdie and Walker)[3], Morphine (Irvine and Patterson)[4], the best results being obtained by the use of morphine and by crystallisation of the zinc ammonium lactate (Purdie[5] and Purdie and Walker[6]). The method adopted for the preparation of the active starting product was a modification of the methods of Purdie and Walker, and Patterson, loc.cit.

The starting material for the work to be described was commercial lactic acid syrup. The rotations of such syrups are often considerably higher than that of pure active lactic acid itself, this being due to the presence of lactide which possesses a high rotation. It is interesting to note that the lactide may exist in the cis and trans forms,

Both the d and l lactides obtained from the cis form have been prepared by Jungfleisch and/

1) J. 87, 1016.

2) C.R. 139 57, 204.

3) J. 61, 754.

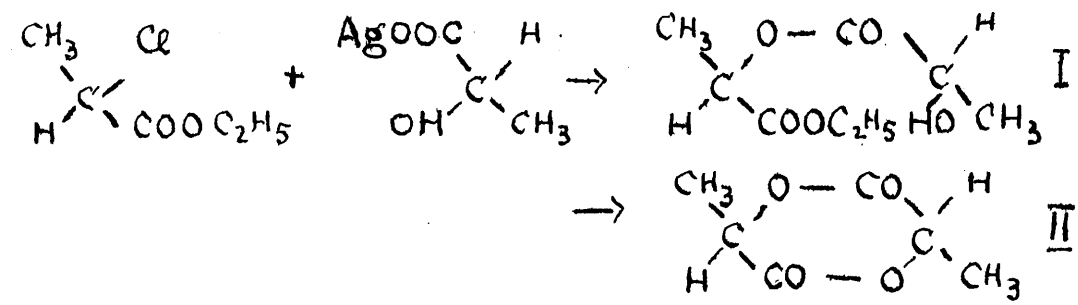
4) J. 89, 935.

5) J. 103, 2263.

6) 63, 1143.

7) J. 67, 618.

Sodchot[1]. It will be seen that the trans modification has a pseudo symmetrical structure, and can only exist in the inactive form. Up to the present, the trans form has not been isolated, the cis-trans conversion having proved unsuccessful. It is suggested that the preparation of the compound could be brought about in the following manner. By the action of ethyl d-chloropropionic acid on the silver salt of l-lactic acid, ethyl d lactyl-l-lactate (T) could be prepared, and this on losing alcohol should give the trans-lactide (II)



A sample of the commercial lactic acid which showed a reasonably high rotation, indicative of a fair excess of the d or l isomer, having been obtained, this was first converted into zinc lactate, the impure active zinc lactate being separated off by fractional crystallisation, and purified by conversion into the zinc ammonium salt. Active zinc lactate cannot be readily purified by crystallisation, since the inactive material is less soluble, and comes out of solution first. The active zinc ammonium lactate, on the other hand, is less soluble in ammonia solution than inactive zinc lactate, and under certain conditions can be purified by repeated/

1) C.R. 141, 111, 1905; C.R. 142, 637, 1906.

crystallisation. Inactive zinc lactate forms no double salt with inactive ammonium lactate.

The conversion of the zinc lactate into zinc ammonium lactate was at first done by partly converting the crude zinc lactate into ammonium lactate by means of ammonium sulphide, and filtering off the precipitated zinc sulphide. The difficulty, however, of getting rid of excess ammonium sulphide, the sulphur attendant on its decomposition, and the pollution by hydrogen sulphide fumes given off by the ammonium sulphide suffered by other supplies of zinc lactate in the course of preparation, were disadvantages to this method. The zinc was therefore precipitated as carbonate with ammonium carbonate, and this method was found to be more satisfactory.

From the moderately active lactic acid syrup, by means of the zinc ammonium salt, methyl lactate was prepared, and from it, the methyl esters of α-acetoxypropionic acid, α-methoxypropionic acid, α-monochloroacetoxypropionic acid, α-dichloroacetoxypropionic acid, α-trichloroacetoxypropionic acid, α-benzoyloxypropionic acid, α-o-nitrobenzoyloxypropionic acid, and α-chlorosulphynyl-propionic acid, were obtained, and the rotations of these substances examined for six different colours of light over a considerable range of temperature.

Freudenberg [1] showed that the four hydroxy acids, l-lactic, l-glyceric, d-malic, and d-tartaric all possess the same relative configuration/,

1) Ber. 1914, 47, 2037.

the hydrogen atoms, the hydroxyl, and the carboxyl groups attached to the asymmetric carbon atom having a similar spatial arrangement on the molecules of these compounds. These relationships were proved by means of reactions which were known to have no effect on the spatial arrangement of the groups on the asymmetric carbon atom. Again, comparison of the rotatory powers of these acids and their simple derivatives showed them to bear similar regularities. Clough [1], by a study of the influence of solvents on the rotatory power of these acids, their esters and their salts, pointed out the similarity in character. He suggested the method of determining the relative configuration of similar α-hydroxy acids with reference to d-tartaric acid. Wood and Nicholas [2] showed the relationship between the position of the region of anomolous rotatory dispersion exhibited by optically active compounds and the configuration of the groups attached to the asymmetric carbon atoms, compounds of d-configuration having the anomaly (when realisable) always occurring in the positive region of rotation, and the crossing of the x axis taking place in one way only, so that $\frac{da}{dx}$ is positive.

The results of this work are in agreement with those of the above workers, and show that the lactic acid, which in the homogeneous condition is dextrorotatory, but which forms a/

1) J. 1918, 113, 528.

2) J. 1928, 1712.

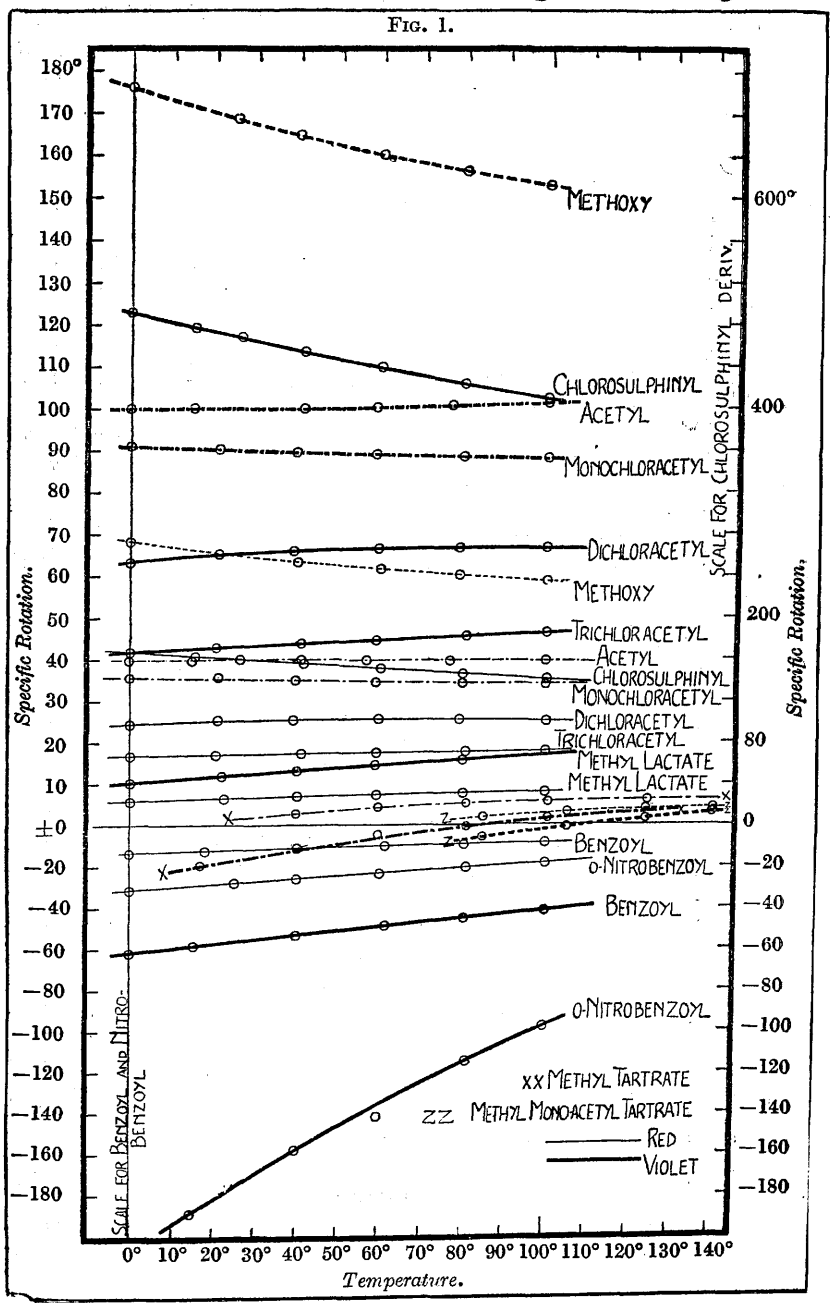
strongly laevorotatory lactide, is configuratively related to the l-tartrates, and should therefore be called l-lactic acid. It was previously referred to as d-lactic acid. The lactic acid with which Patterson and Forsyth worked should accordingly be called d-lactic acid, since it was the isomer which showed relationship to d-tartaric acid. In this connection it may be noted that the configurational sign of the lactic acid contained in all samples of the commercial syrup examined (the rotations of which were mainly due to lactides) was that of the rotation of the syrup itself. That is, laevorotatory syrups contained l-lactic acid in excess. Although in the experiments recorded in the sequel, the isomer that should be regarded as l-lactic acid was used, the sign has been inverted in all figures to make the results comparable with those for the d-tartrates.

Various suggestions have been put forward for a better nomenclature for optically active compounds in general. Wohl and Freudenberg[1] suggested that l-lactic acid should be written "l(+) lactic acid", but this is open to the objection of ambiguity unless the conditions of the positive rotation are more accurately stated. It refers in this case to the rotation of lactic acid in the homogeneous condition at a temperature of 25°; for sodium light, but at other temperatures, in other colours of light, and in certain solvents the rotation could probably be negative. Again the symbol "d (+)

1) Ber. 1923, 56, 309.

tartaric" acid refers to a different condition of the active substance, being applicable to aqueous solutions of tartaric acid at the ordinary temperature. In the homogeneous condition, at about room temperature, ordinary tartaric acid would be probably laevorotatory, at least for red and yellow light[1]. A more definite nomenclature is therefore necessary such as "l ($+\text{homog.}_{\lambda}^{t^\circ}$) lactic acid" or "l ($-p_{\lambda}^{t^\circ}$ = x [solvent]) lactic acid", but this, though it may be definite, has the disadvantage of being somewhat clumsy.

FIG. 1.



In Fig. 1. are shown temperature-rotation curves for the two colours of light, red (λ = 6716 Å.U.) and violet (λ = 4358 Å.U.), the curves for the latter being heavily drawn, and those for the former, lightly. Each set of curves may therefore be taken by itself. Curves for the other colours of light were unnecessary, since in all cases examined, they were distributed fairly evenly between the two extremes shown. In order to facilitate the comparison between the lactates and the tartrates, curves for methyl tartrate and its monoacetyl derivative, for the same colours of light, taken from a recent paper by Wood and Nicholas[1] are included. There are three different scales on the diagram. That on the left margin above the zero line is applicable to all the curves in the upper part of the diagram (including those for methyl-tartrate and acetyltartrate) the scale for the chlorosulphinyl derivative being shown on the right hand margin. The scale on the part of the diagram below the zero line applies only to the benzoyl and the nitrobenzoyl derivatives.

It is apparent from the curves that they resemble fairly closely those for the corresponding tartrates, the curves for methyl lactate itself showing the same type of curvature as do those for the tartrates. At higher temperature, the curves for methyl lactate progress in such a fashion as at least to suggest the possibility of a maximum at a moderately high temperature in just the same way that the corresponding curves for ethyl tartrate intersect at ordinary temperature and proceed to a maximum/

1) J. 1928, 1692.

at high temperature. Perhaps some idea of the behaviour of methyl lactate at high temperature could be obtained by a study of solutions in quinoline or in other such solvents which are known to have the power of elevating the rotation of ethyl tartrate. It will be seen, that whereas the methyl tartrate curve for violet light lies below the corresponding curve for red light, the opposite is the case for methyl lactate. It is however to be noted that the two curves for methyl tartrate tend to intersect at high temperature, whilst the two curves for methyl lactate tendtto intersect and clearly would, at some fairly low temperature in the neighbourhood of -100°. In other words, the region of anomalous dispersion for the simple tartaric esters occurs at a high temperature, and that for the simple lactic esters, if it exists, would be found at a low temperature. In addition the temperature range in the case of the lactates would appear to be greater than that of the tartrates.

When methyl lactate is converted into methyl acetoxypropionate, the change is accompanied by a considerable decrease in rotation, but the noticeable feature about this compound is that the rotation is very little affected by change of temperature within the range under examination. This would point to the presence of maxima or minima, since it is in the neighbourhood of such regions that $\frac{da}{dx}$ is a minimum.

When the acetoxy group is replaced by chloroacetoxy, there is a slight decrease in specific rotation and the curves show negative temperature coefficients.

With the production of the di-chloroacetoxy derivative, there is a further decrease in specific rotation, and the rotations show, in part of the range under examination, definite positive temperature coefficients in contrast with those of the mono-chloroacetoxy compound where the temperature coefficient is negative. From the rotation values for the di-chloroacetoxy derivative, it will be seen that maxima are shown for all colours of light, the tendency being for their occurrence at higher temperature with decreasing wavelength as has been shown to be the case with homogeneous ethyl-d-tartrate[1].

Again in the case of the tri-chloroacetoxy compound, the rotations all show positive temperature coefficients.

Taken in general therefore, it will be noticed from the curves, that whereas the derivatives having considerable negative rotations show a rapid increase in rotation — become less negative — with rise in temperature, those with intermediate rotation such as methyl lactate itself, whose curves would probably reach maxima at temperatures in the region of 150°, show comparatively small changes in rotation. The tendency then appears, that going in order through the series methyl lactate, methyl trichloroacetoxypropionate, methyl di-chloroacetoxypropionate, methyl mono-chloroacetoxypropionate, methyl acetoxypropionate, there is a progression from/

Patterson. J. 1916, 109, 1145.

those derivatives exhibiting positive temperature coefficients, through those exhibiting maxima, to those showing negative temperature coefficients.

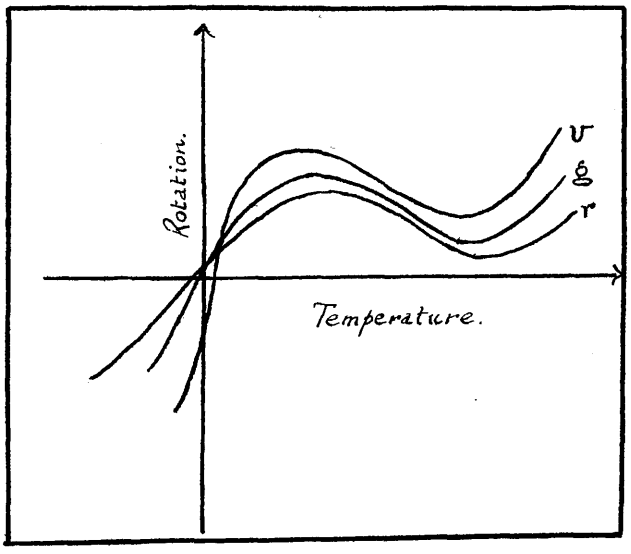
The methoxy and the chlorosulphinyl derivatives had the highest rotations of the substances examined, and it will be seen that the positive temperature coefficient of methyl lactate is in these substances converted into a negative temperature coefficient, the rotations rapidly diminishing as the temperature rises, and suggesting the existence of a minimum at still higher temperatures.

Again, just as in the case of the tartrates, an exactly opposite behaviour is shown by the benzoyl and the nitrobenzoyl derivatives. The introduction of the benzoyl radicle lowers the rotation greatly at ordinary temperatures, and the curves then assume a form very similar to that shown on Fig.1. for the corresponding lactates in which the curves apparently tend to intersect at high temperatures. This behaviour is still more obvious and pronounced in the case of the nitrobenzoyl derivatives.

The curves in Fig.1. having been shown to possess a behaviour in general agreement with that of the d-tartrates, it is for this reason that they are considered analagous to those for the derivatives of d-tartaric acid, and therefore that the particular set shown are those of d-lactic acid. It will be seen that the lactic acid syrup which was used in this work, and which possessed in the crude syrup a negative rotation, is actually l-lactic acid.

Taking into consideration all these curves, it would appear that the general temperature-rotation curves[1] for the lactates are very similar to those for the tartrates (Fig.2.) ; at low temperatures, the rotations should have high negative values, with increasing temperature, the rotations should become less negative, pass through a region at which they intersect one another, reach maximum values, and ultimately tend again towards minimum values.

Fig. 2.



The region of visibly anomalous dispersion was not actually reached in any of the above cases, but it has been shown by Clough[2] to exist in solutions of methyl lactate in water. Now it would be expected from the appearance of these curves that the intersection of the violet and the red curves for methyl lactate, which would occur at a low temperature in the homogeneous ester, should come into the region of ordinary temperatures in a solvent possessing the power of depressing the/

1) J. 1916, 109, 1141.

2) J. 1918, 113, 552.

the rotation of the ester. Ethylene dibromide considerably depresses the rotation of ethyl tartrate, and therefore, a solution of methyl lactate in ethylene dibromide was examined, and as expected, data was obtained which afforded evidence of visibly anolamous dispersion. The results are shown in Fig.3.

Fig. 3.

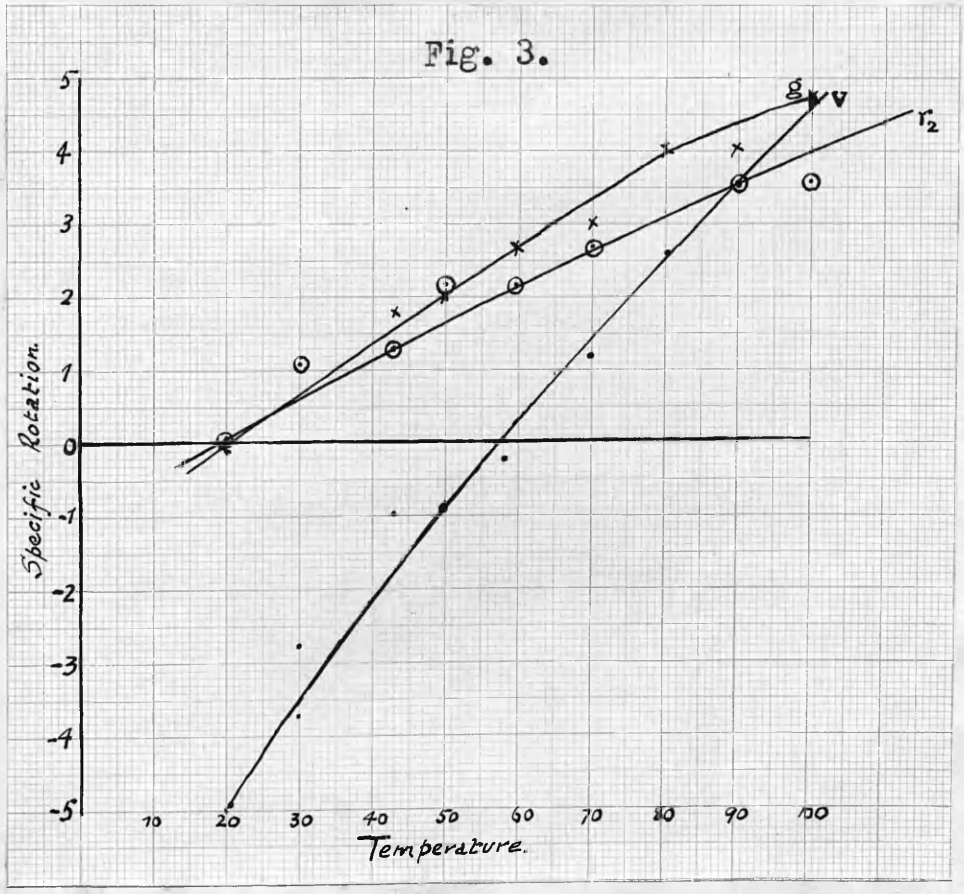


Fig. 4.

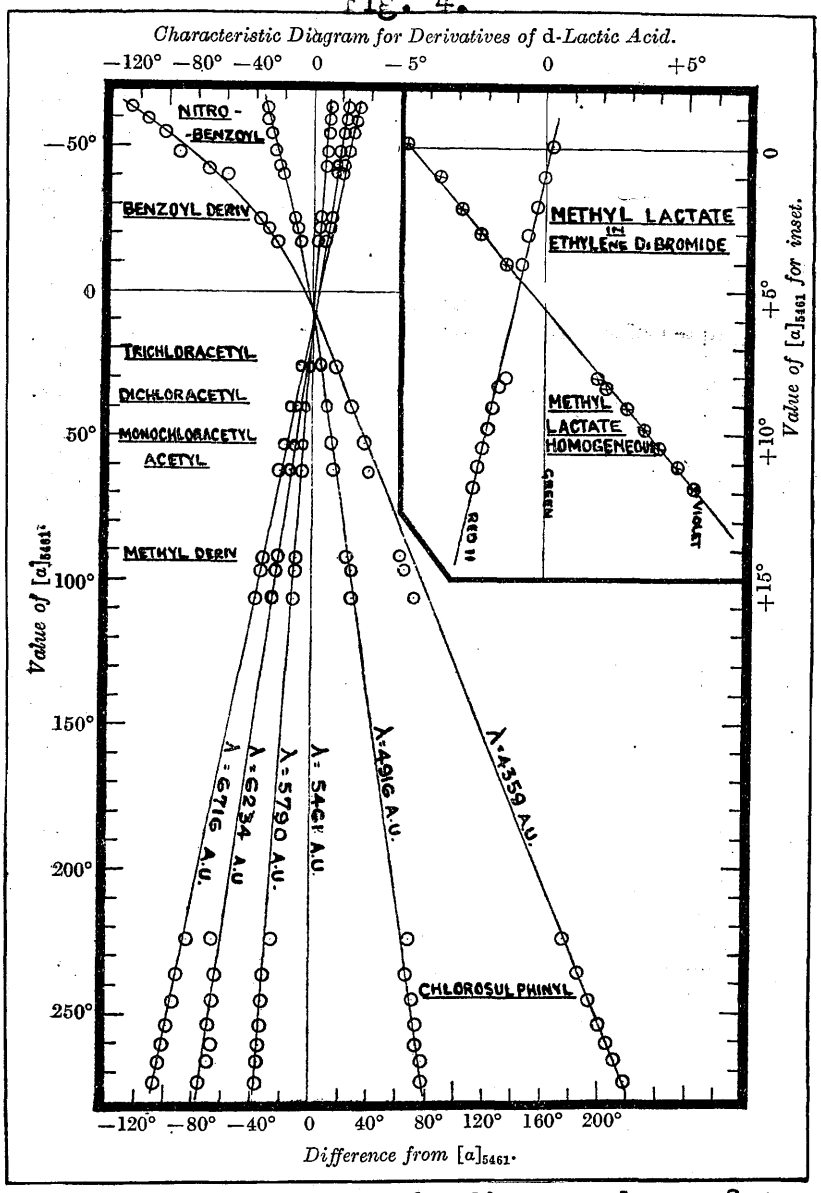


Fig. 4. shows a characteristic diagram drawn from data collected during the course of thw work. In the whole perhaps the results do not lie quite so well upon straight lines as has been found for many other compounds. This is especially the case for violet light notably, but also that for blue requires a decided curvature to fit the experimental points.

The scale in the main part of Fig.4. is much too small for the data for methyl lactate in the homogeneous condition and in solution in ethylene dibromide, to be represented, and therefore a small characteristic diagram for these two conditions is inset in the larger one. It will be seen from this that the region of anomalous dispersion which is not quite reached on the homogeneous ester is reached in the solution in ethylene dibromide. It appears from this diagram that by cooling homogeneous methyl lactate, the rotation for green and violet would become identical at the rotation value of $+5.5^{\circ}$ which would be the rational zero for these two colours.

Experimental.

Several samples of commercial lactic acid syrup from different sources were examined with a view to obtaining a supply which showed a fairly high rotation. Samples having rotations of $\alpha^{18^\circ}_{5461}$ (100 mm.) = $+2.41^\circ$ — $\alpha^{18^\circ}_{5461}$ (100 mm.) = -4.27° were received, it being decided to use that supply possessing the highest negative rotation. Unfortunately, this material could not be supplied in sufficient quantity, so a syrup having rotation $\alpha^{18^\circ}_{5461}$ (100 mm.) = -3.47° was used. The preparation of the zinc lactate was carried out as follows:- 900 c.c. of water having been heated almost to boiling, 750 c.c. of lactic acid syrup was intrdduced, the mass being vigorously stirred. By this means, hydrolysis of the lactide was brought about. When the mixture was boiling, 426 g. zinc oxide were added slowly with stirring, and the solution was boiled till neutral, the bulk of the liquid being maintained by the addition of more water at intervals. This process took about 8 hours. The solution was then allowed to cool, and the inactive zinc lactate which crystallised out was removed by filtration. On concentrating the filtrate and allowing to stand, the active zinc lactate crystallised. The bulk of inactive zinc lactate was extracted with several 2 - litre portions of boiling water till the extracts showed no appreciable rotation. On concentrating these extracts after cooling and removal of inactive zinc lactate, more active salt was obtained, this being added to the main portion of active zinc lactate.

In this manner, about 278 g. active and 900 gs inactive zinc lactate were obtained, so that the original lactic acid syrup must have been composed of about 62% of the lævo and 38% of the dextrorotatory form. The purest sample of zinc lactate obtained showed a rotation of $\alpha^{17^\circ}_{5461} = -0.39^\circ$ (c = 2.5; l = 200 mm.)

The next stage in the process was the conversion of the zinc lactate into zinc ammonium lactate. 100 g. zinc lactate prepared as above were dissolved in 500 c.c. water, 40 g. ammonium carbonate dissolved in 180 c.c. water were added, and the precipitated zinc carbonate filtered off. The filtrate in boiling deposited more zinc carbonate which was also removed by filtration after any excess ammonium carbonate had been destroyed by boiling. To this solution of ammonium lactate, 160 g.(?) (40 g. less than was required to convert all the ammonium lactate into the double salt) dissolved in 750 c.c. water were added. On evapprating and allowing to cool, small crystals of zinc ammonium lactate were deposited. The mother liquor after being concentrated, was seeded with a few crystals of pure active zinc ammonium lactate, and on standing further crops were obtained. Mother liquors from further preparations were treated in the same manner till no more zinc ammonium lactate was deposited. It is important that the first crystallisation should be carried out from a solution containing excess of ammonium lactate, otherwise, the tendency is for the zinc lactate to crystallise instead of the double salt. It was also found/

that solutions of specific gravity 1.20 gave the best yields of the double salt.

The zinc ammonium lactate so obtained was recrystallised from dilute ammonia solution $\frac{N}{2}$ (120 c.c. being used to dissolve 100 g. salt), till the product showed a rotation of $\alpha^{25°}_{5461}$ (200 mm.) - 1.90° for a solution of 1.60 g. in 20 c.c. of dilute ammonia (300 c.c. concentrated ammonia and 1000 c.c. distilled water; Patterson and Forsyth(loc.cit.) From 2 litres of lactic acid syrup, 400 - 450 g. of pure zinc ammonium l -lactate could be obtained in the above manner.

Methyl l-Lactate:

The ester was prepared by the methods of Purdie and Irvine[1] and Patterson and Forsyth[2], the zinc ammonium lactate being previously carefully dried in an air oven till it had lost a weight equivalent to slightly more than 2 mols of water of crystallisation. The methyl alcohol had been purified by standing over quicklime and by distillation. The pure ester boiled at 47°/ 15 mm., the yield being 60%.

The rotation of the preparation was then examined as shown below.

Rotation of methyl l-lactate.

Densities determined:

t	17.6°	22.3°	40.4°	79.6°	100°
d	1.096	1.091	1.071	1.025	1.002.

1) J. 1899, 75, 484.

2) loc.cit.

λ = 6716 Å.U. Red light (r_1)

t	- 7.5°	0°	22.3°	39.9°	58.7°	80.05°	100°
α(160 mm.)	-10.35	10.49	11.15	11.48	11.98	21.41	12.73
[α]	-5.76	5.88	6.39	6.70	7.13	7.57	7.95
[M]	-5.99	6.11	6.65	6.97	7.41	7.87	8.27

λ = 6234 Å.U. Red light (r_2)

t	- 7.5°	0°	22.0°	39.9°	58.7°	80.05°	100°
α(160 mm.)	-11.75	11.93	12.58	13.18	13.72	14.23	14.66
[α]	-6.53	6.68	7.20	7.69	8.17	8.68	9.16
[M]	-6.79	6.96	7.48	8.00	8.50	9.03	9.51

λ = 5790 Å.U. Yellow Light (y.)

t	- 9°	0°	21.9°	39.9°	58.7°	80.05°	100°
α(160 mm.)	-13.18	13.53	14.35	15.08	15.73	16.35	16.87
[α]	-7.32	7.58	8.22	8.79	9.36	10.02	10.53
[M]	-7.62	7.88	8.55	9.15	9.74	10.43	10.97

λ = 5461 Å.U. Green Light (g.)

t	- 10°	0°	21.9°	39.9°	58.7°	80.95°	100°
α(160 mm.)	-14.33	14.89	15.79	16.64	17.39	18.15	18.74
[α]	-7.95	8.29	9.04	9.70	10.35	11.08	11.69
[M]	-8.27	8.63	9.40	10.08	10.77	11.52	12.15

λ = 4916 Å.U. Blue Light (b.)

t	- 7.5°	0°	22 0°	39 9°	58 7°	80 05°	100°
α(160 mm.)	-16.45	16.90	18.31	19.48	20.56	21.50	22.37
[α]	-9.15	9.47	10.48	11.36	12.25	13.11	13.97
[M]	-9.52	9.84	10.90	11.82	12.74	13.65	14.53

λ = 4358 Å.U. Violet Light (v.)

t	-10°	0°	21.9°	39.9°	58.7°	80.95°	100°
α(160 mm.)	17.49	18.57	20.83	22.58	24.23	25.79	26.99
[α]	-9.71	10.40	11.93	13.17	14.42	15.73	16.86
[M]	-10.98	10.82	12.43	13.69	15.00	16.37	17.54

Methyl l-α-Acetoxypropionate.

This substance was prepared by adding slowly 32 g. (twice theoretical amount) of pure acetyl chloride to 21 g. methyl lactate. When the action became quiet, the mixture was heated to boiling under reflux for 1½ hours, and the excess acetyl chloride removed on the water bath. The product was repeatedly fractionated under reduced pressure through a Sydney Young fractionating column till no change in the rotation after successive distillations was observed. The methyl acetoxypropionate boiled at 68°/ 13 mm. and was obtained as a colourless liquid with a faint odour. Yield was 80% of theoretical.

Rotation of Methyl l-α-Acetoxypropionate.

Densities obtained:

t	18.8°	20.4°	39.6°	67°	80°	100°
d	1.089	1.0866	1.0677	1.0350	1.0204	0.9983

r_1.

t	0°	15.0°	41.3°	58.8°	76.8°	100°
α(160 mm.)	-26.64	26.13	25.37	24.85	24.27	23.66
[α]	-40.03	39.83	39.74	39.71	39.49	39.51
[M]	-58.44	58.15	58.00	57.95	57.65	57.65

r 2.

t	0°	15.0°	41.3°	58.8°	76.8°	100°
α(160 mm.)	-31.04	30.44	29.59	28.87	28.36	27.72
[α]	-46.64	46.41	46.33	46.13	46.16	46.28
[M]	-68.09	67.80	67.65	67.35	67.45	67.60

y.

t	0°	15.4°	41.3°	58.8°	76.8°	100°
α(160 mm.)	-36.32	35.71	34.61	33.92	33.21	32.41
[α]	-54.56	54.47	54.21	54.21	54.06	54.11
[M]	-79.65	79.60	79.20	79.20	78.95	79.00

g.

t	0°	15.4°	41.3°	58.8°	76.8°	100°
α(160 mm.)	-41.12	40.47	39.25	38.51	37.77	36.83
[α]	-61.78	61.72	61.47	61.54	61.48	61.50
[M]	-90.25	90.10	89.90	89.95	89.80	89.85

b.

t	0°	15.0°	41.30°	58.8°	76.8°	100°
α(160 mm.)	-51.00	50.03	48.94	48.08	47.37	46.25
[α]	-76.62	76.27	76.65	76.83	77.10	77.21
[M]	-111.75	111.35	111.80	112.25	112.45	112.55

v.

t	0°	15.4°	41.3°	58.8°	76.8°	100°
α(160 mm.)	-66.59	65.58	64.15	62.97	61.92	60.71
[α]	-100.03	100.02	100.47	100.06	100.78	101.35
[M]	-146.10	146.10	146.70	146.10	147.10	148.00

Methyl l-α-chloroacetoxypropionate.

The mono-chloroacetyl chloride used in this preparation was prepared by the action of thionyl chloride on mono-chloracetic acid. Phosphorus pentachloride cannot be used owing to the difficulty of separating the product of the reaction from the phosphorus oxy-chloride formed, both substances having boiling points very near one another.

The mono-chloracetic acid and the thionyl chloride were boiled under reflux till no more hydrogen chloride was liberated, and the product fractionated through a Sydney Young column, the portion boiling at 105°- 106° being retained.

The chloroacetyl chloride prepared as above was heated under reflux with twice the theoretical quantity of methyl lactate for four hours, and the product fractionally distilled under reduced pressure through a column till no further change in rotation was observed. The methyl l-α-monochloroacetoxypropionate was a colourless liquid of b.p. 110°/15 mm. Yield was theoretical. Analysis of the product gave Cl, 19.65%. Calculated result is Cl, 19.77%.

Rotation of Methyl l-α-Chloroacetoxypropionate.

Densities determined:

t	20.5°	40.5°	41.5°	59.25°	80.0°	100°
d	1.2521	1.2287	1.2284	1.2064	1.1830	1.1598

η.

t	0°	21.3°	39.75°	59.0°	80.0	100°
α (60 mm.)	-27.50	27.01	25.88	25.04	24.20	23.81
[α]	-35.91	35.99	35.07	34.58	34.09	34.35
[M]	-65.00	65.15	63.45	62.59	61.70	62.15

r 2.

t	0°	21.3°	39.75°	59.0°	80.0°	100°
α(60 mm.)	-32.12	31.21	30.24	29.29	28.28	27.64
[α]	-41.95	41.58	40.98	40.45	39.84	39.72
[M]	-75.90	75.22	74.17	73.20	72.08	71.88

y.

t	0°	21.3°	39.75°	59.0°	80.0°	100°
α(60 mm.)	-37.63	36.55	35.44	34.47	33.26	32.31
[α]	-49.15	48.70	48.02	47.60	46.86	46.42
[M]	-88.95	88.10	86.85	86.10	84.78	84.00

g.

t	0°	21.3°	39.75°	59.0°	80.0°	100°
α(60 mm.)	-42.64	41.43	40 22	39.08	37.75	36.79
[α]	-55.69	55.19	54.50	53.96	53.18	52.87
[M]	-100.75	99.90	98 65	97.73	96.30	95.75

b.

t	0°	21.3°	39.75°	59.0°	80.0°	100°
α(60 mm.)	-53.26	51.78	50.46	48.89	47.26	45.98
[α]	-69.56	68.98	68.37	67.51	66.58	66.08
[M]	-125.80	124.80	123.65	122.15	120.45	119.55

v.

t	0°	21.3°	39.75°	59.0°	80.0°	100°
α(60 mm.)	-69.70	67.91	66.40	64.79	62.67	61.27
[α]	-9104	90.48	89.97	89.47	88.29	88.04
[M]	-164.55	163.75	162.80	161.95	159.70	159.25

Methyl l-α-Dichloroacetoxypropionate.

This was prepared from methyl lactate and dichloroacetyl chloride (b.p. 107° - 108°) in exactly the same manner as was the monochloroacetoxy derivative from chloroacetyl chloride. The colourless product boiled at 115° / 15 mm. Yield of impure ester was theoretical. Analysis gave Cl, 33.27%. Theory requires Cl, 33.03%.

Rotation of Methyl l-α-Dichloroacetoxypropionate.

Densities determined:

t	22.0°	39.0°	60.5°	81.5°	100°	
d	1.3315	1.3105	1.2842	1.2852	1.2359	

r 1.

t	0°	21.0°	39.0°	59.5°	79.0°	100°
α(60 mm.)	-20.33	20.35	20.07	19.72	19.24	18.88
[α]	-24.94	25.46	25.33	25.575	25.43	25.19
[M]	-53.62	54.74	54.89	55.00	54.88	54.03

r 2

t	0°	21.0°	39.0°	59.5°	79.0°	100°
α(60 mm.)	-23.74	23.83	23.57	23.07	22.59	21.99
[α]	-29.13	29.82	29.97	29.925	29.86	29.67
[M]	-62.63	64.11	64.44	64.35	64.20	63.79

y.

t	0°	21.0°	39.0°	59.0°	79.0°	100°
α(60 mm.)	-27.86	28.03	27.61	27.12	26.57	25.93
[α]	-34.18	35.07	35.11	35.175	35.105	34.965
[M]	-73.49	75.40	75.49	75.64	75.51	75.19

g.

t	0°	21.0°	39.0°	59.5°	79.0°	100°
∝(60 mm.)	-31.64	31.80	31.41	30.87	30.20	29.49
[∝]	-38.82	39.79	39.95	40.05	39.91	39.76
[M]	-83.46	85.55	85.89	86.11	85.81	85.49

b.

t	0°	21.0°	39.0°	59.5°	79.0°	100°
∝(60 mm.)	-39.27	39.67	39.28	38.65	38.15	36.98
[∝]	-48.18	49.64	49.96	50.14	50.41	49.87
[M]	-103.6	106.7	107.4	107.8	108.4	107.2

v.

t	0°	21.0°	39.0°	59.5°	79.0°	100°
∝(60 mm.)	-51.63	52.28	52.17	51.48	50.48	49.51
[∝]	-63.36	65.41	66.345	66.78	66.71	66.77
[M]	-136.2	140.6	142.6	143.6	143.4	143.6

Methyl l-∝-Trichloroacetoxypropionate.

The trichloroacetylchloride used in this preparation was obtained in the same manner as monochloroacetyl chloride, from trichloracetic acid. The product was fractionally distilled, that portion boiling at 117° - 118° being retained.

To prepare the methyl trichloroacetoxypropionate, the acid chloride was boiled under reflux with twice the theoretical quantity of methyl lactate till no more hydrogen chloride was liberated (about 3 hours). The product was fractionally distilled through a column under reduced pressure till no change in the/

rotation of the distillate was observed. The ester boiled at 116° / 13 mm. Yield of impure product was theoretical.

Analysis gave Cl, 42.40%. Theory requires Cl, 42.69%.

Densities determined:

t	21.5°	40.0°	59.0°	81.5°	100°
d	1.3919	1.3678	1.3416	1.3219	1.2893

Rotation of Methyl l-α-Trichloroacetoxypropionate.

r l

t	0°	20.5°	41.0°	59.0°	80.5°	100°
α(60 mm.)	-14.18	14.19	14.04	13.93	13.82	13.69
[α]	-16.64	16.97	17.13	17.31	17.52	17.73
[M]	-41.52	42.34	42.74	43.19	43.71	42.24

r 2.

t	0°	20.5°	41.0°	59.0°	80.5°	100°
α(60 mm.)	-16.60	16.58	16.49	16.37	16.22	16.07
[α]	-19.48	19.83	20.12	20.33	20.57	20.79
[M]	-48.60	49.48	50.19	50.72	51.32	51.87

y.

t	0°	20.5°	41.0°	59.0°	80.5°	100°
α(60 mm.)	-19.45	19.45	19.36	19.23	19.14	18.91
[α]	-22.81	23.26	23.62	23.89	24.27	24.45
[M]	-56.91	58.03	58.93	59.61	60.55	61.00

g.

t	0°	20.5°	41.0°	59.0°	80.5°	100°
α(60 mm.)	-22.06	22.06	21.96	21.83	21.72	21.49
[α]	-25.88	26.39	26.79	27.12	27.54	27.79
[M]	-64.57	65.84	66.84	67.66	68.72	69.34

b.

t	0°	20 5°	41.0°	59.0°	80.5°	100°
α(60 mm.)	-27.39	27.46	27.52	27.36	27.23	27.02
[α]	- 32.13	32.84	33.58	33.99	34.52	34.93
[M]	-80.18	81.00	83.85	84.80	86.13	87.15

v.

t	0°	20.5°	40.0°	59.0°	80.5°	100°
α(60 mm.)	-35.53	35.95	35.89	35.95	35.90	35.74
[α]	-41.68	43.00	43.73	44.66	45.51	46.20
[M]	-104.0	107.3	109.1	111.4	113.5	115.3

Methyl l-α-Methoxypropionate.

The ester was prepared according to the method of Purdie and Irvine[1], and was finally purified by fractional distillation through a column under reduced pressure. It had b.p. 38°/13 mm.

Rotation of Mathyl l-α-Methoxypropionate.

Densities determined:

t	15.0°	24.5°	42·0°	49·5°	61·5°	73·5°	89·0°
d	1.0039	0·9953	0·9765	0·96775	0·9555	0·9408	0·9225

1) J. 1899, 75, 485.

r d

t	0°	25.0°	40.0°	60.0°	79.0°	100°
α(60 mm.)	-41.70	38.74	37.25	35.71	33.83	32.10
[α]	-68.14	64.96	63.51	62.29	60.37	58.82
[M]	-81.05	77.34	75.58	74.10	71.85	69.99

r 2

t	0°	25.0°	40.0°	60.0°	79.0°	100°
α(60 mm.)	-48.63	45.25	43.72	41.59	39.91	37.88
[α]	-79.46	75.88	74.55	72.54	71.22	69.42
[M]	-94.55	90.30	88.75	86.30	84.72	82.59

y.

t	0°	25.0°	40.0°	60.0°	80.0°	100°
α(60 mm.)	-57.17	53.32	51.26	48.78	46.20	44.16
[α]	-93.42	89.40	87.41	85.09	82.53	80.93
[M]	-111.20	106.40	103.90	101.25	98.20	96.22

g.

t	0°	25.0°	40.0°	60.0°	80.0°	100°
α(60 mm.)	-64.94	60.58	58.30	55.29	52.55	50.31
[α]	-106.12	101.57	99.41	96.45	93.87	92.19
[M]	-126.20	120.90	118.25	114.60	111.70	109.70

b.

t	0°	25.0°	40.0°	60.0°	79.0°	100°
α(60 mm.)	-81.48	75.38	72.95	69.98	66.08	63.23
[α]	-133.14	126.39	124.38	122.06	117.92	115.88
[M]	-159.35	150.40	148.10	145.20	140.35	137.95

V.

t	0°	25.0°	40.0°	60.0°	80.0°	100°
α(60 mm.)	-107.86	100.61	96.98	92.02	87.65	83. 67
[α]	-176.24	168.7	165.36	160.51	156.57	153.35
[M]	-209.85	200.70	196.80	191.00	186.30	182.50

Methyl l-α-Benzoyloxypropionate.

The ester was prepared by boiling methyl lactate with twice the theoretical amount of redistilled benzoyl chloride for about 3 hours under reflux. The product was then fractionated from a Claisen flask under reduced pressure till the rotation was constant. The product boiled at 145° /13 mm. and was a colourless viscous liquid with a faint agreeable odour. Yield was 85% of theoretical.

Rotation of Methyl l-α-Benzoyloxypropionate.

Densities determined:

t	16.5°	20.25°	40.0°	60.0°	80.25°	98°
d	1.1474	1.1443	1.1260	1.1068	1.0872	1.0703

r l

t	0°	18.0°	40.0°	61.5°	80.5°	100°
α(60 mm.)	+ 9.32	8.66	7.90	7.08	6.422	5.83
[α]	+ 13.36	12.59	11.69	10.68	9.84	9.10
[M]	+ 27.79	26.20	25.48	22.23	20.46	18.92

r 2

t	0°	18.0°	40.0°	61.5°	80.5°	100°
α(60 mm.)	+11.54	10.82	9.79	8.85	7.99	7.22
[α]	+16.55	15.73	14.49	13.35	12.25	11.26
[M]	+34.42	32.72	31.6	27.39	25.49	23.44

y.

t	0°	15.5°	40.0°	61.5°	80.5°	100°
α(60 mm.)	+ 14.69	13.88	12.54	11.26	10.17	9.185
[α]	+ 21,06	20.14	18.57	16.98	15.59	14.334
[M]	+43.80	41.82	38.61	35.35	31.85	29.47

g.

t	0	15.5°	40.0°	61.5°	80.5°	100°
α(60 mm.)	+17,96	16.98	15.27	13.69	12.40	11.20
[α]	+25.76	24.64	22.60	20.63	19.01	17.48
[M]	+53.45	51.28	47.05	42.85	39.55	36.41

b.

t	0°	15.5°	40.0°	61.5°	80.5°	100°
α(60 mm.)	+26.17	24.75	22.27	19.92	18.08	16.32
[α]	+37.53	35.92	32.97	30.02	27.72	25.46
[M]	+78.10	74.70	68.60	62.42	57.60	52.80

v.

t	0	15.5°	40.0°	61.5°	80.5°	100°
α(60 mm.)	+ 42.98	40.27	36.18	32.47	29.25	26.56
[α]	+ 61.65	58.44	53.55	48.94	44.85	41.45
[M]	+ 128.30	121.55	111.30	103.50	93.50	86.25

Methyl l-α-o-Nitrobenzoyloxypropionate.

O- Nitrobenzoyl chloride (7 g.) and methyl lactate (8 g.) were boiled gently in a flask fitted to an air condenser for 8 hours, and the unchanged methyl lactate was distilled off under reduced pressure. The residue consisting of impure product which distilled at 140°- 200°/6 mm. was dissolved in ether, and shaken with a concentrated/

solution of sodium carbonate to free it from excess nitrobenzoyl chloride. After removal of the ether, the product was distilled several times, the portion boiling at 175° - 180°/16 mm. being retained. On standing, the ester solidified, and was crystallised several times from 40° - 60° petroleum ether in which it is slightly soluble. It had m.p. 36° - 37° and was in the form of colourless plates. Yield of impure ester was 70% of theoretical. Analysis:- found: N, 5.67%. Calculated: N, 5.53%.

Rotation of Methyl l-α-o-Nitrobenzoyloxypropionate.

Densities determined:

t	25.5°	42.0°	60.0°	82.0°	100°
d	1.2692	1.2537	1.2367	1.2177	1.2009

r l

t	14.0°	25.0°	40.0°	60.0°	81.25°	100°
α(40 mm.)	+15.95	14.97	13.61	11.91	10.18	8.98
[α]	+31.14	29.47	27.08	24.08	20.90	18.69
[M]	+78.78	74.56	68.51	60.93	52.90	47.28

r 2

t	14.0°	24.0°	40.0°	60.0°	81.25°	100°
α(40 mm.)	+20.37	18.90	17.19	15.01	12.89	11.38
[α]	+39.76	37.18	34.20	30.34	26.47	23.66
[M]	+100.58	94.08	86.52	76.77	66.97	59.86

y.

t	14.0°	24.0°	40.0°	60.0°	80.0°	100°
α(40 mm.)	+26.30	24.53	21.99	19.36	16.72	14.83
[α]	+51.34	48.26	43.74	39.14	34.30	30.87
[M]	+132.91	122.10	110.70	99.02	86.78	78.11

g.

t	14.0°	24.0°	40.0°	60.0°	81.25°	100°
α(40 mm.)	+ 48.93	46.28	41.89	37.06	31.90	28.35
[α]	+ 95.52	91.06	83.35	74.92	65.50	59.02
[M]	+ 241.68	230.39	210.87	189.52	165.71	149.31

b.

t	14.0°	24.0°	40.0°	60.0°	81.25°	100°
α(40 mm.)	+48.93	46.28	41.89	37.06	31.90	28.35
[α]	+95.52	91.06	83.35	74.92	65.50	59.02
[M]	+241.68	230.39	210.87	189.52	165.71	149.31

v.

t	14.0°	24.0°	40.0°	60.0°	81.25°	100°
α(40 mm.)	+ 97.02	88.55	79.45	70.14	55.79	47.04
[α]	+189.39	174.22	158.07	141.79	114.55	97.92
[M]	+479.91	440.78	399.91	358.73	289.81	247.77

Methyl α-m-Nitrobenzoyloxypropionate.

Methyl lactate (8 g.) was boiled under reflux with m-nitrobenzoyl chloride (7 g.) until no more hydrogen chloride was evolved (about 6 hours). The excess methyl lactate was distilled off under reduced pressure, and on further distillation a product came over at 180°-196°/ 8 mm. An ether solution of this substance was shaken with sodium carbonate solution, and after removal of the ether, the residue was fractionated. The rotation of the product however gradually diminished apparently on account of racemisation. Several further attempts were attended with the same result. Analysis of the product gave N, 5.85%. Calculated N, 5.53%.

It seems curious that the m-nitro-derivative should undergo racemisation, whilst the corresponding o-compound seemed to be quite stable.

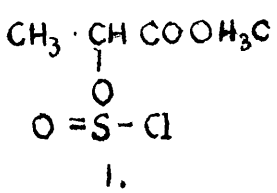
The higher boiling point of the m-compound seems scarcely sufficient to account for this.

The preparation of l-α-p. nitrobenzoyloxypropionate was attempted in the same manner as indicated above for the O-and m-nitrobenzoyl derivatives, but the methyl lactate, and the p-nitrobenzoyl chloride did not seem to react with one another at all, the methyl lactate being recovered from the reaction mixture unchanged. The use of large excess methyl lactate, the employment of higher temperatures, and prolonged heating made no appreciable difference in the final result. The effect of pyridine on the condensation at 0° and 100° was also tried without success.

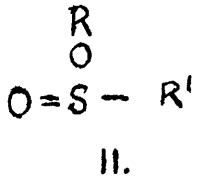
Methyl l-α-Chlorosulphinylpropionate.

This ester was prepared by Frankland and Garner's[1] method for the ethyl derivative. The product, purified by fractional distillation through a column, boiled at 89°/13 mm. Analysis of the ester showed Cl, 18·13%; S, 17·64%. Theoretical; Cl, 19.03%; S, 17.20%.

It has been pointed out that methyl α-chlorosulphinylpropionate I. should contain an asymmetric sulphur atom[2] which might influence its rotation. Several compounds containing trivalent sulphur of the type II have already been resolved.



I.



II.

With regard to the configurational nature of the chlorosulphinyl group in the compound examined, nothing can at present/

1) J. 1914, 105, 1101.
2) Phillips, J. 1925, 127, 2552.
Harrison, Kenyon and Phillips, J. 1926, 2079.

be said.

Rotation of l-α-Chlorosulphinylpropionate.

Densities determined:

t	19.5°	40.0°	59.5°	80.0°	100°
d	1.3420	1.3165	1.2922	1.2645	1.2414

r l

t	0°	16.0°	26.5°	42.0°	60.0°	80.0°	100°
α(60 mm.)	-136.76	131.33	127.79	122.92	118.86	109.80	104.33
[α]	-166.84	162.57	159.70	155.91	150.83	144.66	139.94
[M]	-311.3	303.3	298.0	290.9	281.4	269.5	261.1

r 2

t	0°	16.0°	26.5°	42.0°	60.0°	80.0°	100°
α(60 mm.)	-162.51	158.40	154.21	145.50	138.28	130.50	123.49
[α]	-198.26	196.08	192.73	184.50	178.47	171.70	165.80
[M]	-369.9	365.8	359.6	344.2	333.0	320.3	309.3

y.

t	0°	15.5°	26.5°	41.5°	60.0°	80.0°	100°
α(60 mm.)	-194.04	186.19	180.72	173.34	164.58	155.69	147.34
[α]	-236.72	230.34	225.86	219.80	212.43	204.84	197.81
[M]	-441.7	429.7	421.4	410.1	396.3	382.2	369.1

g.

t	0°	15.5°	26.5°	41.5°	60.0°	80.0°	100°
α(60 mm.)	-223.66	214.85	208.33	199.85	189.66	179.35	166.81
[α]	-272.86	265.80	260.36	253.41	244.800	235.96	223.96
[M]	-509.1	495.9	485.7	472.8	456.7	440.2	417.8

b.

t	0°	16.0°	26.5°	42.0°	60.0°	80.0°	100°
α (60 mm.)	-288.88	278.76	267.57	258.73	245.30	231.51	218.01
[α]	-351.68	345.07	334.40	328.17	316.60	304.59	292.70
[M]	-656.1	643.8	623.9	612.3	590.7	568.3	546.1

v.

t	0°	15.5°	26.5°	42.0°	60.0°	80.0°	100°
α (60 mm.)	-402.95	386.19	375.15	358.90	340.88	321.45	304.42
[α]	-491.59	477.77	468.84	455.22	439.97	422.92	408.71
[M]	-917.2	891.4	874.7	849.3	820.8	789.0	762.8

Anilide of l - lactic acid.

This preparation was carried out as described by Bischoff and Walden[1]. 10 g. methyl lactate and 9 g. Aniline were heated together under reflux at 150° - 160° for 6 hours. The unchanged substituents being removed by distillation. 5 g. methyl lactate were recovered. The brown residue was dissolved in benzene, washed with water and dilute hydrochloric acid, and boiled with animal charcoal. On removal of the benzene, repeated attempts at purification by crystallising ended in failure, and the boiling point, 250° /13 mm. was so high that the risk of racemisation was considered too great for purification by distillation.

o- Toluidide of l-lactic acid.

o-Toluidine, and the requisite quantity of methyl lactate were heated together for varying periods of time, and it was found that the reaction required 10 hours heating before it took place to any appreciable extent. The brown residue left after heating one such preparation for 12 hours was freed from unchanged substituents,

1) Ann. 1894, 279, 73.

taken up in benzene, washed with dilute hydrochloric acid, and boiled with animal charcoal. The yellow oil left on removal of the benzene was crystallised from alcohol and 60° - 80° petroleum ether m.p. was 59°.

On examination it was found that the rotation of the products obtained in the above manner was very small ($\alpha^{18°}_{5461}$ (100 mm.)+0.10 - $\alpha^{18°}_{5461}$ (100 mm.)+0.15; (0.70 g. in 10 c.c. benzene), and with a view to ascertaining if the substance had racemised 1·5 g. were heated with 6 c.c. dilute hydrochloric acid for a considerable time to bring about hydrolysis. The resulting solution was neutralised with zinc carbonate, and the o-toluidine removed with benzene. The aqueous solution of zinc lactate so obtained was evaporated to dryness on the water bath and dissolved in dilute ammonia. The highest rotation obtained for such zinc lactate was,

$$\alpha^{18°}_{5461} \text{ (100 mm.)} - 0.32°.$$

For comparison with this, the rotation of the theoretical amount of zinc l -lactate obtainable from 1.5 g. o-toluidide was dissolved in ammonia of the strength previously used, and the rotation was

$$\alpha^{18°}_{5461} \text{ (100 mm.)} - 1.20$$

It was therefore concluded that the substance had undergone racemisation.

To minimise the risk of racemisation, the preparation was attempted at lower temperature, but the two reactants were in all cases recovered unchanged.

An attempt was also made to isolate the p. nitranilide of l-lactic acid, but continued heating of methyl lactate and p.nitraniline together over long periods of time was in sufficient to cause any/

reaction to take place.

Methyl l-Lactate in Ethylene Dibromide.

p = 2.3043

Densities determined:

t	20.5°	39.0°	59.5°	80.5°	100°
d	2.1193	2.0819	2.0402	1.9963	1.9577

t	[α]r	[α] r 2	[α] y	[α] g	[α] b	[α] v
21.0°	0.00°	-0.05°	+0.05°	+0.16°	+1.04°	+4.97°
30.0	-1.56	-1.085	-0.49	-0114	+1.02	+3.76
43.0	-1.27	-1.31	-1.85	-1.085	-0.37	+0.955
49.0	-	-2.21	-	-2.05	-	+0.91
59.5	-2.17	-2.21	-2.27	-2.62	-1.61	+0.21
70.0	-2.44	-2.70	-2.89	-2.98	-2.61	-1.17
80.5	-2.75	-2.40	-4.13	-4.02	-2.63	- 2.65
90.0	-2.44	-3.54	-3.71	-4.06	-3.32	-3.54
100°	-3.09	-3.535	-4.42	-4.70	-5.03	-4.67

The data for the characteristic diagram (based on [α]g.) were taken from smoothed curves drawn from the above figures for r 2, g. and v. and were as follows (before inversion, see page 6.)

[α] r 2	-0.05°	-0.95°	-1.7°	- 2.4°	-3.15°
[α] g.	+0.10	-100	-2.00	-3.00	-4.00
[α] v.	+5.00	+2.60	+0.80	-0.95	-2.60

Part II.

The Dehydrogenation of Succinic Acid and Aldehydes by Animal Tissue.

Introduction.

Thunberg[1] in 1909 made the discovery that Succinic acid was able to increase the respiration of surviving muscle in as far as the oxygen requirement was concerned. Battelli and Stern[2], who took up a study of this phenomenon were able to show that the property of oxidising Succinic acid was possessed in varying degree by most animal tissue, and they gave the name "Succinoxydone" to the enzyme responsible, pointing out that it was very resistant to washing of the tissue with water, being closely bound up with the cellular structure. In the oxidation of the Succinic acid, oxygen was taken up, but no formation of carbon Dioxide occurred. Malic acid was postulated by Battelli and Stern as the product of the reaction. The real course of the process, however, was established by Einbeck[3], who found that the primary reaction product of the Succinic acid was not Malic acid, but Fumaric acid, a second enzyme, Fumarase, catalysing the formation of Malic acid from Fumaric acid. This view has been accepted by Battelli and Stern[4], H.O. Dakin[5], and Thunberg[6].

1) Skand, Archiv, Phys. 22, 430, 1909.

2) Bioch. Z. 30, 172, 1910.

3) Ber. 90, 301, 1914.

4) Bioch. Z. 31, 478, 1911.

5) Jnl. Biol. Chem. 52, 183, 1923.

6) Skand. Archiv. Phys. 24, 23, 1910; 25, 27, 1911; 33, 223, 1916; 35, 163, 1917; 40, 1, 1920.

Siegfried[1] was able to show that Succinic acid is a product of the physiological decomposition of protein, and this view is generally accepted now, the Succinic acid being probably formed through the breaking down of Amino acids (D.H.Moyle[2]). This worker suggests that Succinic acid may also occur in the decomposition of fatty acids and carbohydrates.

The isolation from tissue of the Succinodehydrase, as the enzyme is called, is attended with many difficulties. E. Ohlsson[3] was able to obtain an active preparation by extracting the minced flesh with $\frac{m}{15}$ sodium phosphate solution, and Widmark[4] by the use of 1.5% sodium carbonate solution. The ferment isolated by these methods, however, was found to be susceptible to shaking, and to lose its activity very easily on coagulation of the associated protein. Accordingly, therefore, for most of the work reported here, the source of the enzyme was minced muscle which had been thoroughly washed with water so that the "self-respiration" of the flesh was reduced so far as to be negligible. By this means, results obtained with the same enzyme preparation could be reproduced in a satisfactory manner.

Battelli and Stern[5] in their study of the Succinic acid oxidation used oxygen as the hydrogen acceptor, whilst Thunberg[6] and his school employed the anaerobic method, with methylene blue as acceptor. Thunberg postulated the enzyme as being of a/

1) Ber. 21, 383, 1895/96.

2) Bioch. Jnl. 18, 351, 1924.

3) Skand. Archiv. Phys. 41, 77, 1921.

4) Ibio. 44, 200, 1923.

5) loc. cit.

6) loc.cit.

/a dehydrase nature according to the Wieland[1] theory, and quite specific for the substrate Succinic acid. Meanwhile, the identity of the Succino dehydrase and the "Succinoxydone" had not been established, but this was done by Fischer[2] and Hahn and Haarmann[3] who proved the identity of the reaction products in the two cases, thus showing that in both reactions, the succinic acid was being acted on by the same enzyme—the Succinodehydrase. Fischer found that the dehydrogenation under anaerobic and aerobic conditions led to the formation of 25 - 30% Fumaric acid, and 70 - 75% Malic acid, carbon dioxide being formed only to a very slight extent.

It was pointed out by Thunberg[4] that the dehydrogenation of Succinic acid with oxygen as hydrogen acceptor is very susceptible to the influence of cyanide, traces of which inhibit the reaction considerably. Strangely, however, in the reaction under anaerobic conditions in presence of methylene blue which leads to the same products, such an influence is not apparent. This difference in behaviour in the two cases was taken by Bach and Michlin[5] as evidence against the identity of the two enzymes concerned, and the possibility of their being two Succinodehydrases was suggested. Wieland and Frage[6], however, by means of a close study of the Kinetics of the two reactions justified the view deduced from the results of Fischer.

1) Ber. 55, 36 39, 1922; Ergeb. d. Physial. 20, 477, 1922.

2) Ber. 60, 2257, 1927.

3) Zeitsch. f. Biol. 86, 523, 1927; 87, 107, 1928.

4) loc. cit.

5) Ber. 60, 827, 1927.

6) Ann. 477, 1, 1929.

In this connection O. Warburg[1] postulated that the inhibitory influence of cyanide on oxidation processes is due to the effect on the oxygen activating component of the systems, and he produced the Thunberg observation as proof of the presence of activated oxygen on the Succinic acid oxidation. warburg, explaining the difference between the effect of cyanide on the anaerobic and the aerobic oxidations, put forward the view that in contrast with the oxidation by means of oxygen, that using methylene blue cannot really be comparable with biological oxidation processes, and that results obtained by this method are not applicable.

This view of Warburg is opposed by Wieland[2] who put forward the theory that Biological oxidations proceed just as well without oxygen, much less activated oxygen, all that is required being a suitable hydrogen acceptor which takes up the hydrogen activated by the particular enzyme responsible. According to the Wieland theory, all biological oxidations lead to the formation of hydrogen peroxide, the function of the catalase being to destroy this as it is formed. The explanation of the Thunberg observation is given as being due to the poisoning of the catalase by the cyanide, the dehydrase action being then inhibited in the aerobic process by the hydrogen peroxide upon which the cyanide poisoned catalase is unable to act. Using methylene blue as hydrogen acceptor, since no hydrogen peroxide is formed, the catalase action does not come into play, and the cyanide is therefore without injurious effect on the system.

1) Bioch. Z. 177, 471.

2) loc.cit.

In the following work, an attempt was made to throw some light on these problems.

Enzyme Material.

As the source for the preparation of a suitable enzyme containing material, the muscle of the left ventricle of horse heart was used (Wieland and Frage, loc.cit.). As soon as possible after the slaughter of the animal, the heart muscle was washed with toluene saturated water, fat and sinews removed, and the remainder put through a flesh mincer three times. The freshly divided tissue was then washed with toluene-saturated distilled water till no more protein was detected in the washings, and filtered off. The amount of wash water used was about 25 times the weight of the minced flesh. In this manner, in 1½ hours after the slaughter of the animal, a large supply of no longer respiring tissue containing about 18% dry weight could be obtained.

This material was very active as regards succino-dehydrase content, and its activity was not lost on drying. The preparation was kept in ice under toluene, and in this manner was found to retain its activity at a more or less constant level during the second to the tenth day after the preparation. After the first day, it was always found that the activity had suffered a considerable decrease, and after the tenth day, a gradual sinking in activity set in, a process which was attended by coagulation of protein in the supernatant toluene-water layer covering the flesh. No injurious effect due to toluene could be detected/.

Results obtained with the same preparation could be repeated, but large variations were noted on material prepared from different sources, a result which is only to be expected, considering the difference in the conditions such as age, health, and nature of the animals from which the hearts were obtained. In experiments on the inhibitions of reactions of the enzyme, control experiments were therefore made, in which the normal course of the reactions was studied, and by this means, since the results were relatively the same, comparable effects could be obtained.

The Dependence of the Activity of the Enzyme on outside Influences.

According to Ahlgren[1] the optimum temperature for Succinodehydrase is 47° - 48°, and for horse muscle, the temperature coefficient is constant between 34 - and 46 . The temperature used in the work described here was 37°, this being maintained constant by means of an electrically heated and controlled thermostat.

The hydrogen ionconcentration for the optimum activity of the dehydrase according to E.Ohlsson[2] is at P.H. 8.6 - 8.7, and dependent on the temperature, so that at lower temperatures the optimum is moved towards the alkaline range. Lehman[3] found it at P.H. 7.3 - 7.9, while Wieland and Frage gave it at 7.3 to 7.8, depending on the concentration of the substrate. The enzyme is however still quite active in the acid range, retaining 25% of its activity at P.H.6.3, but in the alkaline side it loses its/

1) Skand. Archiv. Phys. 1925 Supplement.

2) loc. cit.

3) Bot. Notis. 1922, 289.

activity more quickly, being destroyed completely according to Ohlsson at pH.9.5.

As has been shown by Wieland and Frage (loc.cit.) by a study of the reaction velocity in a Barcroft-Warburg apparatus, the rate of reaction is somewhat greater using pure oxygen then in air. It was also shown by these workers that the velocity is only to a slight extent dependent on the concentration of the substrate.

Comparison of the Effect of Cyanide on the Succino-dehydrase and the Catalase Action.

The measurements in these experiments were made by means of the Barcroft-Warburg apparatus, in which the vessels were fitted with small side tubes, from which one of the reacting substances could be introduced into the main part of the vessel when the contents had reached the temperature of the thermostat. By this means, errors due to extrapolation for the amount of oxygen evolved and taken up during the time the reacting substances were attaining the bath temperature, were avoided. The apparatus held 12 vessels which were shaken at 37°, control experiments being made for the self respiration of the enzyme material, the temperature and barometric change, and the normal uninhibited reaction. The volumes of the vessels were calculated according to Warburg[1].

For the experiments on the dehydrase action, the vessels contained 0.5 g. enzyme material, 4.0 cc. $\frac{m}{15}$ phosphate buffer solution (P.H.7.4), the requisite amount of $\frac{N}{10}$ Hydrogen Cyanide solution, and in the small side tubes, 2.0 cc. $\frac{m}{10}$ sodium succinate solution. Small cups inside the vessels held 0.1 cc. 50% potassium hydroxide solution which absorbed any carbon dioxide which might be evolved. The total volume/

1) Biach. Z. 142. 325. 1925.

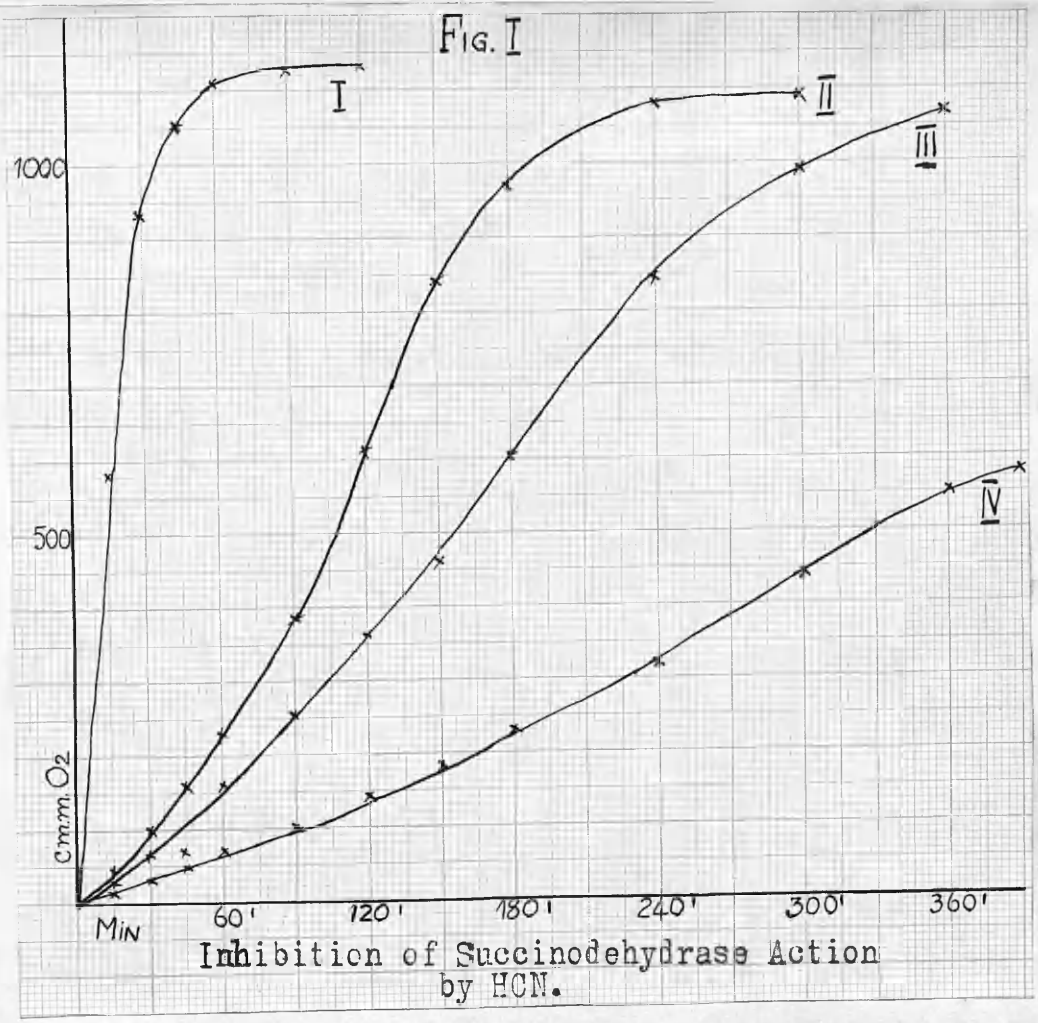
of the contents of each vessel was 10 cc. To equalise the temperature, the vessels were shaken for several minutes with open taps, then the taps having been closed, the sodium succinate solution was run in from the side tubes, and readings of the oxygen uptake taken at regular intervals.

	I.	II.	III.	IV.
Concentration of H.C.N.	0	$\frac{n}{1000}$	$\frac{n}{500}$	$\frac{n}{200}$.

Table 1.

Oxygen Uptake cmm.

Time (min.)	I.	II	III.	IV.
15	586	45	28	14
30	926	97	65	33
45	1053	158	70	51
60	1112	230	160	70
90	1126	385	255	100
120	1126	615	360	140
150		840	460	180
180		970	600	230
210		-	-	-
240		1080	845	320
300		1090	990	440
330		-	1070	550
360		-	-	582
Theoretical	1120	1120	1120	1120



The curves drawn from the results are shown above (Fig.1). The results are in agreement with those of Wieland and Frage (loc. cit.) It will be seen that in the uninhibited reaction, the rate of dehydrogenation is at first linear, gradually falling off with decreasing substrate concentration. From the course of the inhibited reaction, it is apparent that the inhibition does not remain constant for any one concentration of cyanide, but that it gradually decreases as the reaction proceeds. This is specially noticeable in the cases of $\frac{m}{1000}$ and $\frac{n}{500}$ .HCN where the recovery from the initial effects of the HCN is very marked. It is difficult to reconcile these results with the view that the inhibition produced by cyanide on the succino dehydrase action is not due directly to the effect of cyanide on the succinodehydrase, but to the

hydrogen/

peroxide which the cyanide inhibited catalase is unable to remove from the reaction. If this were the case one would expect the rate of the normal and inhibited reactions to be the same at first, and then to fall away gradually in the inhibited reactions as the hydrogen peroxide accumulated.

It is to be noted that although the effect of cyanide is constant for any one enzyme material, it varies with the different preparations, this being due to individual differences in the enzyme surfaces in the various materials. Only with the pure enzyme free from accompanying protein could this effect be excluded.

The experiments on the catalase action were carried out with the same material, and in precisely the same manner as those for the dehydrase, but in this case, instead of sodium succinate solution, the side tubes attached to the Barcroft vessels held 2.0 cc. of $\frac{m}{30}$ hydrogen peroxide solution making the concentration on the total 10 cc. of liquid in each case $\frac{m}{150}$.

	I.	II.	III.	IV.	V.	VI.	VII.
Concentration of HCN.	-	$\frac{n}{1000}$	$\frac{n}{500}$	$\frac{n}{200}$	$\frac{n}{100}$	$\frac{n}{75}$	$\frac{n}{50}$.

Cmm. Oxygen evolved.

Table 2.

Time (min.)	I.	II.	III.	IV.	V.	VI.	VII.
2	580	572	347	240	110	63	30
5	-	615	505	380	-	-	-
7	615	629	567	469	333	235	64
10	615	629	587	524	-	-	-
12	-	-	-	-	466	350	150
15			602	590	-	-	-
17			-	-	547	467	214
20			613	615	-	-	-
22			-	-	605	529	306
25			622	625	-	-	-
27			-	-	633	564	384
30				630	-	-	-
32					656	592	435
42					670	621	535
52					680	635	595
62					-	-	628
72					-	-	636.
Theoretical	700						

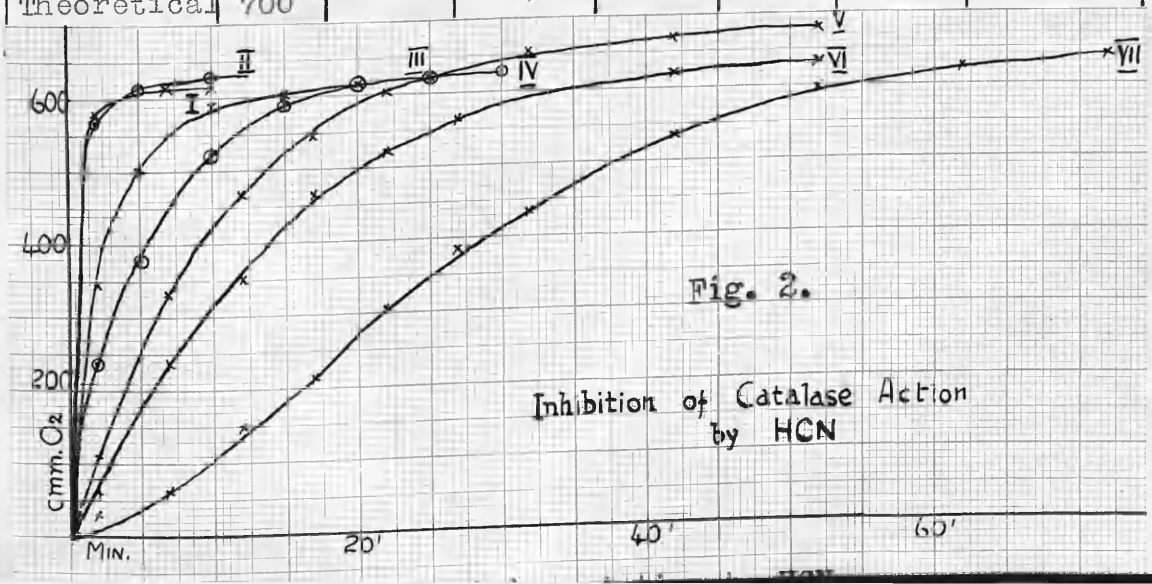


Fig. 2.

Inhibition of Catalase Action by HCN

Catalase is generally recognised as being exceedingly susceptible to the influence of cyanide, and according to Rona, Fiegel and Nakahara[1], the presence of $\frac{n}{100}$ HCN is sufficient to inhibit its action completely. Although this was not found for the catalase contained in the preparation used for this work, yet the effect of the cyanide was quite marked. This difference in results, and the comparative passivity of the catalase in the preparation is probably due to the protective influence of protein contained in the enzyme material, and perhaps also to the large amount of catalase present. It will be seen however, from the results (Fig.2.) that the presence of $\frac{n}{500}$ HCN is sufficient to reduce the rate of reaction to about 1/3 of its normal value, and that $\frac{n}{50}$ causes it to be twelve times as slow.

There does not appear to be any relationship between the inhibition of the catalase as shown above and the corresponding inhibitions obtained for the succinodehydrase action, but it must be remembered that the two cases are not quite analagous, since the behaviour of cyanide inhibited catalase when under the influence of hydrogen peroxide added directly may not be similar to that when the hydrogen peroxide is formed on the enzyme surface as a product of the dehydrogenation of succinic acid by the succinodehydrase.

With a view to showing that hydrogen peroxide is formed on the dehydrogenation of succinic acid by succinodehydrase, the use of cerous hydroxide was tried. This method of identifying and estimating the hydrogen peroxide formed by dehydrogenating enzymes was applied successfully by Wieland and Rosenfeld[1] in connection with Xanthine dehydrase, and it was thought that under suitable conditions an application could be found in the investigation of the succinodehydrase action.

1) Ann. 477, 69, 1929.

Cerous hydroxide which is itself colourless, reacts with hydrogen peroxide to form the yellow-brown ceric peroxide, thus

$$3\,Ce(OH)_3 + 3H_2O_2 \rightarrow 2\,Ce.(OH)_3OOH + 2H_2O,$$

and when the reaction is carried out at p.H 8.0, the conditions are such that autoxidation of the cerous hydroxide does not take place.

Assuming the inhibition produced by cyanide on the succinodehydrase to be due to the effect of cyanide on the catalase rather than directly on the succinodehydrase itself, it was thought that by carrying out the reaction in the presence of hydrogen peroxide and cerous hydroxide, due to the hydrogen peroxide being removed from the reaction by the cerous hydroxide, the reaction could be induced to take its normal course, or, in other words, that the cerous hydroxide would, by taking the place of the catalase, allow the uninhibited course of the reaction to proceed. Although such conditions could not be realised, definite proof of hydrogen peroxide formation, and also evidence of the inhibitory action of hydrogen peroxide on the succinodehydrase action was obtained.

Preparation of the Cerium Reagent.

An $\frac{m}{50}$ solution of cerous sulphate was brought to p.H 8.0 by the addition of the requisite amount of borate buffer solution p H 9.24, and the white precipitate of cerous hydroxide so obtained was used in the following experiments. This reagent was non-autoxidisable, and when treated with hydrogen peroxide assumed a yellow-brown colour due to the formation of ceric peroxide. When shaken in a Barcroft apparatus with the enzyme preparation, then

with/

/with sodium succinate solution, and finally with hydrogen cyanide, no trace of oxygen uptake was apparent in any case.

Control experiments were first carried out to see if it were possible to obtain the formation of ceric peroxide when hydrogen peroxide was added to the enzyme preparation in the presence of cyanide. The following results were obtained. The strength of the hydrogen peroxide used was determined by titration with potassium permanganate solution. The reaction was carried out at 37° and the Barcroft vessels were filled according to the table shown below.

	I.	II.	III.	IV.
Enzyme Preparation	0.5g	0.5g.	0.5g.	0.5g.
Hydrogen peroxide solution $\frac{N}{100}$ cc.	2.0	2.0	4.0	4.0
cc. $Ce(SO_4)_2$ solution $\frac{m}{50}$	-	2.0	-	2.0
cc. Borate buffer solution	2.0 pH 8.0	2.0 pH 9.24	2.0 pH 8.0	2.0 pH 9.24.
cc. HCN $\frac{N}{10}$	0.2	0.2	0.2	0.2
cc. H_2O.	5.3	3.3	3.3	1.3
cmm. Oxygen evolved. Time (Min.)				
2	42	6	101	10
5	70	12	158	13
8	93	12	177	16
10	-	-	186	19
15	96	12	189	19
25	99	12	189	19
30	104	12	189	19.
Theoretical.	117		234	

At the end of the experiment, the contents of vessels II and IV. were yellow in colour due to the ceric peroxide formation, and it is evident from the results that the affinity of cerous hydroxide for hydrogen peroxide is greater than that of the catalase when cyanide is present at the above concentration. Owing to the reducing property of the flesh in the enzyme preparation, and the presence of fumaric acid formed in the dehydrogenation of succinic acid when this substrate was present, the application of the usual methods (by potassium permangate or iodometrically) of estimating the ceric peroxide formed was not possible. This difficulty, however was avoided by the use of titanous chloride in acid solution, a definite volume of this reagent being added to the reaction mixture at the end of the experiment, and the excess titrated with ferric iron solution till the brown colouration with potassium rhodamide was obtained. In II. and IV. of the above experiments using $\frac{n}{50}$ solutions of these reagents, the theoretical amount of hydrogen peroxide was found in combination with the cerous hydroxide at the end of the experiment.

The investigation of the succinodehydrase action in the presenc of cerous hydroxide and cyanide gave the following results. The Barcroft vessels were filled according to the Table shown below.

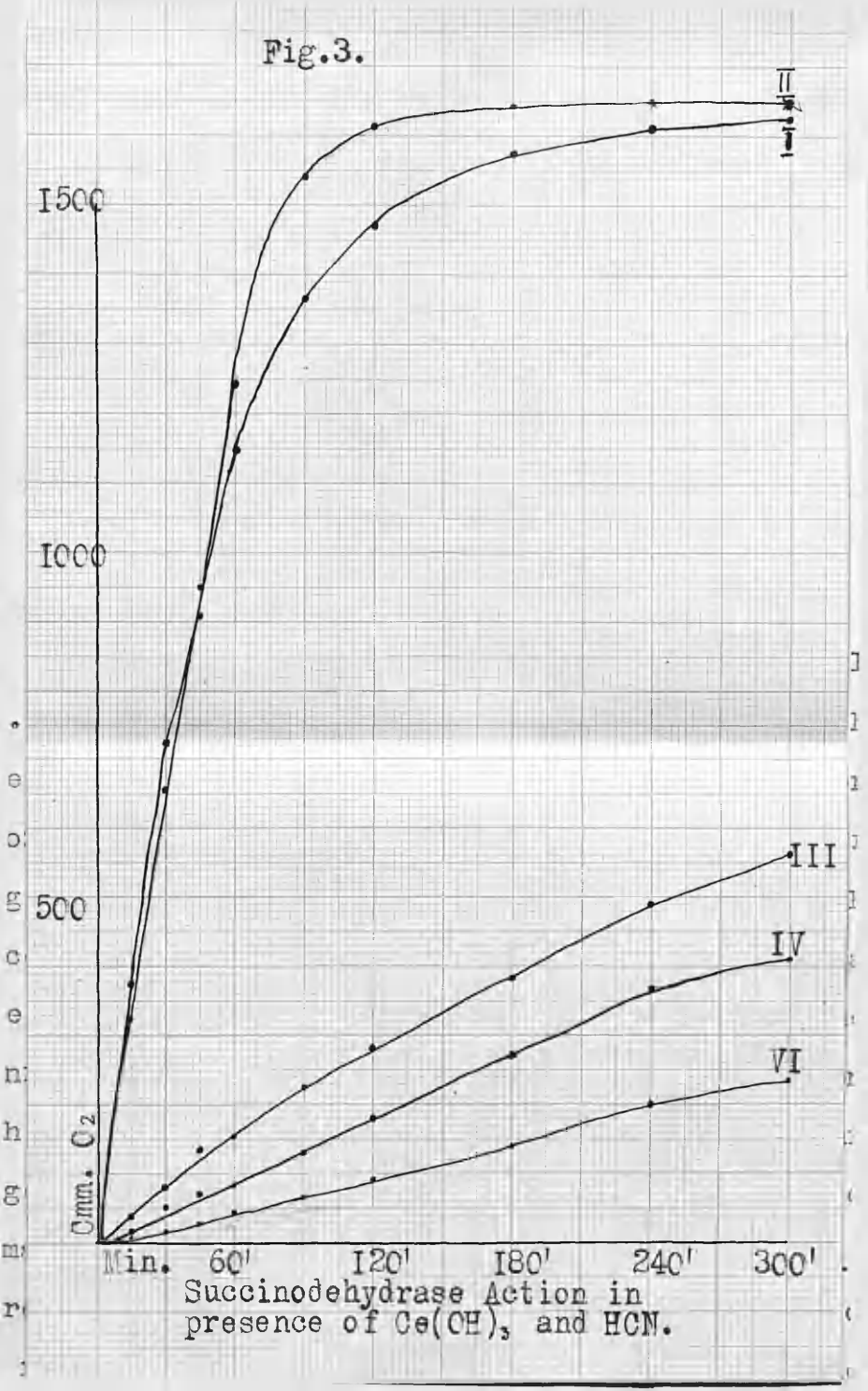
Table 3.

	1.	11.	111.	1V.	V.	V1.
Enzyme preparation.	0.5g.	0.5g.	0.5g.	0.5g.	0.5g.	0.5g.
cc.Borate buffer solution.	2.0 pH 8.0	2.0 pH 9.24	2.0 pH 9.24	2.0 pH 8.0	2.0 pH 9.24	2.0 pH 8.0
cc.$\frac{m}{50}$ Ce $(SO_4)_2$ solution.	-	2.0	2.0	-	2.0	
cc.Sodium Succinate solution.$\frac{m}{N}$	2.0	2.0	2.0	2.0	2.0	2.0
cc.$\frac{1}{10}$ HCN.	-	-	0.1	0.1	0.2	0.2
cc. H_2O	5.5	3.5	3.4	5.4	3.3	5.3

Cmm. Oxygen.

Time (min.)	1.	11.	111.	1V.	V.	V1.
15	378	324	39	21	59	12
30	726	653	92	45	107	18
45	950	908	135	72	154	30
60	1146	1242	152	84	193	45
90	1365	1540	225	132	261	66
120	1470	1610	281	180	316	90
180	1575	1640	384	273	414	141
240	1610	1650	489	369	515	198
300	1625	1653	560	508	584	234
Theoretical.	1792.					

Fig.3.



Succinodehydrase Action in presence of $Ce(OH)_3$ and HCN.

At the end of the experiment, the flesh particles in vessels III. and IV. were yellow in colour in contrast with those in the other vessels, showing apparently that the ceric peroxide was absorbed in the enzyme material. It will be seen from the curves (Fig.3.) drawn from the data obtained, that although in the presence of ceruim hydroxide and cyanide the theoretical oxygen uptake was not realised, yet a large increase over the uptake with cyanide alone was obtained and again it will be seen that when both are present, the cyanide has practically no influence on the oxygen uptake. It will also be observed that the course of the normal reaction in presence of cerous hydroxide (II) shows an increased velocity towards the end of the reaction over that of the reaction without cerium hydroxide (I), a phenomenon which is difficult to explain unless by assuming that the cerous hydroxide is protecting the succinodehydrase in some way - namely, by removing hydrogen peroxide which the catalase, weakened, by this time, is unable to do quickly enough to prevent its inhibiting influence on the succinodehydrase.

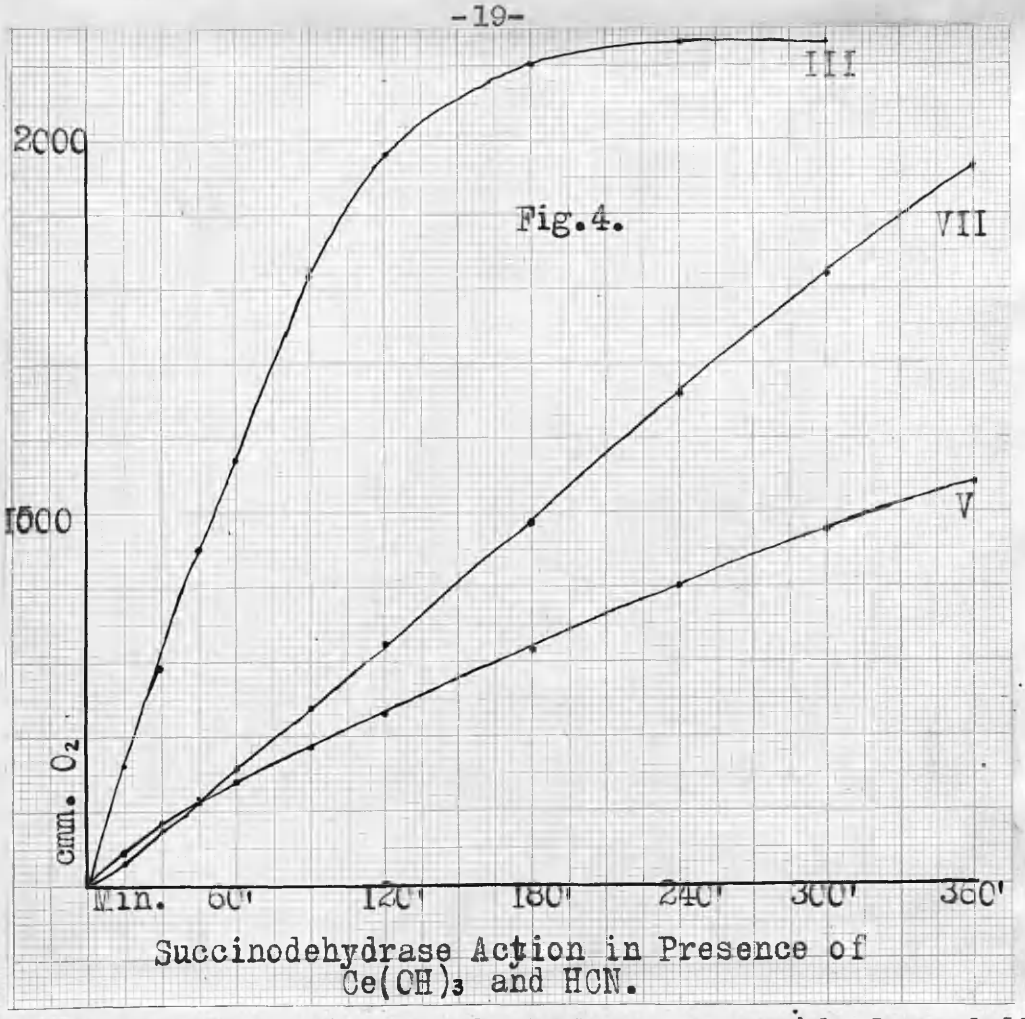
The above experiment was repeated with new enzyme material, and the hydrogen peroxide estimated as described above. The Barcroft vessels were filled according to the table.

	1.	11.	111.	1v.	V.	V1.	V11.	V111.
Enzyme Preparation.	0.5g	0.5g.	0.5g.	0.5g.	0.5g.	0.5g.	0.5g.	0.5g.
Cc. Borate buffer solution.	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Cc. $\frac{m}{50}$ $Ce_2(SO_4)_3$ solution.	2.0	2.0	2.0	2.0	2.0	2.0	-	-
Cc. $\frac{m}{10}$ Sodium succinate solut.	-	-	2.0	2.0	2.0	2.0	2.0	2.0
Cc. $\frac{n}{10}$ HCN solutt.	-	-	-	-	0.2	0.2	0.2	0.2
Cc. H20	5.5	5.5	3.5	3.5	3.3	3.3	5.3	5.3

Cmm. Oxygen

Table 4.

Time (Mins)	1.	11.	111.	1v.	V.	V1.	V11.	V111.
15	-	-	324	340	89	78	62	57
30	-	-	582	647	165	141	147	145
45	-	-	890	955	218	194	238	236
60	-	-	1145	1210	279	244	316	322
90	-	-	1630	1704	374	328	485	496
120	-	-	1960	1985	463	412	645	672
150	-	-	2155	2140	548	490	818	850
180	-	-	2215	2190	632	563	980	1019
240	-	-	2260	2235	802	721	1324	1380
300	-	-	2260	2235	950	863	1642	1709
360	-	-	-		1081	998	1930	1985.
Theoretical.			2240.					



Succinodehydrase Action in Presence of $Ce(OH)_3$ and HCN.

In the estimation of the hydrogen peroxide I. and II. were used as controls, the ceric peroxide in V. and VI. being determined.

	I.	II.	V.	VI.
cc. N.H202 found.	0.017	0.017	0.069	0.064.
cc. N.H202 present	-	-	0.052	0.047
equivalent cmm.oxygen.	-	-	293	265
% of oxygen taken up used in H202 formation.	-	-	31.7	26.6.

It would appear from the above results that the enzyme pre-

/preparation is not so susceptible to the influence of cyanide as the one previously used, since this time in the presence of cerium hydroxide and cyanide the oxygen uptake was considerably less than when cyanide alone was present. It will be seen, however, from the curves in Fig.4. that when both were present (V.) the initial velocity of reaction was slightly greater than when only cyanide was added (VII.). The results obtained with the first preparation were repeated, however, with the second preparation using larger quantities of cyanide. Each determination was made twice, the results being only shown for one series, since they were, within the experimental error, the same.

Fig.5.

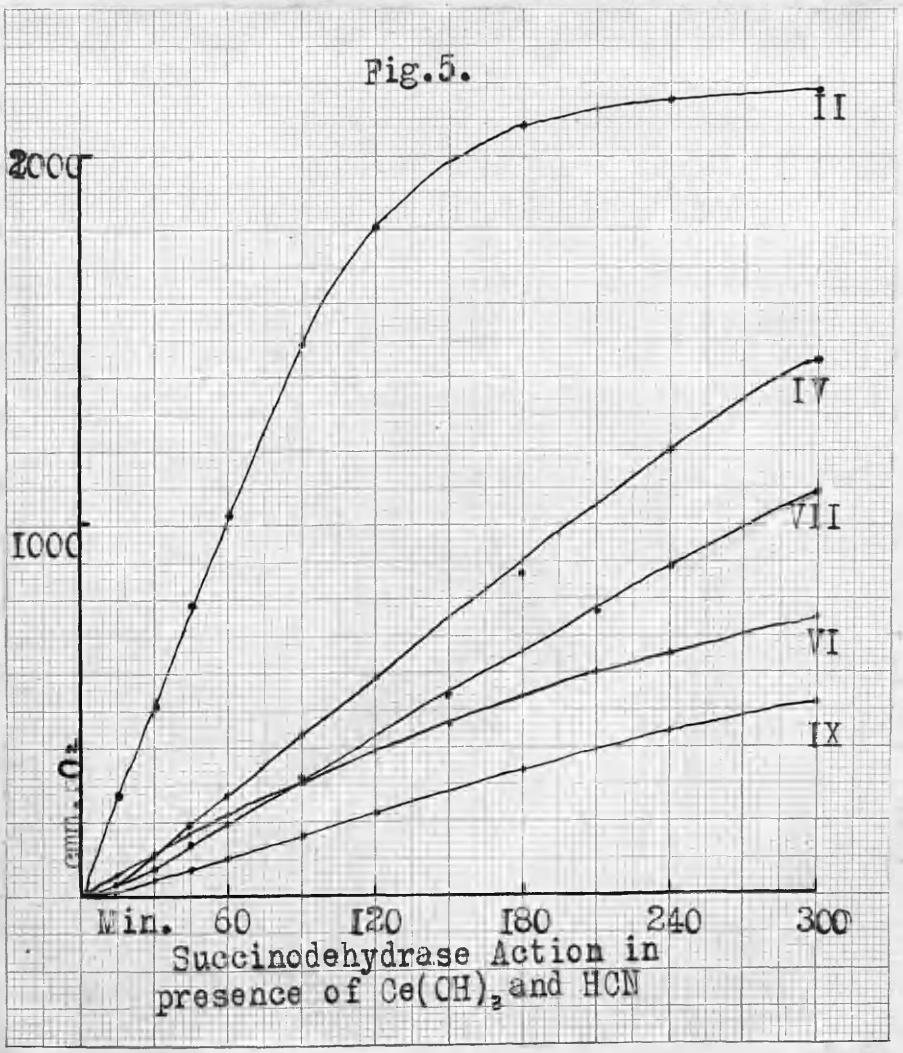


Table 5.

	1.	11.	111.	1v.	v.	V1.	V11.	V111.	1X.
Enzyme preparation.	0.5g.	0.5g	0.5g	0.5g	0.5g	0.5g	0.5g	0.5g	0.5g
c.c.Borate buffer solution.	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
c.c. $\frac{m}{50}$ cerium sulphate soln.	-	2.0	2.0	-	-	2.0	-	2.0	-
c.c. $\frac{m}{10}$ sodium succinate.	-	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
c.c. $\frac{N}{10}$ HCN.	-	-	0.2	0.2	-	0.4	0.4	1.0	1.0
c.c.H20.	5.5	3.5	3.3	5.3	5.5	3.1	5.1	2.5	4.5

Cmm. Oxygen.

Time (mins.)	I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.
15	-	279	54	28	225	60	36	60	12
30	-	516	108	116	432	111	84	117	46
45	-	789	159	192	661	171	145	162	77
60	-	1032	213	271	881	216	198	210	107
90	-	1492	314	432	1310	309	316	291	171
120	-	1805	398	590	1625	387	428	363	223
150	-	1998	476	741	1842	463	538	429	278
180	-	2080	555	865	-	-	-	496	337
210	-	2140	-	1056	2062	603	765	-	-
240	-	2160	694	1195	2102	648	890	612	438
300	-	2180	795	1440	2140	746	1095	708	521.

Estimation of hydrogen peroxide.

	1.	11.	111.	1v.	V.	V1.	V11.	VIII.	IX.
cc.N H202 found.	0.017	0.035	0.049	0.037	0.038	0.054	-	0.051	0.047
cc.N H202 present.	-	0.018	0.032	0.020	0.021	0.037	-	0.034	0.030
equivalent cmm. oxygen.									
% of 02 taken up used in formation of H202.			22.8			27.5		27.0	

It will be seen from the results that again, as in the case of the first enzyme preparation, the amount of cyanide within the limits of $\frac{n}{500}$ to $\frac{n}{100}$ has no effect on the oxygen uptake in the presence of cerous hydroxide. This could be due to two reasons, Firstly, the cerous hydroxide might prevent the action of the cyanide due to some protective effect exerted by it on the enzyme surface, or secondly, the catalase action having been excluded due to the cyanide, the inhibition could be due to the adsorption on the enzyme surface of the ceric peroxide formed, the action of the substrate being thus inhibited. If the first was the case, then one would expect that the cerous hydroxide would also inhibit the action of the substrate - a condition which is not in agreement with the results obtained when the reactions in the presence and absence of cerous hydroxide were compared (Fig.3.) Of the two, the second condition is the more probable. From the appearance of the reaction mixture at the end of the experiments when both cerous hydroxide and cyanide were present, it could be seen that the ceric peroxide was formed on the surface of the enzyme preparation, and the constant result (27%) for the amount of hydrogen peroxide formed would appear to be in agreement with this conclusion.

The increased uptake of oxygen in the presence of both cerous hydroxide and cyanide at certain concentrations of cyanide over that when cyanide alone is present, and the fact that in every case where both are present the initial reaction velocity is greater than when cyanide alone is present would point to the fact that hydrogen peroxide has an inhibitory effect on the succinodehydrase action, and that this is the reason for the difference/

/difference between the aerobic dehydrogenation, and the anaerobic dehydrogenation in presence of methylene blue with regard to the effect of cyanide.

The reason why no greater results than 27% were obtained for the amount of hydrogen peroxide formed is not clear. One would expect that with increasing amounts of cyanide, the amount of ceric peroxide would also increase.

The Influence of Carbon Monoxide on the Succinodehydrase action.

Keilin[1] from a study of the action of carbon monoxide on certain oxidising enzymes found that the action of the indophenol oxidases of both yeast and muscle and the oxidation of catechol by the oxygenase of the potato was strongly inhibited. Hydrogen cyanide in these cases was also found to have a strong inhibitory action. Haldane[2] showed that the inhibitory effect on the activity of moths and cress plants was probably due to the poisoning of an oxidation catalyst present in the cells. Again Warburg[3] pointed out the analogy between the inhibiting effect of cyanide and carbon monoxide on the respiration of yeast, postulating that in both cases the inhibition was due to the effect on the iron-containing oxygen transporting component of the respiratory ferment resulting in its inactivation. Up to this point it seemed that the carbon monoxide and the cyanide act in a similar way, the cyanide of the two having the more marked effect, fairly large amounts of carbon monoxide being necessary to produce results obtained with small amounts of cyanide. Dixon 4) however, showed that there was no such inhibiting effect of carbon monoxide on the dehydrogenation of aldehydes, by the aldehydrase present in milk, and on the succinic acid oxidation by the ferment contained in muscle. In the following work, the effect of carbon monoxide on the succinic acid dehydrogenation was again carried out using another enzyme material, and employing a different technique, from that used by Dixon in his research.

1) Nature, 119, 670.
2) Nature, 119, 352; Bioch. J. 21, 1068.
3) Bioch. Z. 177, 471.
4) Bioch. " 1211, 1927.

The carbon monoxide was prepared in the usual way by the action of concentrated sulphuric acid on formic acid, the gas evolved being purified by passing through strong sodium hydroxide solution, strong sulphuric acid, alkaline potassium permanganate solution, and water. The experiment was conducted in the Barcroft-Warburg apparatus, the vessels being provided with small side tubes which could be closed by means of ground glass stoppers.

Various mixtures of carbon monoxide and air were introduced into a long graduated gas cylinder, and the Barcroft vessels having been charged as noted below, with the exception of the sodium succinate solution, the gas mixture was led through the vessels from the containing cylinder for 15 minutes, so that they contained the same proportion of air and carbon monoxide as had been present in the containing cylinder. At the end of this time, the gas being still allowed to pass, the sodium succinate solution was introduced through the side tubes, the stoppers replaced and the apparatus lowered into the thermostat. When the temperature of the contents of the vessels had reached that of the thermostat, readings of the oxygen uptake were taken at regular intervals. A control experiment without carbon monoxide was made in each case.

Each Barcroft vessel held 0.5g enzyme preparation, 4 cc. $\frac{m}{15}$ phosphate buffer solution p H.7.4, and 5 cc. $\frac{m}{50}$ sodium succinate solution, the total volume being made up in each case to 10 cc. with distilled water saturated with toluene.

The gas mixtures were as follows:-

	I.	II.	III.	IV.	V.	VI.	VII.
Volume % of air	100%	80	80	50	50	25	25
Carbon Monoxide	-	20	20	50	50	75	75

Table 6.

cmm. Oxygen.

Time (Mins.)	I.	II.	III.	IV.	V.	VI.	VII.
15	467	351	350	196	218	173	152
30	851	704	680	444	513	330	320
45	1010	942	938	722	694	509	499
60	1046	1000	1019	823	839	664	664
75	1087	1050	1043	887	903	711	807
90	1101	1070	1065	937	952	881	909
120	1104	1107	1067	1000	985	957	984
150	1104	1107	1067	1010	-	985	1011
180	-	-	-	1029	997	1007	1038
210	-	-	-	1040	1025	1035	1038
240	-	-	-	1040	1025	1035	1038
Theoretical.	1120.						

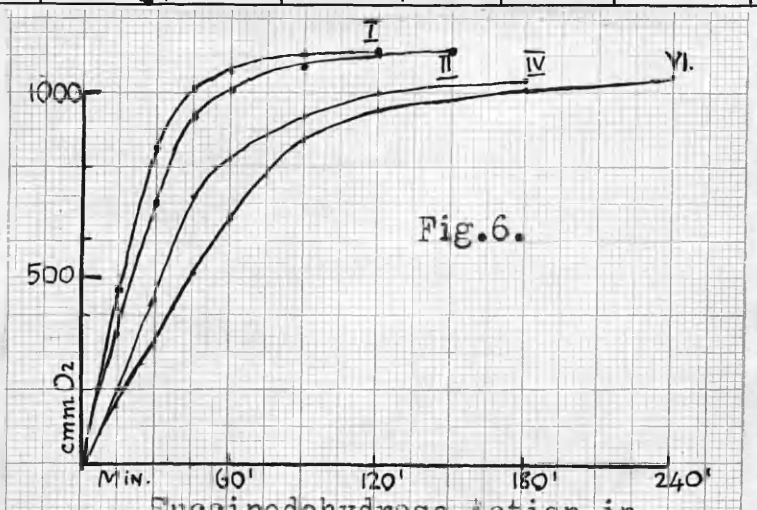


Fig.6.

Succinodehydrase Action in presence of CO.

It will be seen from the results that although the reaction velocity falls off with decreasing oxygen concentration, in every case the theoretical oxygen uptake is obtained. No inhibiting influence due to the presence of carbon monoxide is apparent.

A further experiment was then carried out with the same enzyme material. In this case pure carbon monoxide was passed from the generating flask into the Barcroft vessels, sodium succinate solution added, and the reaction mixture shaken up for 135 minutes under an atmosphere of carbon monoxide. At the end of this time, a stream of air was quickly passed through the vessels to expel the carbon monoxide, and the activity measured in the usual way.

I. normal experiment with 5 cc. $\frac{m}{50}$ sodium succinate solution.

II. and III. as I. in an atmosphere of carbon monoxide

IV. control experiment to which the sodium succinate was not added till the carbon monoxide was expelled from II. and III.

cmm. Oxygen. Table 7.

Time (mins.)	1.	11.	111.	1V.
15	428	5	-7	
30	820	5	-12	
45	978	5	-14	
60	1019	5	-16	
90	1085	13	-14	
120	-	13	-19	
135	-	0	0	
150		344	384	439
165		689	752	744
180		831	914	863
195		877	998	936
210		941	1034	971
225		956	1055	989
240		063	1064	1011
255		987	1064	1026.
Theory	1120.			

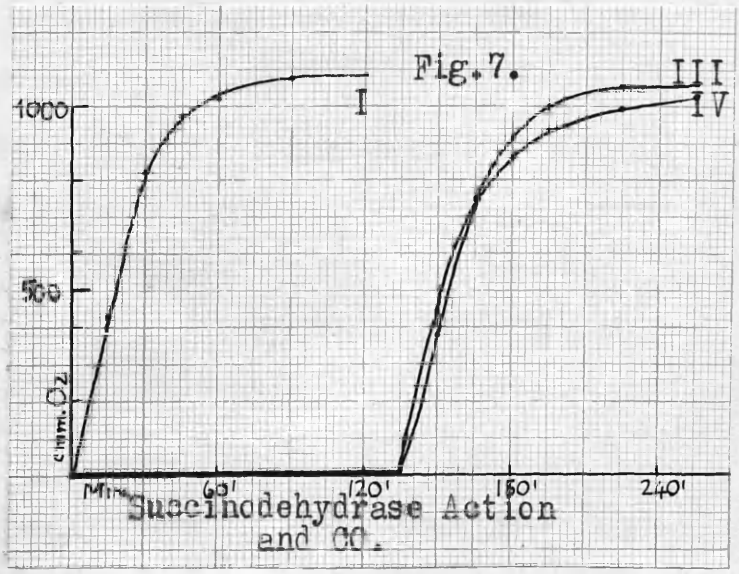


Fig.7.

Succinodehydrase Action and CO.

If, as Warburg believes, the actions of cyanide and carbon monoxide are analagous, we might expect that some inhibition of the succinodehydrase action due to carbon monoxide would be apparent. The results given above show definitely that the carbon monoxide has no such effect. In fact, as is shown in fig. 7, even after the enzyme preparation had been shaken in an atmosphere of pure carbon monoxide for over two hours, it was still as capable of dehydrogenating succinic acid as another sample of the enzyme preparation which had been shaken for the same length of time in air. The negative values in column III. above are probably due to small quantities of CO_2 evolved by the enzyme preparation, since the usual small amount of potassium hydroxide was not introduced into the small cups of the Barcroft vessels owing to the danger of combination with the carbon monoxide.

If it is assumed that the action of carbon monoxide and cyanide are analagous in so far as their inhibitory action is due to their effect on the iron containing oxygen transporting system, then it would follow from the above results that the view that an iron complex is the oxygen carrier in the succinodehydrase action does not hold. It is apparent that the action of cyanide is upon some mechanism of the dehydrogenation process upon which the carbon monoxide has no effect, and from the results of the work done on the influence of cyanide, it would appear that the catalase is the susceptible part of the system.

The Influence of Quinone and Hydroquinone on the Succinodehydrase action.

Through the inability of quinone to act as hydrogen acceptor in the action of succinodehydrase, Wieland and Frage (loc. cit.) were led to a study of the effect of quinone and hydroquinone on the enzyme preparation obtained from horse heart muscle. They found that quinone caused an inhibition of the succinic acid dehydrogenation, and was at the same time strongly absorbed on the tissue. Hydroquinone, when added to a solution containing the enzyme preparation and succinic acid was found in some cases to cause an increase, and in most cases, a considerable decrease in the oxygen requirement, although the formation of quinone could be detected in the resulting mixture. These facts were further investigated in the following work.

Hydroquinone as Hydrogen Donator.

The experiments were carried out using the Barcroft-Warburg apparatus, the usual washed muscle preparation being the source of the enzyme. The conditions of the experiments were the same as previously reported, except that the hydrogenion concentration was changed over slightly to the acid side (p H 6.8) in view of the susceptibility of hydroquinone to autoxidation in alkaline solution.

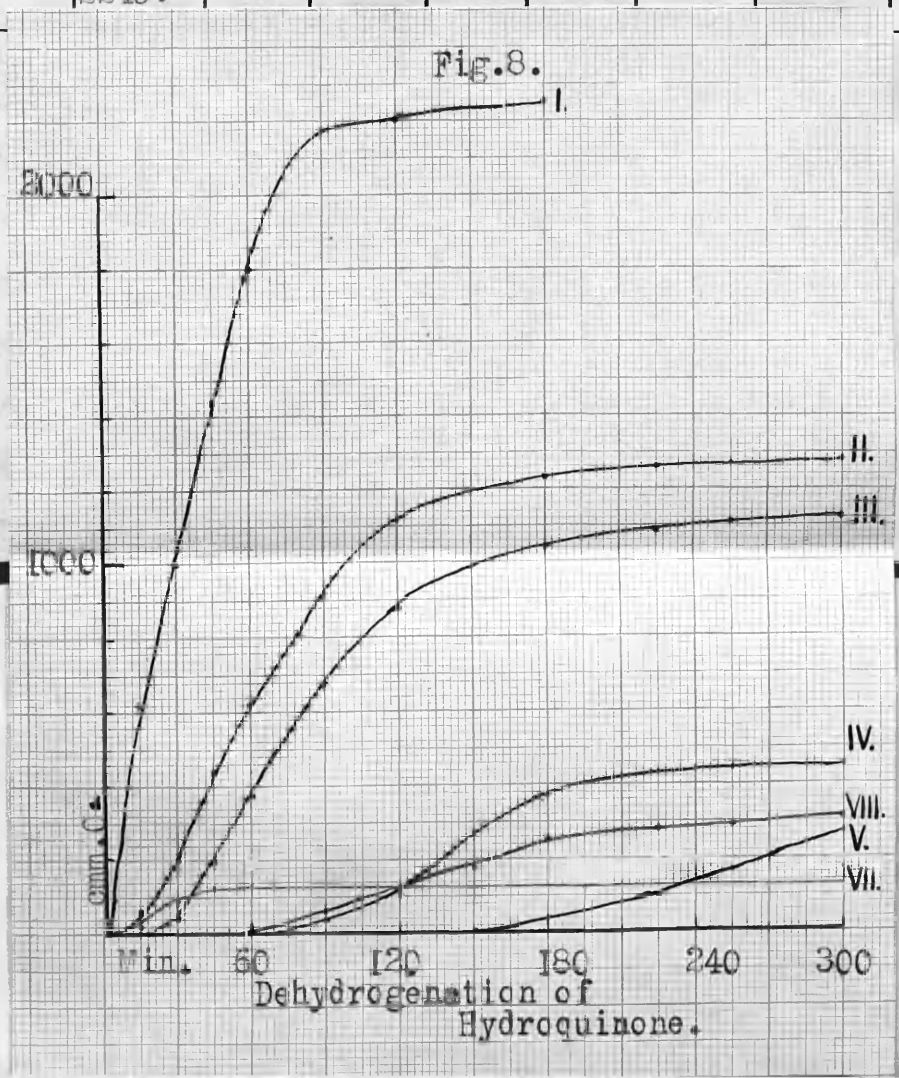
Each vessel, with the exceptiono of Vl., to which no enzyme was added, held 0.5 g enzyme preparation. 1. is the control experiment for the succinodehydrase action, the concentration of sodium succinate being $\frac{m}{50}$, T = 37°.

	1.	11.	111.	1v.	V.	V1.	V11.	V111.
Conc. of Hydroquinone.	-	m/25	m/50	m/250	m/500	m/50	m/50	m/250
Conc. of Quinone.	-	-	-	-	-	-	m/50	m/250

c.mm. Oxygen. Table 8.

Time (min).	1.	11.	111.	1V.	V.	V1.	V11.	V111.
15	623	57	0	0	0	0	15	0
30	1003	203	45	0	0	0	91	0
45	1443	443	199	0	0	0	125	0
60	1796	619	376	7	0	0	129	4
90	2180	922	675	38	0	0	131	58
120	2205	1118	880	105	0	-	131	117
150	2265	1190	995	270	2	-	131	184
180	2265	1230	1042	371	37	-	-	251
225		1255	1083	428	99	-	-	279
255		1268	1110	443	173	-	-	284
300		1279	1117	451	269	0	131	308
Theoretical	2240.							

Fig.8.



Dehydrogenation of Hydroquinone.

It is apparent from the above results (Table 8.) that the course of the reaction with hydroquinone goes very slowly at first, even in cases where a relatively high concentration of hydroquinone is present, that a maximum velocity is reached and maintained for a considerable time, the reaction gradually becoming slower till no more oxygen is taken up. In some cases only is the theoretical oxygen requirement of the added hydroquinone satisfied. Where the original concentration of the added hydroquinone is above $\frac{m}{100}$, oxygen is taken up till the point is reached indicating that $\frac{m}{100}$ hydroquinone has been oxidised, where the original concentration of the hydroquinone is below this figure, the theoretical oxygen uptake is reached.

As is shown by columns VII and VIII, quinone has an inhibitory effect on the hydroquinone oxidation. Since it can be shown that quinone is formed in this reaction, it would appear that this compound is responsible for the inhibitory effect which prevented the hydroquinone in some cases from being completely oxidised. Further, it would appear that $\frac{m}{100}$ quinone is the amount necessary to bring the reaction to a standstill. Repeated experiments gave the same results and confirmed this view.

The Influence of Cyanide on the Dehydrogenation of Hydroquinone. -

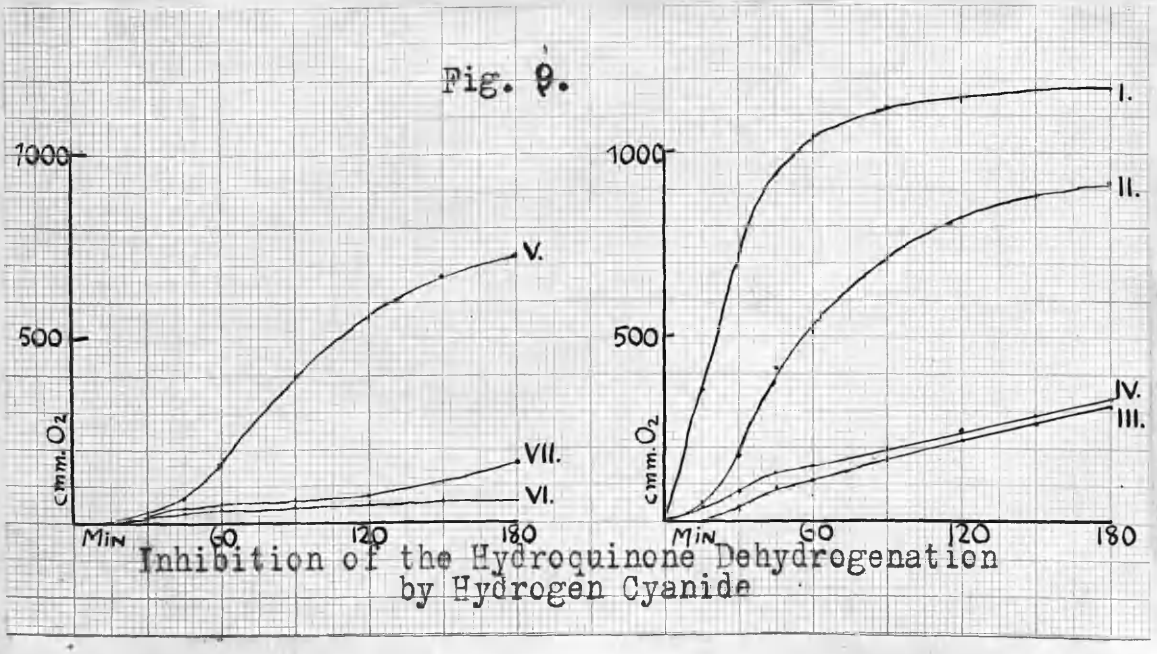
In view of the fact that the autoxidation of hydroquinone is not susceptible to cyanide poisoning, the dehydrogenation of hydroquinone was carried out in presence of added amounts of hydrogen cyanide. Two different enzyme preparations A and B were used, each vessel containing 0.5 g. enzyme preparation. 1. represents the control experiment containing $\frac{m}{100}$ sodium succinate, p H = 6.8, T = 37°.

	A			B		
	1.	11.	111.	1v.	V.	V1.	V11.
Hydroquinone conc.	-	m/25	m/25	m/25	m/100	m/100	m/100
HCN conc.	-	-	n/400	n/1000	-	n/200	n/500.

cmm. Oxygen. Table 9.

Time (mins.)	1.	11.	111.	1V.	V.	V1.	V11.
15	360	52	2	40	0	0	2
30	722	179	42	80	29	15	24
45	941	407	90	126	68	26	35
60	1043	506	111	145	161	35	45
90	1118	713	170	201	394	43	61
120	1140	822	223	250	557	47	72
150	-	879	266	292	671	58	106
180	1170	917	314	335	720	60	158
Theoretical.	1120.						

Fig. 9.



Inhibition of the Hydroquinone Dehydrogenation by Hydrogen Cyanide

In contrast with the values obtained by Wieland and Frage (loc.cit.) for the influence of cyanide on the dehydrogenation of hydroquinone, the above figures (Table.9.) show that system is much more susceptible to the influence of cyanide than the succinic acid dehydrogenation.

Succinic Acid and Hydroquinone acting together as Substrate.

The effect of hydroquinone and sodium succinate acting together as substrate and the influence of quinone on the succinic acid and dehydrogenation was the subject of the following work.

0.5 g. enzyme preparation. p.H 6.8, T = 37°.

Volume of solution in all cases was 10 cc.

Concentration	1.	11.	111.	1V.	V.	V1.	V11.	V111.
Sodium Succinate	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{50}$
Hydroquinone	-	$\frac{m}{25}$	$\frac{m}{50}$	$\frac{m}{250}$	$\frac{m}{500}$	$\frac{m}{1000}$	-	-
Quinone	-	-	-	-	-	-	$\frac{m}{50}$	$\frac{m}{250}$.

cmm. Oxygen. Table 10.

Time (mins.)	I.	II.	III.	IV.	V.	VI.	VII.	VIII
15	580	385	658	751	672	681	0	0
30	982	566	925	1164	1029	1052	0	17
45	1395	828	1118	1640	1447	1478	0	43
60	1603	1100	1250	2020	1780	1825	0	62
90	2135	1492	1641	2385	2145	2250	0	98
120	2318	1731	1948	2618	2300	2402	0	122
150	2327	1820	2115	2745	2384	2490	0	139
180	-	1875	2182	2830	2402	2530	0	156.
Theoretical	2240.							

With small quantities of hydroquinone it is apparent that when both hydroquinone and succinic acid are acting together, the reaction takes place without injurious effect to either of the systems, the theoretical oxygen requirements for both substrates being satisfied; when, however, the concentration of hydroquinone reaches values greater than $\frac{m}{100}$, the inhibition becomes marked, and the oxygen uptake decreases with increasing quantity of hydroquinone. The inhibitory effect of quinone on the succinic acid dehydrogenation pointed out by Wieland and Frage (loc.cit.) is shown by the results in VII and VIII (Tab.10.)

A comparison of the effects of quinone on the two systems shows that the succinic acid dehydrogenation is the more susceptible. This may be taken as evidence that the two systems are quite distinct from one another, and that different enzymes are responsible.

As a further demonstration of the fact that the complete dehydrogenation of $\frac{m}{100}$ hydroquinone was sufficient to inhibit completely the succinodehydrase reaction, the following experiment was carried out. A new enzyme preparation was used.

0.5 g. enzyme preparation. p.H 6.8, T = 37°.

	1.	11.	111.
Hydroquinone concentration.	-	$\frac{m}{100}$	$\frac{m}{100}$

The Barcroft vessels filled as indicated above were shaken at 37° for two hours, when to each, sodium succinate solution was added so that the concentration was $\frac{m}{50}$, and the oxygen uptake measured in the usual way. Volume of solution in each case was 10 cc.

Cmm. Oxygen. Table 11.

Time (mins.)	1.	11.	111.
15	541	20	21
30	766	48	53
45	1030	71	73
60	1236	92	90
90	1540	121	113
120	1715	145	136.
Theoretical.	2240.		

It can be seen from the results that when the succinate was added the dehydrogenation of the hydroquinone had been sufficient to almost completely inhibit the succinodehydrase action.

In order to find if the enzyme preparation lost its activity towards succinic acid and hydroquinone at the same rate, the following experiment was carried out. A preparation of the enzyme material was preservedunder toluene in an ice box at 0°, and the activity towards the two substrates measured from time to time, under the usual conditions, by means of the Barcroft-Warburg apparatus. The following table shows the rates of decrease in activity. 0.5 g enzyme preparation was used in each case and volume of solution was 10 cc.

Table 12.

No. of days after preparation of enzyme material.	c.mm. Oxygen m/50 taken up by hydroquinone after 120 mins. at p H 6.8 and 37°.	c.mm. Oxygen taken up by m/50 sodium succinate after 120 min. at p H 6.8 & 37°.
4	1025	2280
5	960	2100
6	940	2140
11	980	440
17	660	140.

It is evident that the activity towards succinate falls away very much more rapidly than that towards hydroquinone, the latter maintaining the same value from the 4th to the 11th day after the preparation of the material. It would therefore appear that there are two different enzymes concerned in the two cases, and that of the two, the enzyme responsible for the hydroquinone dehydrogenation is the more stable. The conclusion that there are two different

enzymes/

/enzymes is also supported by the fact that the extent of the inhibition produced by cyanide is not the same in the two systems.

In order to find if the enzyme preparation was able in the presence of hydroquinone to bring about the dehydrogenation of such substances as Methyl glyoxal, pyruvic acid and acetic acid, quantities of these substances were shaken with the enzyme preparation and varying amounts of hydroquinone in the Barcroft apparatus at 37° and p.H 6.8. In no case however, was any influence apparent on the oxygen uptake due to the presence of these substances.

Tyrosine and other Phenolic substances as Hydrogen Donators.

It has been shown by various workers including Bach [1], Onslow and Robinson [2], and many others that tyrosinase is capable of oxidising, besides tyrosine, such other compounds as catechol, p-cresol, di-hydroxy-phenylalanine and hydroquinone. In view of this, it was thought possible that the action of the enzyme preparation used in this work might be due to the so-called tyrosinase action, and the following series of experiments was carried out. Varying quantities of catechol, p cresol, di-hydroxyphenylalanine and tyrosine were shaken with the enzyme preparation in a Barcroft apparatus at p H 6.8 and 37°, the following figures showing the greatest extents to which oxidation of the various substances was apparent after a peridd of 3 hours. All preparations examined showed the normal activity towards hydroquinone.

Catechol	10%
p cresol	10%
Tyrosine	2%
2,3,di-hydroxyphenylaline	nil.

1) Bioch. Zeit. 60, 221. 2) Bioch. Jnl., 420, 1925

It would appear that the activity towards hydroquinone is not caused by tyrosinase, the enzyme responsible being apparently specific for the hydroquinone. It may be noted in this connection, that Bertrand [1] was able to effect a separation of Laccase and Tyrosinase occurring in Russula delica. The laccase was shown to be specific for hydroquinone and pyrogallol, but had no effect on tyrosine.

In contrast with the results obtained above for the simultaneous dehydrogenation of hydroquinone and succinic acid when both together were present as substrate, the presence of catechol was found to have a very considerable inhibitory effect on the dehydrogenation of succinic acid.

Each Barcroft vessel held 0.5 g enzyme preparation, p H 6.8, T = 37°.

Concentration	I.	II.	III.	IV.	V.
Succinate sodium	$\frac{m}{50}$	$\frac{m}{50}$	-	$\frac{m}{50}$	-
Catechol	-	$\frac{m}{50}$	$\frac{m}{50}$	$\frac{m}{250}$	$\frac{m}{250}$

1) Bull. Soc. Chem. Serie 3 Bd.13, 361, 1896.

2) Bull. Soc. Chem. Serie 3 Bd.17, 621, 1897.

cmm. Oxygen.

Table 13.

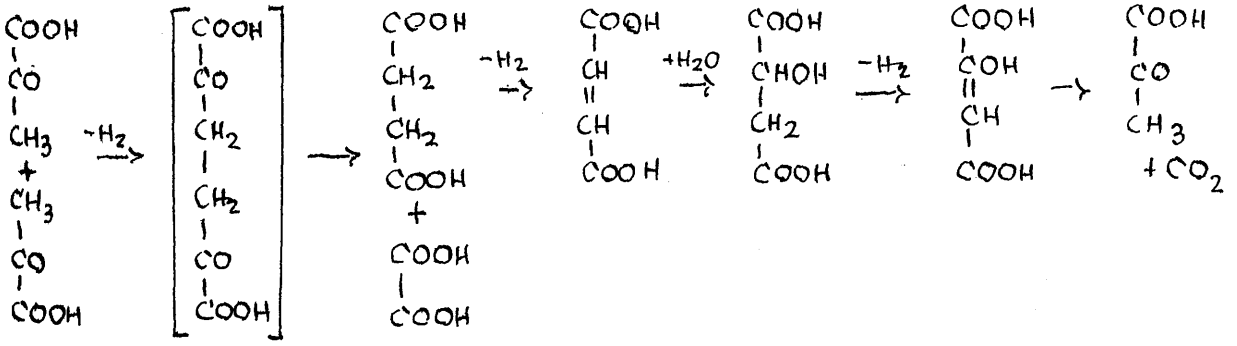
Time (mins.)	I.	II.	III.	IV.	V.
15	165	88	27	133	6
30	328	163	54	268	11
45	506	198	81	402	17
60	670	226	102	524	23
90	952	267	152	695	43
120	1182	304	191	791	55
150	1370	338	233	873	72
180	1525	370	254	861	85.
Theoretical.	2240.				

Methyl Glyoxal, Acetic Acid and Pyruvic Acid as Hydrogen Donators.

Loeniessen[1] working on the products of organic oxidation brought about by physiological processes taking place in the body of a dog was able to identify succinic acid and formic acid as the products formed by the oxidation of pyruvic acid. In these experiments, conducted in vivo, definite quantities of pyruvic acid were injected into the anaesthetised animal, the succinic acid and formic acid being detected and estimated in the usual way. This discovery gave rise to a theory for the breaking down of hexoses in animal metabolism, the process being represented as follows:-

$$\text{SUGAR} \longrightarrow 2\,[CH_3COCHO] \longrightarrow 2\,[CH_3COCOOH]$$

methyl glyoxal — pyruvic acid



The process was assumed to be continuous with repeated formation and breaking down of pyruvic acid.

In view of the above, the minced muscle of horse heart, both in the washed and unwashed condition, was heated with varying amounts of methyl glyoxal, pyruvic acid and acetic acid in a Barcroft apparatus at p H 7.4 and 37° to find if any oxidation of/

1) Klin. Wochenschrift 211, 1930.

/of these substances took place. With quantities of methyl glyoxal varying from $\frac{m}{50}$ to $\frac{m}{250}$ and the usual 0.5 g muscle tissue, no uptake of oxygen was apparent after 4 hours. With pyruvic and acetic acids using washed tissue, the results were again negative, but with the unwashed muscle, an oxygen uptake equivalent to about 5% and 2% respectively of the added substrates was obtained. This however, was too small to admit identification of the products.

The Press Liquor.

With a view to obtaining a more suitable enzyme containing material than the previously used flesh preparation, a press liquor was prepared. The left ventricle of horse heart immediately after the slaughter of the animal was minced three times, wrapped in linen, and subjected to a pressure of 300 Kg. per sq. cm. for 1 hour in a steel press. During the operation, the receiver for the liquor was kept at 0° in ice, and after centrifuging off any solid matter, the blood coloured solution was saturated with toluol, and preserved at 0° till used. In this manner, from 500 g. muscle, 160-180 g. of liquor could be obtained.

The press liquor was not so active as an equal weight of the previously used enzyme preparation, and had the disadvantage of having a considerable self-respiration. Also, the activity towards succinic acid decreased very quickly, being reduced to one half of the original activity after standing, 24 hours at 0° under the presence of toluene. In the case of hydroquinone, however, the decrease in activity was not so marked, and after falling slightly during the first 24 hours, it was maintained at a fairly constant level for several days. This increase in activity towards succinic acid was always accompanied by the precipitation of quantities of/

/of protein which themselves showed no activity, this being probably a manifestation of the phenomena already pointed out in this field by Ohlsson, Widmark, and Gronvall (loc.cit.) who found that the succinodehydrase was unstable in solution, its activity being lost due to the coagulation of either the enzyme itself or the associated protein.

The activity of press liquors made in the above manner varied considerably according to the condition of the starting material, but the following results obtained using one preparation, were general for those examined. The experiments were carried out on the Barcroft-Warburg apparatus on the same day as the press liquor was prepared. At the end of the experiments, the Barcroft vessels were in every case observed to contain quantities of coagulated protein.

5.0 cc. press liquor used.(IV and VIII held 0.5 g. of previously used washed muscle preparation from the same source.)

T = 37°.

p H of succinic acid 7.4.

p H. of hydroquinone 6.8

Volume of solution in each case was 10 cc.

Concentration	I.	II.	III.	IV.	V.	VI.	VII.	VIII.
Sodium Succinate	$\frac{m}{50}$	$\frac{m}{50}$	-	$\frac{m}{50}$	-	-	-	-
Hydroquinone	-	-	-	-	$\frac{m}{50}$	$\frac{m}{50}$	-	$\frac{m}{50}$.

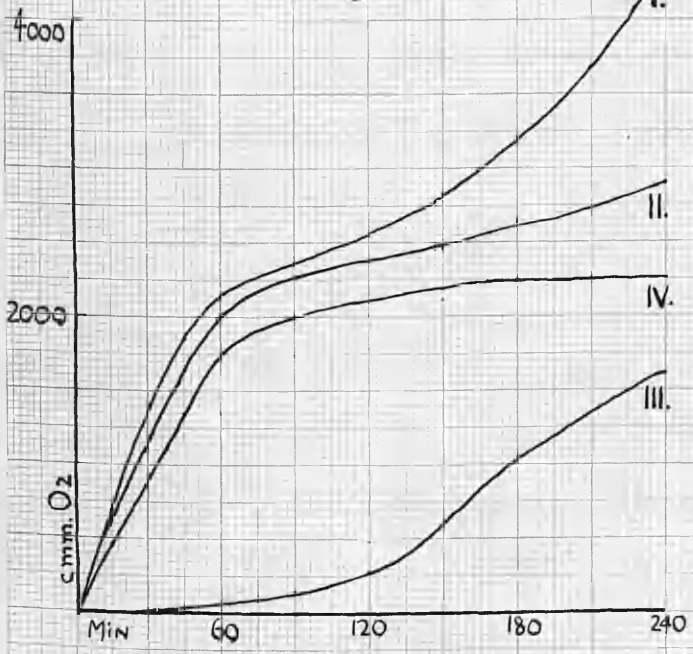
In all cases except II and VI, CO_2 absorption by 50% KOH.

cmm. Oxygen.

Table 14.

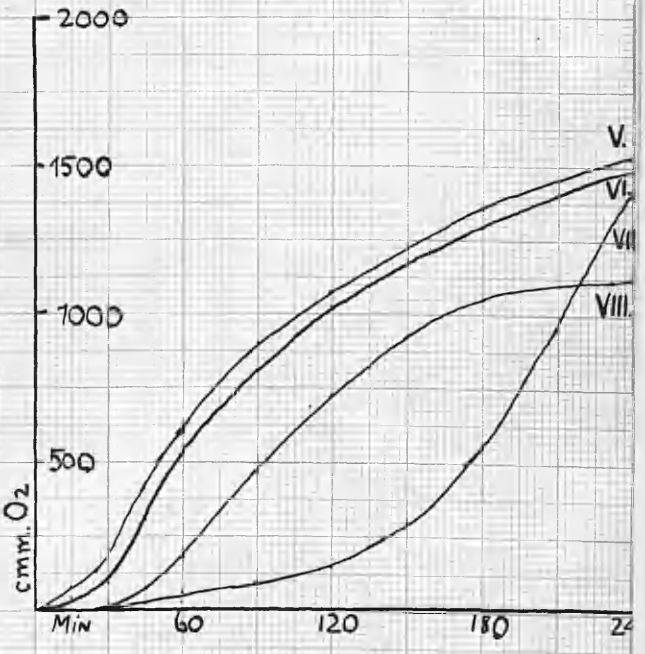
Time (mins)	I.	II.	III.	IV.	V.	VI.	VII.	VIII.
15	630	625	4	465	84	40	0	0
30	1340	1114	15	952	179	98	0	18
45	1862	1646	34	1352	433	304	27	78
60	2125	2010	60	1727	616	538	59	186
90	2360	2270	141	1995	900	810	95	468
120	2540	2366	237	2113	1080	1020	144	729
150	2820	2469	585	2180	1230	1175	294	919
180	3240	2654	1032	2247	1364	1290	564	1049
240	4410	2892	1605	2262	1530	1485	1413	1120.

Fig. 10.



Dehydrogenation of Succinic Acid by Press Liquor.

Fig. 11.



Dehydrogenation of Hydroquinone by Press Liquor.

Making allowance for the self-respiration, it would appear that 0.5 g. of the usual flesh preparation (dry weight 0.1 g.) is only slightly less active towards succinic acid and hydroquinone than 5.0 cc. of the press liquor (dry weight 0.3 g.) It will be seen from the curves (Figs.10 and 11.) that the self respiration III and VII increases rapidly after 60 minutes, maintaining a linear velocity during the remainder of the time under observation. Corresponding to this increase in the self respiration, there is an increase in the speed of uptake of oxygen in the experiments in which sodium succinate is present. This phenomenon is however absent in the cases where hydroquinone is added, a fact which can be accounted for by the inhibitory action of the quinone formed as a product of the dehydrogenation of hydroquinone. It will be noticed from the results, that in the case of succinate, a considerable quantity of CO_2 was evolved, while with hydroquinone, such an effect was only apparent to a very slight degree, this difference again being probably due to the inhibitory action of the formed quinone.

By quickly drying the press liquor in a vaccum desiccator over caustic soda and concentrated sulphuric acid, a light brown coloured product was obtained, which on being ground to a fine powder in a mortar, was found to have a negligible self respiration, and to retain the activity quite satisfactorily. The activity towards succinic acid, however, was not quite so great as that of the corresponding amount of press liquor, and considerably less than that of the dry preparation obtained from 0.5 g. of the previously used flesh preparation.

5 cc. press Liquor gave 0.29 g. dry material.

0.5 g. washed muscle preparation gave 0.1 g. dry material.

The Barcroft vessels were filled as follows:-

I. 0.5 g. washed muscle preparation.

II. 0.1 g. dry material from I.

III. 5.0 cc. press liquor.

IV. 5.0 cc. press liquor.

V. 0.29 g. dried press liquor.

Each vessel, with the exception of IV, which was the control experiment to determine the self respiration, held $\frac{M}{50}$ sodium succinate, p H was 7.4, T, 37°.

Volume of solution in each case was 10 cc.

cmm. Oxygen. Table 15.

Time (min.)	I.	II.	III.	IV.	V.
15	325	408	431	3	205
30	635	713	793	8	385
45	1053	1124	1326	28	564
60	1414	1502	1770	60	746
90	2030	2027	2148	132	1061
120	2267	2370	2268	271	1296
Theoretical.	2240.				

It is interesting to note that whereas the dryingof the washed muscle preparation was attended by an increase in the activity, in the case of the press liquor, a considerable reduction was the result. It would appear that there is some difference in the state in which the enzyme occurs in the two cases, and in view of this it was thought that if the enzyme could be/

/be separated from part of the associated protein, it might be obtained in a more stable form. Attempts were therefore made using the press liquor to see if it were possible to effect a purification by precipitation and absorption methods.

Attempts at Purification of Succinodehydrase.

With a view to obtaining a more active and purer form of the succinodehydrase, a series of experiments were carried out using the press liquor as the starting material. The treatment and testing of the various products unless otherwise stated, was done on the same day as that on which the horse heart had been obtained, and the press liquor was prepared as soon as possible after the slaughter of the animal.

By precipitation of the centrifuged press liquor with an equal volume of acetone, products were obtained which were active towards hydroquinone, but inactive towards succinic acid.

Using an equal volume of saturated ammonium sulphate solution, it was found that the succinodehydrase could be precipitated, while the enzyme responsible for the dehydrogenation of hydroquinone remained in solution.

100 cc. of the press liquor were treated with 100 cc. saturated ammonium sulphate solution, the precipitate certrifuged off, and made up to 30 cc. with distilled water. In I and III of the following experiments made on the Barcroft apparatus, 1 cc. of the above suspension was used. II and IV show for comparison the values obtained with 5.0 cc. of the original press liquor.

I. and II held $\frac{m}{50}$ sodium succinate p H 7.4.

III and IV held $\frac{m}{50}$ hydroquinone p H 6.8.

Volume of solution in each case was 10 cc.

cmm. Oxygen. Table 16.

Time (mins.)	I.	II.	III.	IV.
15	231	431	0	31
30	441	793	0	86
45	691	1326	0	240
60	801	1770	2	474
90	1238	2148	11	847
120	1440	2267	23	1175
150	1560	2275	33	1205
180	1649	2283	42	1293.

The figures shown in column II. were obtained by subtracting the uptake of oxygen representing the self respiration of 5 cc. of press liquor from that obtained when sodium succinate was present.

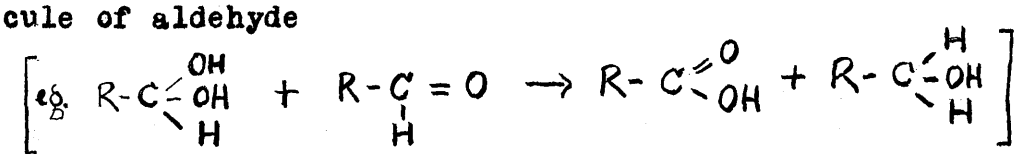
In view of the above results, it was thought that washing the precipitate enzyme with small amounts of distilled water might affect a separation of the associated ammonium sulphate, but when this was done, it was found that although a considerable purification of the enzyme was obtained, the products were very unstable. On being dried or allowed to stand overnight at 0°, the activity decreased very rapidly, and in the case of solutions, coagulation of the protein accompanied by inactivation of the enzyme could not be prevented.

Purification of the enzyme by absorption from the press liquor using varying amounts of Kaolin at p H 4.6 was also tried, but again the instability of the enzyme was made apparent. The enzyme was absorbed, and could be eluted with $\frac{n}{50}$ ammonia solution quite/

/quite satisfactorily, but the activity of the solutions so obtained decreased rapidly on standing as was experienced above in the case of the ammonium sulphate precipitates. It would appear that that the succinodehydrase can only exist in a stable form when attached to the cell structure, and the activity of the dried press liquor already noted is no doubt due to the activity of the enzyme attached to cells present therein.

THE DEHYDROGENATION OF ALDEHYDES BY ANIMAL TISSUE.

SCHMIEDEBERG [1] in 1881 found that surviving kidney had the power of oxidising salicylaldehyde into salicylic acid, and JACOBI [2] who verified this work, also showed that the property was possessed by various other organs including, liver, kidney, lungs, and spleen. Later PARNAS [3], and BATTELLI and STERN [4] pointed out that these organs also possessed the power of bringing about the Cannizzaro reaction, and to the enzyme responsible, Parnas gave the name "Aldehydmutase", while Battelli and Stern identified it with the Schmiedeberg enzyme. Further relations between the "Aldehydrase", and the "Aldehydmutase" were not forthcoming till WIELAND [5], postulating that the mutase reaction was only a form of the aldehydrase reaction in which the hydrogen acceptor was another molecule of aldehyde



showed, using milk in a study of

(1) the Cannizzaro reaction with Salicylaldehyde in absence of oxygen,

(2) the Schardinger reaction with methylene blue,

(3) total oxidation in presence of oxygen,

---

1) A.Pth. 14, 288, 1881.
2) Ibid 29, 386, 1891.
3) Bioch Z. 28, 274, 1910.
4) Bioch Z. 29, 130, 1910.
5) Ber. 47, 2085, 1914.

that the progressive inactivation of the ferments concerned in each case followed the same course. It is therefore postulated that there is no acceptor specificity for the aldehydrase, and that the same ferment which dehydrogehates aldehydes in presence of oxygen performs the schardinger reaction, and the Cannizzaro reaction.

With a view, therefore, to comparing the actions of the enzyme in animal tissue responsible for the direct oxidation of aldehydes and the disproportioning of aldehydes into the alcohol and acid, it was proposed to examine these two actions, the first by the usual methods depending on the rate of decolourisation of methylene blue in presence of aldehyde ,or the oxygen requirement of the aldehyde as measured on the Barcroft-Warburg Apparatus, and the second by means of titrating the acid as it was formed under anaerobic conditions according to the Cannizzaro reaction.

Preparation of Suitable Enzyme Material.

Knowing the heart to be associated with sugar metabolism it was thought possible, though no mention of this could be found in the literature, that aldehydrase might be present there. Accordingly the press liquor from horse heart prepared as described previously was examined for the presence of aldehydrase using the Barcroft-Warburg apparatus,

Each vessel held 5.0 cc. press liquor, the quantity of acetaldehyde necessary to give the concentrations noted on following page, and 4.0 cc. $\frac{m}{15}$ phosphate buffer solution ph.8.0, the total volume of solution being made up in each case to 10 cc. with/

with toluene saturated distilled water. Temperature was 37°.

	I	II	III	IV.
Concentration of Acetaldehyde.	$\frac{m}{10}$	$\frac{m}{20}$	$\frac{m}{50}$	-

Cmm. oxygen. Table 17.

Time (min).	I	II	III	IV.
15	2	4	6	7
30	26	32	33	38
45	46	56	61	63
60	74	80	86	90
90	122	128	137	160
120	170	174	188	232
180	262	252	272	521
240	306	298	322	1020.

It is evident from the above results that acetaldehyde inhibits very strongly the self respiration of the press liquor. It cannot however be concluded that the aldehyde is not being dehydrogenated, as the effects due to the self respiration and the presence of aldehyde may not be additive. It may be that the aldehyde is adsorbed on the surface of the enzymes, thus preventing the action of the substrates responsible for the self respiration.

An attempt to find if the press liquor contained aldehydrase was also carried out using the method depending on the time of decolourisation of methylene blue in an atmosphere of nitrogen. With acetaldehyde, positive results were obtained, but for salicylaldehyde, the time of decolourisation was exactly the same/

same as that for the press liquor alone without added substrate. This negative result with acetaldehyde may be explained by the slow rate of reaction of this substrate.

Experiments made with a view to following the course of the Cannizzaro reaction as catalysed by the aldehydrase, by titrating the formed acid with Barium hydroxide solution showed that the press liquor was unsuitable for a study of the reaction by this method being too highly buffered, and too deeply coloured.

The press liquor was however shown to contain the enzyme capable of promoting the Cannizzaro reaction by means of the following experiment. 100 cc. of fresh press liquor, saturated with toluene, were introduced into a round bottomed flask fitted with a rubber stopper containing two tubes with stopcocks. The flask was placed in a thermostat at 37°, and freed from oxygen, by repeated evacuation and filling with nitrogen. 1.055 g. salicylaldehyde dissolved in 160 cc. $\frac{m}{15}$ phosphate buffer solution, ph.8.0, were introduced, and evacuation and filling with nitrogen repeated. After 24 hours, the reaction was stopped by the addition of 50 cc. $\frac{n}{4}$ sulphuric acid, and the reaction mixture extracted for 12 hours with ether. The ether extract was then examined for salicylaldehyde, salicylic acid and salicyl alcohol according to the method of Wieland [1]. The separation was not carried out quantitatively, as it was found that the salicylaldehyde could not be completely extracted/

---

1) Ber. 1914, 47, 2085.

extracted from the reaction mixture, but 0.012 g. salicylic acid and a trace of the alcohol were isolated and identified.

Washing of minced horse heart muscle was found to remove the aldehydrase, as the succinodehydrase containing preparation used earlier in this work was incapable of catalysing the dehydrogenation of aldehydes either according to the Cannizzaro reaction or, with uptake of oxygen, into the corresponding acid.

Liver having been shown to contain considerable quantities of aldehydrase by various workers including Parnas and Battelli and Stern (loc.cit.), it was thought that a preparation better suited for a study of the enzyme could be made. Accordingly, half an hour after the slaughter of the animal, a piece of horse liver was minced three times, and shaken for 1 hour with an equal weight of $\frac{m}{15}$ phosphate buffer ph.6.2 (the hydrogen ion concentration of horse liver) under presence of toluene. The activity of the liquid obtained after centrifuging towards salicylaldehyde and acetaldehyde was then determined at ph.8.0 and 37^{0} by means of the Barcroft apparatus.

Each vessel held 2 cc. phosphate solution extract and 4.0 cc. $\frac{m}{15}$ phosphate buffer solution ph.8.0, the total volume being made up to 10 cc. with toluene saturated water.

	I	II	III	IV	V	VI
Conc.acetaldehyde	-	-	-	$\frac{m}{10}$	$\frac{m}{20}$	$\frac{m}{50}$
Conc.salicylaldehyde	-	$\frac{m}{100}$	$\frac{m}{165}$	-	-	-

Cmm.oxygen. Table 18.

Time (min.).	I	II	III	IV	V	VI
15	0	4	8	117	117	94
30	6	8	13	197	186	151
45	24	11	20	248	226	182
60	44	15	24	281	259	211
90	53	18	26	339	293	270
120	68	22	35	380	372	324
180	123	28	42	457	468	414

It will be seen from the results, that again, as in the corresponding experiment made with the press liquor from horse heart, the effect of salicylaldehyde was to inhibit the self respiration, but on the other hand, in this case acetaldehyde caused an increase in the oxygen uptake. It would appear also that the course of the reaction with acetaldehyde is attended with a large decrease in the activity of the enzyme. Again, however, as in the case of the press liquor from horse heart, in spite of the inhibition of the self respiration, the Cannizzaro reaction could take place.

100 cc. of the above phosphate extract, saturated with toluene, were added to 1.0 g. salicylaldehyde dissolved in 150 cc. $\frac{m}{15}$ phosphate buffer solution ph.8.0, in the absence of oxygen, and the reaction mixture was allowed to stand for 16 hours at 37°. On treating the ether extract of the resulting product according to the method previously used, 0.04 g. salicylic acid, and 0.01 gm. salicylalcohol were obtained and identified.

With a view to obtaining an enzyme preparation which would possess no self respiration, the use of acetone as a precipitating agent was tried. Fresh horse liver having been minced three times, was/

was extracted for 1 hour on a shaking machine with an equal weight of toluene saturated water. The aqueons solution was then centrifuged off, precipitated with acetone, and the precipitate, having been washed with 25% aqueons acetone, was dried in a vacuum desiccator. From 100 cc. aqueons extract of liver, 8 g. dry precipitate would be obtained, and this preparation was found to retain its activity for several days when preserved under light petroleum ether B.P. 20° - 40°.

The activity of the aqueous extract of liver and the acetone precipitate when determined on the Barcroft apparatus gave the following results:-

I and II contained 5.0 cc. aqueons extract.

III, IV, V, VI, VII, VIII contained 0.5 g. acetone precipitate.

Volume of solution in each vessel was 10 cc. pH.8.0, T, 37°.

	I	II	III	IV	V	VI	VII	VIII
Acetaldehyde concentration	$\frac{m}{20}$	0	$\frac{m}{20}$	0	$\frac{m}{20}$	0	$\frac{m}{20}$	0
HCN concentration	0	0	0	0	$\frac{n}{500}$	$\frac{n}{500}$	$\frac{n}{200}$	$\frac{n}{200}$

cmm.oxygen.

Table 19.

Time (min.)	I	II	III	IV	V	VI	VII	VIII
15	576	129	245	12	236	3	225	0
30	787	192	310	24	296	6	279	0
45	1014	246	361	44	340	9	318	0
60	1150	283	390	51	371	14	345	3
90	1371	356	471	77	435	24	395	6
120	1535	430	527	94	478	35	441	10
150	1661	502	575	110	529	48	489	15
180	1755	604	617	133	561	64	528	20

It is evident from the foregoing results that by precipitation with acetone, a considerable decrease in the self respiration was effected, allowing the course of the aldehydrase reaction to be studied more closely. It will be seen that the reaction goes very quickly at first, falling away rapidly at the end of the first 15 minutes. The reason for this is not obvious, and whether it is due to destruction of the enzyme it is not possible to say. The figures in columns VI and VIII show that cyanide at the concentrations used is without appreciable effect on the dehydrogenation.

Further attempts at isolation of the enzyme were unsuccessful. Quantities of the aqueous extract of minced liver were shaken with pure olive oil at pH. 8.0, but the residue left after extracting with ether the curd-like substance which separated on centrifuging, and the remaining solution were found to be inactive, the enzyme having probably been destroyed by the shaking.

Adsorption of the enzyme from the aqueous extract of liver with kaolin at ph. 4.6 was also carried out, but treatment of the kaolin with $\frac{n}{50}$ ammonia solution, and $\frac{m}{50}$ phosphate buffer solution pH. 8.0 failed to elute the enzyme in sufficient quantity to make the method of practical value. It would appear that the Oxidases as a class are not so suited to purification by adsorption methods as the other classes of enzymes, and their purification will always be attended with difficulty till a new method is discovered which is applicable where the usual methods are not.

Concentration of Substrate and Rate of Reaction.

The effect of concentration of substrate on the rate of reaction of acetaldehyde and salicylaldehyde was determined using the Thunberg method employing the time of decolourisation of methylene blue, according to the improved method described by Wieland and ROSENFELD [1]. The enzyme material was obtained by extracting the acetone precipate prepared as described above with a solution containing equal volumes of $\frac{n}{100}$ ammonia solution and $\frac{m}{15}$ phosphate buffer solution ph.8.0 10 cc. of the extracting solution per 1 g. of enzyme material were used. It is to be noted that precipitation of the enzyme by bringing this solution to ph.4.6 with acetate buffer resulted in its inactivation.

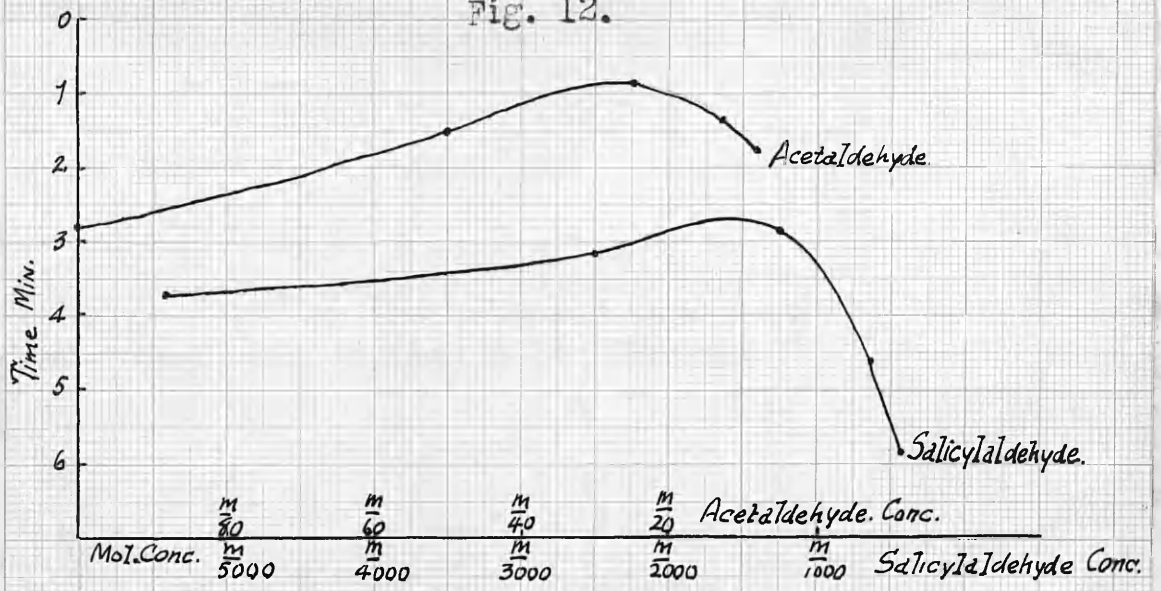
In each of the following tests 5.0 cc. extract were taken, and toluene water was added so that in each case the total volume after adding substrate and methylene blue amounted to 10 cc. The same enzyme material was used throughout the experiment.

Table 20.

acetaldehyde concentration	$\frac{m}{8.3}$	$\frac{m}{12.5}$	$\frac{m}{25}$	$\frac{m}{50}$	$\frac{m}{100}$
Time of decolourisation of 1 mg. M.B.	1'45"	1'20"	50"	1'30"	2'50"
Salicylaldehyde concentration	$\frac{m}{417}$	$\frac{m}{625}$	$\frac{m}{1250}$	$\frac{m}{2500}$	$\frac{m}{5400}$
Time of decolourisation of 1 mg. M.B.	5'50"	4'35"	2'50"	3'10"	3'40"

1) Ann. 477, 32, 1929.

Fig. 12.



Rate of reaction of Aldehydrase and Concentration of Substrate

From the results (Fig.12), it can be seen that definite optimum concentrations exists for both aldehydes examined, the rate of reaction falling more quickly at concentrations above that at those below the optimum. This is especially marked in the case of salicylaldehyde where the velocity of reaction remains almost constant between the concentrations $\frac{m}{1250}$ and $\frac{m}{5400}$. Again it will be seen that at the optimum concentration of both substrates, the reaction with acetaldehyde proceeds almost three times as quickly as that with salicylaldehyde.

The Cannizzaro Reaction.

An attempt to follow the course of the Cannizzaro reaction was carried out using the enzyme preparation obtained by extracting the acetone precipitate of the aqueous extract of liver, prepared as described above, with $\frac{n}{100}$ ammonia solution, 5 g. of the solid being treated with 30 ccs. of ammonia solution.

4 Thunberg tubes were filled according to the table shown below.

	I	II	III	IV
Enzyme solution	5.0 cc	5.0	5.0	5.0
$\frac{m}{15}$ phosphate buffer solution pH. 8.0	-	-	2.0 cc	2.0 cc.
Toluene saturated water	4.0 cc	4.0 cc	0.6 cc.	0.6 cc
Phenolphthalein	1 drop	1 drop		

The tubes were placed in a thermostat at 37°. After repeated evacuation and filling with nitrogen, tubes I and II were titrated with $\frac{n}{20}$ Barium hydroxide from 2 cc. burettes placed in the stoppers, till the red colouration indicating that the hydrogen ion concentration of pH. 8.0 had been reached. A stream of nitrogen still being passed into tubes I and III, 1 cc. $\frac{m}{1}$ acetaldehyde solution was introduced and the pH. of I again brought to 8.0 by the addition of a few drops of $Ba(OH)_2$ solution from the burette.

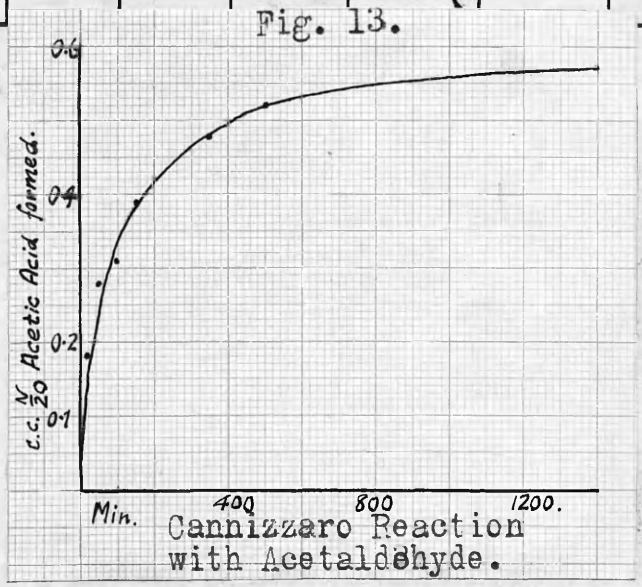
The/

The nitrogen stream was then stopped, and at regular intervals, baryta solution was added to I and II so that the red colouration of the phenolphthalein was maintained. The following table shows the results.

Table 21.

Time (min.).	15	55	90	150	375	500	1440
Tube I cc. $\frac{N}{20}$ $Ba(OH)_2$	0.18	0.66	0.72	0.84	0.94	0.98	1.03
Tube II (control) cc $\frac{N}{20}$ $Ba(OH)_2$	-	0.38	0.41	0.45	0.46	0.46	0.46
cc. $\frac{N}{20}$ acetic acid formed	0.18	0.28	0.31	0.39	0.48	0.52	0.57

Fig. 13.



Cannizzaro Reaction with Acetaldehyde.

Although the reaction might not have been completed after 1440 minutes, it could not be followed any further owing to the precipitation of protein which obscured the colour of the phenolphthalein. The destruction of the enzyme responsible for the cannizzaro reaction was however shown to be attended by the destruction/

destruction of that responsible for the dehydrogenation of aldehyde with methylene blue as hydrogen acceptor by a comparison of the activity of the enzyme in tubes III and IV. These tubes having been kept at 37° alongside tubes I and II served as a control for the activity of the enzyme after the cannizzaro reaction had taken place, and 1 cc. $\frac{m}{1}$ acetaldehyde having been introduced into tube IV, the times of decolourisation of 1 cc. (1 Mg.) methylene blue were determined in the usual way.

	III	IV
Time of decolourisation (min.)	15'	2'50"

A control experiment without acetaldehyde was not decolourised in 45 minutes.

The above can be taken as evidence that the two actions of aldehydrase are brought about by the same enzyme.