XLI. SOME OXIDATION PROCESSES OF NORMAL AND CANCER TISSUE.

By ALFRED FLEISCH.

From the Biochemical Department, University of Cambridge.

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SINCE every difference between normal and cancer tissue may be of importance for cancer research, I compared some of the enzymic processes in normal muscle tissue with those of Jensen's rat sarcoma¹. Furthermore the function of glutathione with the thermostable sarcoma residue was investigated on the same lines as those employed by Hopkins [1921, 1922] in the case of the thermostable muscle residue.

METHODS.

The methods used for these investigations were those of oxygen uptake in a Barcroft apparatus of the form used in this laboratory and the methylene blue technique developed by Thunberg [1920].

A set of 4 Barcroft apparatus immersed side by side in the same water-bath was used. All were shaken by an electric motor at the same rate. The water-bath fitted with an efficient stirrer was kept in all experiments at $35^{\circ} \pm 0.01$. The flat-bottomed flasks of the Barcroft apparatus guaranteed a quick supply of oxygen. The reacting mixture was introduced into the right-hand flask, whilst the left bottle contained the same quantity of distilled water. Potash for the absorption of CO_2 was in every case placed in the receptacles provided. The oxygen uptake of the control experiment was determined simultaneously in a second apparatus. It is necessary, of course, to allow about seven minutes for the attainment of equilibrium in the bath before the flasks are connected with the manometer and the readings begun. Consequently, the oxidation had proceeded to a certain degree before the readings were commenced. In some experiments, however, the material to be oxidised was added after equilibrium was attained by using a special method. In this case an exactly quantitative measurement of oxygen uptake was possible. The calibration of the Barcroft apparatus was made by the simple and exact method related by Münzer and Neumann [1917].

All volumes are reduced to 0° , 760 mm. When not otherwise stated the reacting mixture had a p_{H} of 7.6 buffered with phosphate. In the methylene

¹ The strain used was one supplied to Prof. Hopkins by Dr J. H. Murray.

blue experiments after the solutions and the tissue had been added in the vacuum tubes all tubes were evacuated simultaneously by the same pump until the contained liquids boiled. Then the taps were closed and the tubes placed in a water-bath at 35°. The time of the beginning and of the end of the decolorisation was noted.

For a comparison with sarcoma a washed preparation from a normal tissue, especially rat muscle, was used; 6g. muscle of a freshly killed rat were cut with scissors during eight minutes, then generally washed three times for ten minutes each with a 50-fold bulk of distilled water, by using an electric shaker. After each washing the water was removed by pressing through linen. The sarcoma cannot conveniently be cut up in the same way. It was therefore pressed through wire gauze of 0.8 mm. mesh. The washing applied to the sarcoma was the same as for muscle.

For the experiments with glutathione the preparation was made in the manner described by Hopkins. The cut tissue was washed seven times with water. The first washing lasted only one minute, the second three minutes, and so on, increasing until one hour was used for the last washing. Then the tissue was boiled up, pressed through linen, treated with alcohol for 15 minutes and dried *in vacuo* over sulphuric acid, and finely powdered. This product in correspondence with the terminology used by Hopkins is here called the thermostable muscle- or sarcoma-residue.

ACTION OF GLUTATHIONE.

As Hopkins [1921, 1922] has pointed out, the thermostable muscle residue has only a small oxygen uptake when acting alone but a very large one if glutathione is added. The thermostable sarcoma residue gives the same results, no essential difference having been found. The figures of two comparable experiments with controls (1 c, 2 c) are quoted in Table I.

Table I.

Number of experiment ...	1	1 c	2	2 c
Muscle residue in g.	0.2 g.	0.2 g.	—	—
Sarcoma residue in g.	—	—	0.2 g.	0.2 g.
Phosphate $M/100$ p_H 7.6	0.2 cc.	0.2 cc.	0.2 cc.	0.2 cc.
Glutathione solution 0.8 % neutralised	1.0 cc.	—	0.1 cc.	—
Ringer's solution p_H 7.6	1.6	2.6	1.6	2.6
Cmm. O_2 taken up after 1 hour	119	37	152	29
2 hours	166	54	206	47
3 "	187	66	234	66
4 "	207	79	248	84
5 "	223	90	263	99

The phenomenon observed by Hopkins, that phosphate buffer in rather high concentration inhibits the speed and the total amount of oxygen taken up is also observed in the case of the sarcoma residue. A phosphate concentration of 1/15 molar in the reacting mixture for instance reduced the oxygen uptake to about the half.

SPONTANEOUS OXIDATION OF NORMAL TISSUE.

Several authors have compared the oxygen uptake of normal tissue and cancer tissue. Russel and Gye [1920] as well as Watermann and Dirken [1921] found that tissue of a growth uses more oxygen and for a longer time than normal tissue. My experiments gave approximately the same results. It must be mentioned, however, that the results of different experiments are not quite consistent. Obviously the amount of disintegration suffered by the tissue has a notable influence on the speed of oxygen uptake.

Fig. 1. Composition of the reacting mixture. 0.4 g. of muscle or sarcoma + 0.2 cc. of $M/2$ phosphate, p_H 7.6.

Curve marked	<i>M</i>	<i>M</i> + HCN	<i>S</i>	<i>S</i> + HCN
HCN <i>M</i> /200 ¹	—	1.0	—	1.0
H ₂ O	2.4	1.4	2.4	1.4

¹ 0.5 cc. $M/100$ KCN was added and then neutralised by adding 0.5 cc. $M/100$ HCl.

In Fig. 1 the results of identical experiments are shown. The oxygen uptake of muscle tissue (curve *M*) shows great speed in the beginning, which soon becomes slower. The sarcoma (curve *S*), however, has an oxygen uptake which is slower at the beginning but at a constant rate during the whole course of the experiment, so that after three hours the sarcoma has absorbed more oxygen than the muscle. When the curve *S* is still rising the curve *M* has become nearly horizontal.

Furthermore the effect of cyanide was investigated and is illustrated in Fig. 1. It is evident that cyanide in a concentration of *M*/600 reduces the oxygen uptake in both cases, muscle as well as sarcoma, to approximately one-third.

The results from unwashed tissue obtained by the methylene blue technique *in vacuo* are shown in Table II.

Table II. 0.4 g. of muscle or sarcoma + 0.2 cc. *M*/2 phosphate, p_H 7.6 + 0.2 cc. *M*/2000 methylene blue.

Number of experiment	Muscle				Sarcoma			
	1	2	3	4	1	2	3	4
HCN <i>M</i> /200	—	0.6	—	—	—	0.6	—	—
HCN <i>M</i> /20	—	—	0.3	0.6	—	—	0.3	0.6
H ₂ O	0.6	—	0.3	—	0.6	—	0.3	—
Beginning of decolorisation in minutes	2.5	1.5	2.5	3	6	5	5	7
End of decolorisation in minutes	5	3	3.5	5	8	6.5	8	10

Comparing the methylene blue reduction by muscle (*M* 1) and sarcoma (*S* 1) we see that the muscle tissue reduced the methylene blue in 2.5–5 minutes, whilst an equal weight of sarcoma took 6–8 minutes. This result is in accordance with that of Drew [1920] and with those obtained with the oxygen uptake technique, where in the beginning muscle is more active than sarcoma.

But a surprising result is obtained in the experiments where cyanide is added. A relatively small concentration of HCN, *M*/467 in the experiments *M* 2 and *S* 2 produces an acceleration of the methylene blue reduction. Only in the presence of a very large cyanide concentration as in the experiments *M* 4 and *S* 4 is a small retardation of the reduction produced.

I should like to insist upon this result, that HCN produces an inhibition of the oxygen uptake but an acceleration of the methylene blue decolorisation. Obviously the methylene blue technique of Thunberg is here not equivalent to the oxygen uptake method. Later on there will be an opportunity to discuss this difference further.

THE OXIDATION OF CITRIC ACID BY WASHED TISSUE.

Citric acid added to a washed muscle tissue is oxidised. Battelli and Stern [1911, 2] have shown this by measurements of oxygen uptake and Thunberg [1920] by the methylene blue technique. Some results, consistent with many others, are shown in Table III.

As Thunberg pointed out, washed tissue alone may show a slow spontaneous reduction of the methylene blue. This "residual reduction" in Exp. *M* 3 (Table III) occupied 40 to 105 minutes in the case of muscle and 30 to 36 minutes in the case of sarcoma tissue (Exp. *S* 1). Citric acid added to muscle (*M* 2) produces a marked acceleration of the methylene blue reduction, so that the decolorisation begins after 13 and is finished after 30 minutes.

Table III. 0.2 g. muscle or 0.5 g. sarcoma + 0.2 cc. *M*/2 phosphate, p_H 7.6 + 0.2 cc. *M*/2000 methylene blue.

Number of experiment	Muscle				Sarcoma			
	1	2	3	4	1	2	3	4
HCN <i>M</i> /100	—	—	0.2	0.2	—	—	0.4	0.4
Citric acid <i>M</i> /10	—	0.2	—	0.2	—	0.2	—	0.2
H ₂ O	0.6	0.4	0.4	0.2	0.6	0.4	0.2	—
Beginning of decolorisation in minutes	40	13	105	13	30	33	33	33
End of decolorisation	105	30	(360 +)	48	36	55	70	100

The conditions of the experiments *M* 1 and *M* 2 being the same there can be no doubt that the citric acid is the material oxidised.

The experiments *M* 3 and *M* 4 show the influence of cyanide in the case of muscle. It is obvious that HCN (Exp. *M* 3) inhibits the residual reduction of the washed muscle tissue very strongly. This inhibition of the residual reduction was regularly observed in all experiments with washed muscle as well as with washed sarcoma. The difference between washed tissue and unwashed tissue as shown by the non-inhibition of the methylene blue reduction by cyanide in the case of the latter is noteworthy. Exp. *M* 4 shows that citric acid is also oxidised by muscle in presence of HCN. The reduction time is 13–48 minutes as compared with 105 to more than 360 minutes in the identical experiment *M* 3 without citric acid.

Working with sarcoma the results with citric acid are quite different (*S* 1–*S* 4 in Table III). It is very remarkable that citric acid in this case produces no acceleration of the reduction. Sometimes citric acid even inhibits the residual reduction of washed sarcoma. Thus in Exp. *S* 2 the time for complete decolorisation in presence of citric acid was 55 minutes as compared with 36 minutes without citric acid (*S* 1). Citric acid, as might be expected, has no effect when HCN is present (*S* 3 and *S* 4). This inefficiency of sarcoma in respect of the oxidation of citric acid was consistently found in many experiments. Sometimes the sarcoma was washed only once for some minutes instead of three times, sometimes the p_H of the reacting mixture was 6.0 instead of 7.6, the result being always negative. A different result was obtained with the oxygen uptake method as will be shown.

A further investigation with muscle dealt with the optimum p_H for the oxidation of citric acid. It was found with the methylene blue technique that the optimum is on the acid side at about p_H 6.0. For instance, the reduction times in a series of experiments with washed muscle and citric acid were 21–37 minutes at p_H 7.6; 12–21 minutes at p_H 6.5; 14–27 minutes at p_H 5.0.

Fig. 2. Composition of reacting medium.

Washed muscle	0.5	0.5	0.5	0.5
Phosphate $M/2$	0.2	0.2	—	—
p_H 6.0	—	—	0.2	0.2
Phosphate $M/2$	—	—	0.2	0.2
p_H 6.5	—	0.2	—	0.2
Citric acid $M/10$	—	0.2	—	0.2
neutral	—	—	—	—
H_2O	2.3	2.1	2.3	2.1

 Fig. 4. Composition of reacting medium.
0.5 g. of sarcoma + 0.2 $M/2$ phosphate,
 p_H 7.6.

Succinic acid	—	—	0.1	0.1	0.1
neutral $M/5$	—	—	—	—	—
HCN $M/100$	—	—	0.3	0.3	—
Methylene blue	—	—	—	0.5	—
$M/100$	—	—	—	—	—
H_2O	2.3	2.2	1.9	1.4	—

 Fig. 3. Composition of reacting medium. 0.4 g. muscle + 0.2 cc. $M/2$ phosphate, p_H 7.6.

Succinic acid	—	0.1	0.1	0.1
neutral $M/5$	—	—	0.7	0.7
HCN $M/100$	—	—	—	0.5
Methylene blue	—	—	—	0.5
$M/1000$	—	—	—	—
H_2O	2.4	2.3	1.6	1.1

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 Fig. 5. Composition of reacting medium.
0.4 g. of muscle + 0.2 cc. $M/2$ phosphate,
 p_H 7.6 + 0.5 cc. $M/100$ HCN.

H_2O	0.8	0.9	1.3	1.4
Toluidine blue	1.0	1.0	—	—
$M/500$	—	—	—	—
Soluble blue $M/200$	—	—	0.5	0.5
Succinic acid $M/5$	0.1	—	0.1	—

The same result was obtained with the oxygen uptake method. Working with washed muscle at p_H 7.6 the quantity of oxygen taken up by the citric acid is only very small. For instance, in an experiment at p_H 7.6 with citric acid 36 cmm. O_2 were taken up in two hours, while in the control experiment without citric acid 22 cmm. O_2 were absorbed in the same time. The rate of oxygen uptake at p_H 6.0 and 6.5 is much quicker as shown by Fig. 2.

Whilst both control experiments at p_H 6.0 and 6.5 show only the small O_2 uptake of 29 cmm. in three hours, the experiments with citric acid, and especially that at p_H 6.0 (full curve 6.0 C 1 in Fig. 2), show a good oxygen uptake.

Although sarcoma was found absolutely inefficient in oxidising citric acid by using methylene blue as a hydrogen acceptor, there is evidence that citric acid is oxidised by sarcoma when molecular oxygen is used. A thoroughly washed sarcoma does not show a distinctly higher oxygen uptake with citric acid than without it. But if the sarcoma is washed for a very short time only, say two minutes, then we get an unmistakable oxygen uptake due to the oxidation of citric acid. In one of the series, for instance, the control experiment took up 174 cmm. in $2\frac{3}{4}$ hours whilst the identical experiment but with citric acid took up 260 cmm. in the same time. Consequently 86 cmm. O_2 were due to the oxidation of the citric acid. But the addition of citric acid to preparations from the same individual sarcoma as that used in these experiments did not accelerate methylene blue reduction in the least.

The indications mentioned above, that the methylene blue technique of Thunberg is not equivalent to the oxygen uptake method, are thus extended.

Battelli and Stern long ago pointed out that the agent which oxidises citric acid is different from the enzyme oxidising succinic acid ("succinoxydase"). A tissue preparation loses its power to oxidise citric acid after standing for some hours, whilst the power to oxidise succinic acid remains constant for about two days. Moreover, the mechanism for the oxidation of citric acid is much less sensitive to HCN than is succinoxydase. I have observed this with the oxygen uptake method and also with the methylene blue technique. Washed rat muscle, for instance, to which citric acid had been added, reduced methylene blue when fresh in 13–30 minutes; after standing for four hours it required 40–115 minutes to reduce the same amount, while the data for the residual reduction (*i.e.* without citric acid) are 40–105 when fresh and 70–115 after four hours. After standing for 24 hours the decolorisation took place with and without citric acid in exactly the same time, *i.e.* 136–256 minutes. The oxidising power for succinic acid, however, was not diminished after 24 hours. Washing has a similar effect. The better a muscle preparation is washed the smaller becomes its power to oxidise citric acid, whilst washing may even raise the power to oxidise succinic acid. Furthermore, the oxidation of citric acid in the Barcroft apparatus is not inhibited by HCN $M/1000$, whereas the oxidation of succinic acid is completely suppressed by the same cyanide concentration. Moreover the optimum for the oxidation of citric acid is at p_H 6.0, whilst Ohlsson [1921] has found the optimum for the succinoxydase at p_H 8.7.

My results fully confirm those of Battelli, Stern and Thunberg in showing that the enzyme for the oxidation of citric acid is different from the succinoxidase. I have gone into this matter at some length because the difference between sarcoma and muscle in respect of the anaerobic oxidation of citric acid is a point of some interest.

GLUTAMIC, LACTIC, MALIC, FUMARIC AND MALEIC ACIDS.

These acids were investigated mainly with the methylene blue technique using washed tissue. Glutamic acid was found to be the most effective oxygen acceptor with sarcoma as well as with muscle. The reduction time is always diminished (*M* 4 and *S* 4 in Table IV) when glutamic acid is added to tissue.

Lactic acid accelerates the methylene blue reduction if added to muscle. For instance, the reduction time with lactic acid (*M* 3 in Table IV) is 25–41 minutes whilst the residual reduction (*M* 1) takes 53–64 minutes. A somewhat different result is obtained with sarcoma.

Table IV. 0.2 g. muscle or 0.4 g. sarcoma + 0.2 cc. *M*/2 phosphate, p_H 7.6 + 0.2 cc. *M*/1000 methylene blue.

Number of experiment	Muscle				Sarcoma			
	1	2	3	4	1	2	3	4
Malic acid <i>M</i> /10	—	0.2	—	—	—	0.2	—	—
Lactic acid <i>M</i> /5	—	—	0.1	—	—	—	0.1	—
Glutamic acid <i>M</i> /5	—	—	—	0.1	—	—	—	0.1
H ₂ O	0.6	0.4	0.5	0.5	0.6	0.4	0.5	0.5
Beginning of decolorisation in minutes	53	25	25	16	23	40	20	15
End of decolorisation	64	*	41	41	29	*	29	23

* means that the decolorisation is not complete after 120 minutes.

Sometimes the acceleration of the reduction is distinct although very small, sometimes, however, lactic acid has no effect at all. There can be no doubt, that lactic acid is less effective with sarcoma than it is with muscle.

As regards malic acid, I observed when working with muscle that the decolorisation begins earlier (*M* 2, Table IV) but finishes later than the residual reduction. With sarcoma, however, malic acid was always found to inhibit the residual reduction.

Fumaric and maleic acids have never been found active in reducing methylene blue; on the contrary they regularly inhibit the residual reduction of washed tissue whether it be sarcoma or muscle.

The oxygen uptake method was also tried in the case of these acids, but the quantity of oxygen taken up was so small that clear results could not be obtained.

THE OXIDATION OF SUCCINIC ACID AND ITS MECHANISM.

The oxidation of succinic acid in the presence of washed muscle tissue has often been investigated, especially by Battelli and Stern [1911, 1] working with an oxygen uptake method, and by Thunberg [1918, 1923], Widmark [1921] and Ohlsson [1921] working with the methylene blue technique of

Thunberg. Battelli and Stern [1914] and later on also Widmark and Ohlsson worked with a muscle extract, in which the enzyme was in suspension. Einbeck [1919] confirmed the results of Battelli and Stern, by showing that one molecule of succinic acid takes up one atom of oxygen to yield fumaric acid. Fumaric acid in a second stage by taking up water is transformed into malic acid. In every case 25 % of fumaric acid remains unchanged.

Succinic acid was found to be a very powerful reducing substance for methylene blue if added to washed muscle. No other material investigated has a similar efficiency. In the experiment shown in Table V, succinic acid reduced 0.3 cc. of a $M/1000$ methylene blue solution in 4–18 minutes ($M\ 2$), whilst the reduction in the control without succinic acid ($M\ 1$) was not perceptible after 70 minutes. Cyanide ($M\ 3$) is seen to have no influence on this quick decolorisation.

Table V. 0.2 g. of muscle + 0.3 cc. $M/1000$ methylene blue or 0.3 g. of sarcoma + 0.2 cc. $M/1000$ methylene blue, with in each case 0.2 cc. of $M/2$ phosphate, $p_H\ 7.6$.

Number of experiment	Muscle.			Sarcoma			
	1	2	3	1	2	3	4
Succinic acid $M/5$	—	1	0.1	—	—	0.1	0.1
HCN $M/100$	—	—	0.2	—	0.3	—	0.3
H ₂ O	0.5	0.4	0.2	0.6	0.3	0.5	0.2
Beginning of decolorisation in minutes	*	4	4	33	33	16	16
End of decolorisation		18	18	83	200	37	83

* means more than 70 minutes.

HCN whether in small or in large concentrations ($M/1000$ – $M/50$ have been tried) has no distinct effect upon the reduction due to succinic acid when added to washed muscle. An evaporation of the HCN by the evacuation of the tubes is certainly not responsible for this negative effect. In some experiments the tubes were not evacuated but the air replaced by hydrogen and the result was the same.

The effects obtained with sarcoma are somewhat different. First of all sarcoma is in the oxidation of succinic acid much less active than muscle. In the experiment $S\ 3$ of Table V the reduction time is 16–37 minutes as compared with 4–18 minutes for muscle ($M\ 2$), although more sarcoma and less methylene blue is used in the Exp. $S\ 3$ than in $M\ 2$. All results obtained with the methylene blue technique confirm this fact, that sarcoma has a much smaller oxidising power for succinic acid than muscle. A further difference exists in that cyanide appears to show an inhibitory effect on sarcoma. For instance, the reduction time in Exp. $S\ 3$ without HCN is 16–37 minutes against 16–83 minutes in $S\ 4$ with cyanide. This inhibitory effect of cyanide is however not quite constant; sometimes it was found to be more marked, sometimes less marked, than in the Exps. $S\ 2$ and $S\ 4$ of Table V. The difference may depend upon the degree to which the tissue has been deprived of its original reducing power by washing. If the inhibitory effect of cyanide be caused by a direct

action of HCN on the succinoxidase, *i.e.* the specific enzyme attacking the molecule of succinic acid, the fact would be remarkable since there is no similar effect of HCN upon the succinoxidase of muscle. It is possible on the other hand that the observed inhibitory effect of HCN may be due to the inhibition of the residual reduction of the washed tissue itself. As shown by Exps. *S 1* and *S 2* (Table V), and as I have indeed always found, the residual reduction is inhibited by cyanide. We must imagine that in Exp. *S 3* of Table V only one part of the methylene blue is reduced by the hydrogen coming from succinic acid; the other part of the methylene blue is decolorised by the washed tissue itself. Consequently it is obvious that the reduction time must become longer if this residual reduction is diminished by cyanide. The residual reduction when sarcoma is concerned appreciably influences the observed reduction time since the succinoxidase of the sarcoma is not very active. This is not the case with muscle, where the active enzyme decolorises the methylene blue so quickly that the residual reducing power of the tissue can have only a slight influence. The complication due to the presence of two reducing factors (succinoxidase and residual reduction) prevents the forming of a definite conclusion. However, a quantitative comparison of all my experimental results seems to make doubtful any inhibition by cyanide on the succinoxidase of sarcoma.

Great activity of the succinoxidase is observed when succinic acid is shaken in the Barcroft apparatus with minced and washed rat muscle. In a typical experiment the control with muscle alone gave only the small oxygen uptake of about 80 cmm. in three hours (full curve Contr. in Fig. 3) while muscle and succinic acid together (full curve Succ. in Fig. 3) showed an oxygen uptake of 164 cmm. in the first 15 minutes. This extremely quick oxygen uptake was finished after one hour when about 220 cmm. O_2 had been absorbed. As Battelli and Stern and Einbeck have shown, succinic acid, $C_4H_6O_4$, when oxidised by tissues is transformed into fumaric acid, $C_4H_4O_4$.

One molecule of succinic acid therefore uses one atom of oxygen for the oxidation of hydrogen. The theoretical oxygen uptake (Theor. O_2 in Fig. 3) for the amount of succinic acid used in my experiments (0.1 cc. of a $M/5$ solution) should be 224 cmm. O_2 (reduced). This result has been obtained regularly in all experiments, showing that the method used is extremely reliable. Special precautions must of course be taken to avoid the oxidation of the succinic acid before the taps of the apparatus are closed. For this purpose the succinic acid was placed in small vessels in the flask of the Barcroft apparatus and only allowed to mix with the tissue in its buffer fluid after the taps were closed and thorough shaking had begun.

If cyanide be added to the system (tissue *plus* succinic acid) the rapid oxygen uptake disappears and we get a curve (dotted curve, Succ. + HCN in Fig. 3) which is nearly the same as that of the control (tissue alone). There can be no doubt that the oxidation of succinic acid is inhibited completely by cyanide. As to the effective concentration of cyanide it was found that

HCN in concentration of $M/3000$ in the reacting mixture inhibits the oxidation of succinic acid remarkably; $M/1500$ HCN prevents the oxidation completely.

The fact that cyanide inhibits oxygen uptake but not methylene blue reduction by succinic acid suggested experiments in which the influence of methylene blue upon oxygen uptake was studied. An addition of methylene blue to a flask containing muscle, succinic acid, and cyanide restores the oxidation of succinic acid as is shown by the dotted curve *succ. + HCN + meth.* of Fig. 3. It is certain that succinic acid is oxidised by this system, since the theoretical amount of oxygen (224 cmm.) is taken up. Furthermore this methylene blue effect is only seen in the complete system as used for the experiment "*succ. + HCN + meth.*" in Fig. 3. The same combination but without muscle or without succinic acid takes up no oxygen. Methylene blue added to muscle plus succinic acid without cyanide never accelerates but rather inhibits the rate of oxygen uptake. The meaning of these experimental results will be discussed later on.

Similar experiments were carried out with sarcoma. A typical result corresponding to many others is shown in Fig. 4. Whilst the control experiment without succinic acid (full curve *contr.*) has only a small oxygen uptake, the addition of succinic acid (full curve *succ.* in Fig. 4) produces a quick uptake until the theoretical amount of 224 cmm. O_2 is absorbed. However, sarcoma never shows such velocity of oxygen uptake as muscle. Cyanide (dotted curve *succ. + HCN*) also inhibits the oxidation of succinic acid completely as it does with muscle. But there is this noteworthy difference that with sarcoma methylene blue scarcely restores the oxidation of succinic acid when inhibited by cyanide (compare Fig. 3 muscle with Fig. 4 sarcoma).

THE QUANTITATIVE RELATION BETWEEN METHYLENE BLUE REDUCTION AND OXYGEN UPTAKE.

As I have pointed out already the methylene blue technique of Thunberg gives different results from the oxygen uptake method. This is especially the case when cyanide is applied. Apart from differences of a quantitative kind I have shown above that sarcoma oxidises citric acid in presence of molecular oxygen but not anaerobically in presence of methylene blue, while muscle of course oxidises citric acid in both cases freely. This might lead to the supposition, that the enzyme attacking the citric acid is different in sarcoma and muscle respectively. I would suggest however that a special factor exists responsible for the transport of hydrogen to methylene blue. We may speak of this as the "hydrogen transport factor." The assumption of such a hydrogen transport factor seems to me to be inevitable considering the quantitative relation between methylene blue reduction and oxygen uptake. This quantitative relation changes according to the treatment of the tissue. Some examples based upon investigations carried out with succinic acid will illustrate this fact.

Chopped rat muscle was washed twice, the first time for two and the second time for ten minutes. This muscle preparation "A" in oxidising succinic acid in the Barcroft apparatus gave rise to an initial speed of oxygen uptake corresponding to 212 cmm. in one hour. When the oxidation was wholly inhibited by the addition of sufficient cyanide and then so far as possible restored by addition of methylene blue, the speed of oxygen uptake due to the oxidation of succinic acid was 28 cmm. in one hour. The quotient of these two results, *i.e.*

$$\frac{\text{Cmm. O}_2 \text{ (succinic acid + HCN + methylene blue)}}{\text{Cmm. O}_2 \text{ (succinic acid)}},$$

which may be called the quotient meth. blue/O₂, gives a measure of the facility with which the hydrogen coming from succinic acid is transported to the methylene blue. A high value of the quotient meth. blue/O₂ means that this hydrogen is readily transported to the acceptor. It may be mentioned that the value of this quotient is nearly independent of the amount of succinoxidase present. This fact has been proved by using different amounts of the same muscle preparation.

In the experiment just mentioned the value is

$$\frac{\text{Methylene blue}}{\text{O}_2} = \frac{28}{212} = \frac{1}{7.6}.$$

The muscle A was then washed once more for 2.5 hours. With this well-washed muscle, B, identical observations were made. The values obtained with muscle B were 264 cmm. O₂ for the direct oxidation of succinic acid and 14 cmm. O₂ when HCN and methylene blue were added to the system. Whilst the value for the direct oxidation rose from 212 to 264 cmm. the value for the oxidation by methylene blue fell from 28 to 14 cmm. Consequently the quotient meth. blue/O₂ fell from 1/7.6 to 1/19. This means that the facility of hydrogen transport to methylene blue is diminished by thorough washing but not transport to oxygen. The same results as described for muscle were obtained with sarcoma.

Identical results were also obtained by using Thunberg's technique where the direct oxidation of hydrogen is prevented not by cyanide but by the anaerobic conditions. In the experiment mentioned above in which the speed of oxygen uptake increased by washing from 212 to 264 cmm. the speed of methylene blue reduction in vacuum tubes decreased to about a quarter of its original value (reduction time for 0.3 cc. *M*/1000 methylene blue 36 minutes with the thoroughly washed muscle B against 10 minutes with muscle A).

Since I have made the same observation consistently in many experiments I conclude that a special hydrogen transport factor is responsible for the transport of hydrogen to methylene blue. The nature of such a factor in the reducing system is of course obscure. It would seem that continued washing with water removes it from the tissue or destroys it.

The quotient meth. blue/O₂ enables us to compare the activity of different organs in respect of the transport of hydrogen to methylene blue. I have

found for rat muscle washed in the usual manner values between 1/12 and 1/13; for frog muscle 1/12 (one experiment); for rat liver 1/20–1/24. Very unusual results were obtained in the case of sarcoma. The figures for this vary more widely than they do for muscle. Furthermore the figures obtained for sarcoma were with few exceptions very much smaller than for any other tissue. Values of 1/100 are quite usual. These strikingly small values for the quotient meth. blue/O₂ give an explanation for the phenomenon described above, that in the case of sarcoma methylene blue scarcely restores the oxidation of succinic acid when inhibited by cyanide. Apparently sarcoma possesses the hydrogen transport factor, which I have presumed to exist, in much smaller amount than other tissues.

Such a view may also explain the fact mentioned above, that citric acid with sarcoma does not reduce methylene blue though it takes up some oxygen in the Barcroft apparatus.

Whatever this hydrogen transport factor may be, its probable existence and its variability throw some light on Thunberg's methylene blue technique and its varying quantitative relation to the oxygen uptake method.

THE OXIDATION MECHANISM OF SUCCINIC ACID AS BASED UPON ACTIVATION OF BOTH HYDROGEN AND OXYGEN.

The oxidation of succinic acid is a very proper object for investigations of the oxidation mechanism because we know important details of the chemical changes taking place. As Thunberg points out and Einbeck has proved, succinic acid losing two atoms of hydrogen is directly transformed to fumaric acid without addition of water. Thunberg therefore rightly calls this reaction the type of hydrogen activation and hydrogen transport in the sense of Wieland. It may be remembered that this hydrogen can be transported to methylene blue or to molecular oxygen as hydrogen acceptor.

Yet it seems to be extraordinary that this oxidation of succinic acid is inhibited by cyanide and restored by methylene blue. There can be no doubt that cyanide in small concentrations is a specific inhibitory substance for oxidation processes. Warburg [1921, 1923] considers that such oxidations depend upon catalysis by iron. The inhibitory effect of cyanide is due to the formation of a compound of HCN and iron; cyanide in fact inhibits the activation of oxygen by iron.

As to methylene blue, Meyerhof [1917, 1918] some years ago brought forward the hypothesis that this substance may be able to replace a normal factor in an oxidising mechanism when the latter has been inhibited or destroyed. Whilst the respiration of living micro-organisms is inhibited 10–40 % by methylene blue, the respiration of the same organisms treated with acetone and heated rises in the presence of methylene blue. The more the respiration is inhibited by heating, the stronger is the recuperative power of methylene blue. Furthermore Meyerhof showed that methylene blue can

restore to a certain degree the respiration inhibited by cyanide. In the same way Aschmarin [1922] considers methylene blue as an analogue to an oxidising enzyme.

Thunberg [1918] made the same observations as I, namely, that in the case of succinic acid cyanide does not inhibit the methylene blue reduction but only the oxygen uptake. Thunberg discusses the question as to whether cyanide might inhibit the enzyme succinoxidase, but the answer is a negative one, since the succinoxidase is able in presence of cyanide to transport the hydrogen to methylene blue. Therefore Thunberg inclines to the view that cyanide influences the oxygen, *i.e.* a hypothetical special form of oxygen, which alone can be directly used for the oxidation. As to the power of methylene blue to restore the oxidation inhibited by cyanide Thunberg gives no explanation, but he excludes a direct "inactivation" of the cyanide by methylene blue.

On the latter point my observations confirm Thunberg's view. I regularly found in my experiments that the amount of cyanide added is without influence upon the subsequent restorative power of methylene blue, so long as the HCN-concentration in every case be strong enough to suppress completely the direct oxidation of succinic acid. For instance two identical experiments containing the same amount of methylene blue, succinic acid and muscle show an oxygen uptake of 96 and 100 cmm. respectively, although the cyanide concentration in one experiment was $M/430$ and $M/100$ in the other. The restitution depends on the concentration of methylene blue only. I found that a five-fold amount of methylene blue approximately doubles the speed of oxygen uptake due to the oxidation of succinic acid in the presence of cyanide.

All the results discussed suggest the following theory concerning the oxidation mechanism of succinic acid. The specific enzyme which we may call, with Thunberg, "succino-dehydrogenase" (we reserve the name "succinoxidase" for another sense) induces a dehydrogenisation of succinic acid according to the formulae:

If the process takes place in the absence of oxygen but in the presence of methylene blue, the next step will be

The whole process is written here in two phases, although both reactions are probably coupled at the same surface. For the speed of the reaction (2) the "hydrogen transport factor" discussed in the previous section is of importance.

If the process on the other hand takes place in the presence of oxygen we shall have instead of (2) the reaction

In my opinion for this reaction (3) a catalyst X for the activation of

oxygen is necessary. This catalyst may be imagined to be an independent oxidising enzyme or it may be iron in the sense of Warburg.

All experimental results show that the reduction of methylene blue, involving the reactions (1) and (2), is not influenced by cyanide, which therefore inhibits neither the function of the enzyme "succino-dehydrogenase" nor the dissociation of the hydrogen from (enzyme H_2). On the other hand cyanide inhibits the reaction (3) completely. Consequently in the reaction (3) there must be a factor, X , necessary for this reaction which is sensitive to HCN. Since cyanide is known to be a specific inhibitory substance for oxidising enzymes or oxygen activators we may believe that X is an oxygen activator. We come then to the view, that the hydrogen of (enzyme H_2) cannot be oxidised by molecular unactivated oxygen. Consequently two enzymes are necessary for the oxidation of succinic acid, i.e. the specific enzyme "succino-dehydrogenase" which mobilises the hydrogen of the succinic acid molecule and the probably unspecific oxygen activator X . It will be pointed out later on that these two enzymes may in fact be only one enzyme with two different functions.

If in an aerobic experiment the oxygen catalyser X , and therefore the oxidation of succinic acid, is inhibited by cyanide, there exists a possibility of restoring this oxidation by adding methylene blue. Then the reactions (1) and (2) proceed in spite of the presence of cyanide, and the methylene blue is reduced. Since molecular oxygen is present the autoxidisable methylene white formed is continuously re-oxidised. Thus methylene blue replaces the catalyser X ; it forms a substitute for an oxidising enzyme.

There are some other arguments to support this theory.

If the reaction (2) is in reality responsible for the power of methylene blue to restore the oxidation of succinic acid, then a parallel must exist between:

- A. The power of a tissue to reduce methylene blue in presence of succinic acid *in vacuo*, and
- B. The speed of oxygen uptake in an aerobic experiment with the same tissue in which the direct oxidation of succinic acid is inhibited by cyanide, but the indirect oxidation restored by addition of methylene blue.

It is doubtless somewhat difficult to compare the results of A with those of B, because in my experiments bearing on A no velocity curve was obtained but only the time necessary for the reduction of a certain quantity of methylene blue. Since however we only want relative figures the following procedure is sufficient. From the reduction time of the experiments done under the heading A the number of cmm. O_2 was calculated, corresponding to the amount of methylene blue which would be reduced in one hour (1 cc. $M/1000$ methylene blue is equal to 11.2 cmm. O_2). For the comparison we then take the quotient

$$\frac{\text{Cmm. } O_2 \text{ in 1 hour of experiment B}}{\text{Cmm. } O_2 \text{ in 1 hour of experiment A}} = \frac{B}{A}.$$

In 13 pairs of experiments with different tissues we got for B/A the following values:

Rat muscle, from 1.4 to 2.3; average 2.0.

Sarcoma, from 1.8 to 2.7; average 2.3.

Rat liver, from 2.1 to 2.3; average 2.2.

Foetal rabbit muscle (one experiment), 2.1.

Although the different experiments show a very marked difference in the actual power of methylene blue reduction, the values of the quotient B/A are very consistent. This is I think a strong argument in favour of the view, that, in the experiments of types A and B the same reaction (2) is taking place. Consequently the reaction (2), which is not inhibited by cyanide, is responsible for the power of methylene blue to re-establish the oxidation of succinic acid by a tissue under the influence of cyanide.

This theory involves the assumption that methylene blue is acting only as a hydrogen acceptor and that this function is responsible for the results observed. Lipschitz [1921, 1923] when describing his use of dinitrobenzene was inclined to minimise the significance of methylene blue reduction; but I quite agree with the view of Winterstein [1923] that every one of these reducible substances has its special advantages and disadvantages.

It seemed worth while however to decide whether other reducible substances have the same influence as that described for methylene blue. Toluidine blue, soluble blue and thionine were investigated to find out if they also can restore the oxidation of succinic acid when inhibited by cyanide. The experiments gave a clearly positive answer. Obviously there are differences in the activity, and methylene blue was found to be more active than the others. But all four substances are able to restore the oxidation of succinic acid by a tissue inhibited by cyanide. The theoretical oxygen uptake (224 cmm. for 0.1 cc. *M*/5 succinic acid) is ultimately reached in their presence. As soon as this is reached the oxygen uptake stops. In Fig. 5 two curves obtained with toluidine blue and soluble blue are shown.

In claiming that a "succino-dehydrogenase" and an oxygen activator *X* are necessary for the oxidation of succinic acid I have assumed that two distinct catalysts are actually present. It may be of course that the functions represented as separate in the scheme given above are in reality attached to one enzyme. If so it would be reasonable to name such an enzyme with the united functions "succinoxydase" in opposition to the hypothetical "succino-dehydrogenase" which was assumed to exert only one function. To settle this point an endeavour was made to find some treatment for the tissue which should destroy the factor *X* while leaving the succino-dehydrogenase intact. If this endeavour were successful we could prove the presence of the "succino-dehydrogenase" by restoring the oxidation with methylene blue. The different methods used were:

- (1) Washed muscle was allowed to stand at room temperature for varying periods.

(2) Washed muscle preparations were heated to 50–56° for different lengths of time.

(3) Investigations were made at different hydrogen ion concentrations.

These and other attempts gave the same negative result. It was never possible to destroy or to diminish the activity of an oxygen activator without diminishing the “succino-dehydrogenase” effect at the same time. Consequently we think that only one catalyst, succinoxidase, is present, which unites the two different functions. Yet as has been shown the two functions are certainly different, since one only is inhibited by cyanide. If we look at the question from a biological point of view, it seems to be reasonable that only one enzyme should be present. Undoubtedly energy is used to split off hydrogen from the succinic acid molecule. On the other hand energy is produced by the oxidation of this hydrogen to water. It is probable that reactions so related are catalysed by one and the same agency.

At present a discussion is in progress as to whether the oxidation processes in the organism are caused by an “oxydase” system of the type described by Engler, Bach and Chodat, or by an activation and transport of hydrogen on the lines suggested by Wieland and Thunberg. The experiments described in this paper lead to the conclusion that the activation of both hydrogen and oxygen is necessary at least for the oxidation of succinic acid. Although it does not follow that this complex mechanism is necessary for all biological oxidations, it seems to me that this conception of a double activation (of hydrogen as well as of oxygen) may have significant bearings.

SUMMARY.

A comparison was carried out between some oxidation processes due to normal tissue on the one hand and by sarcomatous tissue on the other. The methods used were measurements of oxygen uptake in the Barcroft differential apparatus and the methylene blue reduction technique of Thunberg in vacuum tubes.

(1) Glutathione has with the thermostable sarcoma residue the same effect as that found by Hopkins for the thermostable muscle residue.

(2) Unwashed sarcoma tissue reduces methylene blue more slowly than muscle. Likewise sarcoma exhibits a lower initial velocity of oxygen uptake than muscle; but the total amount of O₂ taken up weight for weight is greater for sarcoma tissue. Cyanide inhibits the oxygen uptake but not the methylene blue reduction of unwashed tissue, sarcoma as well as muscle.

(3) Citric acid accelerates the methylene blue reduction of washed muscle, but not of washed sarcoma. Cyanide has no influence. Muscle as well as sarcoma oxidises citric acid in the Barcroft apparatus. This oxidation is not sensitive to HCN; the optimum is at p_H 6.0.

(4) The methylene blue technique gave the following results with washed tissue. Glutamic acid is oxidised by muscle and by sarcoma. Lactic acid is less actively oxidised by sarcoma than by muscle. Fumaric, maleic and malic

acids have no distinct effect on the decolorisation of methylene blue by either tissue.

(5) Succinic acid is oxidised freely whether methylene blue or oxygen be the hydrogen acceptor. Muscle was found to be much more active in this respect than sarcoma. The quantity of oxygen used corresponds to one atom oxygen to one molecule succinic acid. Whilst HCN has no effect on the methylene blue reduction, the oxygen uptake by succinic acid is inhibited by cyanide. But the oxidation of succinic acid (with O_2) can in the case of muscle be restored if methylene blue is added to the system. This indirect oxidation of succinic acid induced by the methylene blue occurs to a much less extent, if at all, in the case of sarcoma.

(6) There is evidence that a "hydrogen transport factor" exists which is of importance for the transport of hydrogen to methylene blue. This "hydrogen factor" is distinct from the specific enzyme "succinoxidase." It can be washed out and its concentration is much smaller in sarcoma than in muscle tissue.

(7) The mechanism of the aerobic oxidation of succinic acid is investigated and discussed. The experiments lead to the assumption that the enzyme succinoxidase possesses two different functions: (a) the splitting off of 2H from the succinic acid molecule-activation and transport of 2H and (b) the activation of oxygen. The activation of oxygen as well as the activation of hydrogen is necessary for the oxidation of succinic acid.

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