CXLII. SOME OBSERVATIONS ON THE OXIDATION OF PHENOLS BY TISSUES AND ON THE SIGNIFICANCE OF SURFACES FOR BIOLOGICAL OXIDATIONS.

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PHENOLS, which are so important in the oxidations of plants, would be also of great importance for oxidation in animals, if, as Szent-Györgyi [1924; 1925, 1, 2] asserts, it could be proved that they increase lactic acid oxidation in mammalian muscle; for the conditions of lactic acid oxidation in mammalian muscle are still undefined, though of great significance for the physiology of muscle. Szent-Györgyi's conclusion that a co-enzyme is concerned in its oxidation and his suggestion that this co-agent may be a phenol are of much interest. The intention of the work to be described was to repeat the experiments of the above author in the hope of linking them with other work which is in progress in this laboratory. I must state in advance, however, that to judge from my own experiments as described in this paper it is not possible to increase the oxidation of lactic acid either by ice-water extract of muscle (which according to Szent-Györgyi contains the co-enzyme), or by *p*-phenylenediamine. Szent-Györgyi [1925, 1, 2] ground the muscle he employed with glass-powder and the powder was contained in the systems of which the oxygen uptake was determined. It is clear, however, that the introduction of a foreign surface of the kind may appreciably alter conditions in a system. The following experiments were therefore carried out:

- (i) the effect of boiled ice-water extract on minced muscle;
- (ii) the influence of some phenols on the oxygen uptake of muscle and of reduced glutathione;
- (iii) the influence of various surfaces on the oxidation of phenols;
- (iv) the oxidation of phenols in "mixed systems" such as Szent-Györgyi's.

I.

The muscle, pig's diaphragm, was treated carefully in accordance with Szent-Györgyi's method [1925, 1, 2]. The muscle, freed from sinew and fat, was minced with a chopper on a block, then washed in cool distilled water twice, from 15 to 20 minutes each time, filtered through linen and pressed between filter-papers and finally pounded in a mortar with glass-powder which had been previously washed ten times in distilled water. The oxygen

uptake of this muscle, so treated, was measured in Barcroft's differential manometer at 37°–38°, and the decoloration of methylene blue was observed anaerobically in Thunberg's tubes.

The ice-water extract was made in the following way: 10 g. minced muscle were mixed with 10 cc. water at 1°–3°, the whole put in ice for 1–2 hours and frequently stirred. It was then filtered through linen, squeezed and the coloured solution was boiled, filtered again through paper and made up to four times the amount of the original extract with a phosphate buffer solution of p_H 7.4.

Working with this glass-powder-muscle was not satisfactory, because, as Szent-Györgyi found, the results were not consistent (Table I); but working

Fig. 1.

with the muscle without glass-powder the agreement was quite constant (cf. Table II). From Table II we see firstly that muscle washed as above always takes up some oxygen and in fairly constant amount, but only in phosphate solution, never in 0.9 % NaCl solution (unpublished experiments); secondly, that the muscle prepared in this way oxidises lactic acid constantly, though in small amounts which increase with the lactic acid concentration.

Table I shows that the oxygen uptake of the system muscle + glass-powder + lactic acid was indeed occasionally materially increased by the addition of the boiled water-extract; but we noticed that sometimes the oxygen uptake of the glass-powder-muscle was decreased by the addition of lactic acid (cf. Fig. 1).

Table I.

37°; $p_H = 7.4$.

Material		Duration of experiments		
		2½ hrs.	4½ hrs.	5½ hrs.
		Oxygen uptake mm. ³		
1 g. glass-powder-muscle				
1	+2.5 cc. boiled water-extract	...	...	...
1	+0.3 cc. 0.25 <i>M</i> lactic acid	...	...	...
1	+2.5 cc. boiled water-extract + 0.3 cc. 0.25 <i>M</i> lactic acid	176	208	315

Table II.

38°; $p_H = 7.4$.

Material		Duration of the experiments in hours											
		3½	5½	4	4	4	4	4	4	4	4	4	4
		Oxygen uptake mm. ³											
0.7 g. washed muscle													
0.7	+2.5 cc. extract	40	50	40	35	35	58	35	51	48	61	52	60
0.7	+0.3 cc. 0.25 <i>M</i> lactic acid	154	264*	164	180	266*	408**	134	—	—	—	—	—
0.7	+2.5 cc. extr. + 0.3 cc. 0.25 <i>M</i> lactic acid	64	—	70	64	—	62	74	—	—	—	—	—
0.7	+0.6 cc. 0.25 <i>M</i> lactic acid	196	—	211	152	—	462	136	—	—	—	—	—
0.7	+2.5 cc. extr. + 0.6 cc. 0.25 <i>M</i> lactic acid	—	104	—	111	—	—	—	—	—	95	100	110
0.7	+0.9 cc. 0.25 <i>M</i> lactic acid	—	268	—	—	347	—	—	—	—	—	—	—
0.5	+0.6 cc. 0.5 <i>M</i> lactic acid	—	—	—	—	—	—	—	138	133	—	152	—
0.5	+0.6 cc. 1.0 <i>M</i> lactic acid	—	—	—	—	—	—	—	—	—	—	—	184
0.5	+0.6 cc. 1.0 <i>M</i> lactic acid	—	—	—	—	—	—	—	—	—	—	239	—

The values in brackets denote final concentrations of lactic acid; the oxygen uptake was complete; the extract was diluted 1 in 4; extr. * 1 in 3; extr. ** 1 in 2.

In anaerobic experiments it was observed that the capacity for methylene blue reduction was greatly increased by adding lactic acid to the mixture of muscle and glass-powder, but the increase was very small when lactic acid was added to muscle without glass-powder (cf. Table III). This result will be discussed later.

Table III. *Anaerobic experiments.* p_H 7.4; 37°.

Meth. blue 1 : 2000	Muscle (washed)	Lactic acid 0.3 M	Ice-water extract boiled	Phosphate buffer	Decoloration time in minutes
cc.	g.	cc.	cc.	cc.	
0.3	1	0.25	—	1.00	6
0.3	1	—	—	1.25	64
0.3	1	—	1	0.25	3
0.3	1	0.25	1	—	4
0.3	0.7	0.25	—	1.0	14
0.3	0.7	—	—	1.25	14
0.3	0.7	—	1	0.25	4.5
0.3	0.7	0.25	1	—	4.5
0.5	1	—	—	4.5	25
0.5	1	1.0	—	3.5	22

Meanwhile, it is well to point out that the aqueous extract of muscle is very rich in H-donators; it gives, of course, a strongly positive nitroprusside reaction. The extract itself is not autoxidisable under the conditions which obtain in the Barcroft manometer, but takes up oxygen under the catalytic influence of haemoglobin; *e.g.* 2 cc. extract + 0.5 cc. haemoglobin solution (0.5 %) + 2.5 cc. buffer solution take up 94 mm.³ O₂ in 5 hours, but 0.5 cc. haemoglobin solution (0.5 %) + 2.5 cc. buffer take up under the same circumstances 30 mm.³; the extract alone is not autoxidisable.

As it was not possible to obtain consistent results with the glass-powder-muscle, I made the following experiments on muscle, prepared in the way stated above, but without glass-powder.

Naturally it seemed possible that the haemoglobin present in the aqueous solution might play a part in the effects of the extract on muscle; I therefore made some experiments on the influence of haemoglobin on the oxygen uptake by muscle (cf. Table IV). The haemoglobin preparation I owe to the kindness of Mr G. S. Adair; from this haemoglobin I made methaemoglobin by adding a trace of potassium ferricyanide and removing the latter by dialysis.

Table IV. 37°; p_H = 7.4.

0.5 g. washed muscle	Haemo- globin	Methae- moglobin	0.5 g. muscle + haemoglobin	0.5 g. muscle + methae- moglobin	Duration of the experiments
Oxygen uptake mm. ³					
60	12*	20	195*	199	5 hours (complete)
54	15**	18	130**	118	4 „ (nearly complete)
48	17***	20	215***	222	5 „ (complete)
Concentration of methaemoglobin 2 %; of haemoglobin* 0.1 %; of haemoglobin** 0.5 %; of haemoglobin*** 2 %.					

It will be seen that haemoglobin and methaemoglobin both catalyse the oxygen uptake of washed muscle to the same extent, as Robinson [1924] also found when working on unsaturated fatty acids. That this effect of methaemo-

globin is purely catalytic follows from the fact that the haemoglobin concentration is of no influence in this process. But in four experiments I found no increase of lactic acid oxidation on the addition of haemoglobin or methaemoglobin. I may further point out that some preliminary experiments showed that methaemoglobin reacts with glutathione. That methaemoglobin may be reduced by GSH¹ has been incidentally pointed out by Holden [1924]; I have confirmed his observation as follows: 1 cc. 8 % methaemoglobin solution + 50 mg. GSH ceased to show the characteristic absorption bands within 2–2½ hours at 37°. This reduction is also brought about by washed muscle added to the methaemoglobin solution.

II.

I have made a number of experiments dealing with the influence of *p*-phenylenediamine on the oxygen uptake of washed muscle (studied first by Batelli and Stern [1912]), since Szent-Györgyi states that this substance increases the oxidation of lactic acid by muscle. I found in fifteen consistent experiments that this is not the case. Fig. 2 shows that the oxygen uptake

Fig. 2.

of the systems, muscle + *p*-phenylenediamine and muscle + *p*-phenylenediamine + lactic acid, is additive. In all the experiments the oxidation of muscle + *p*-phenylenediamine is at first diminished by addition of lactic acid and after 2–2½ hours increased. Both influences depend on the concentration of the lactic acid, and increase with increase of the latter. So there seems to be no probability that the oxidation of lactic acid by muscle is increased as the result of the presence of *p*-phenylenediamine.

It was further necessary to try to make clear the mechanism of the oxidation of *p*-phenylenediamine by muscle and to compare it with the oxidation of other aromatic substances, such as phenols. Table V shows that in the concentrations given the oxygen uptake of *p*-phenylenediamine in the presence of muscle is nearly proportional to its concentration. The diamine in the concentrations employed has itself only a small uptake (cf. Table VI). This uptake is not affected by *M*/500 KCN.

¹ GSH = reduced glutathione.

Table V. $p_H = 7.4$; 38° .

Material	Oxygen uptake** mm. ³
0.5 g. washed muscle	61
0.5 " " in 0.001 % <i>p</i> -phenylenediamine*	102
0.5 " " in 0.0035 " " "	218
0.5 " " in 0.05 " " "	300
0.5 " " in 0.1 " " "	573, 573, 552, 550, 550
0.5 " " in 0.15 " " "	800
0.5 " " in 0.2 " " "	1010, 1020
0.5 " " in 0.33 " " "	1510

* Final concentration; ** oxygen uptake in all experiments complete.

Table VI. $p_H = 7.4$; 38° .

The concentrations given denote *final* concentrations.

Material	Oxygen uptake mm. ³	Duration of the experiments
0.5 g. washed muscle	61	5 hours (nearly complete)
0.5 " " + 0.1 % <i>p</i> -phenylenediamine	573	
0.5 " " + 0.33 % GSH*	182	
0.5 " " + 0.1 % <i>p</i> -phenylenediamine + 0.33 % GSH	773	
0.1 % <i>p</i> -phenylenediamine	39	2 hours (complete)
0.1 % <i>p</i> -phenylenediamine	29, 35	
0.1 % " " + 0.33 % GSH	208, 196	
0.33 % GSH	150, 150	
0.2 g. muscle-powder	24	4 hours (complete)
0.2 " " + 0.1 % <i>p</i> -phenylenediamine	69	
0.2 " " + 0.33 % GSH	226	
0.2 " " + 0.1 % <i>p</i> -phenylenediamine + 0.33 % GSH	210	

* The GSH preparation was in fact only 40 % reduced.

Hours
Fig. 3.

Very peculiar are the relations of *p*-phenylenediamine to GSH. Autoxidation of GSH is considerably accelerated (Fig. 3) by 0.1 % *p*-phenylenediamine in the absence of muscle and perhaps also increased in amount; but the oxygen uptake of the system muscle + GSH is perceptibly augmented by *p*-phenylenediamine (cf. Table VI). As follows from some observations carried out conjointly with Dr M. Dixon, however, it seems that a compound of these two substances is formed; because, since the anaerobic decoloration of methylene blue by GSH is likewise accelerated by *p*-phenylenediamine, it seems as though *p*-phenylenediamine activates the hydrogen of the GSH group. Methylene blue (0.5 cc.) + 0.1 cc. 1 % GSH + 4.8 cc. buffer solution ($p_H = 7.5$) was

decolorised in 100 minutes; whilst 0.5 cc. methylene blue + 0.1 cc. GSH + 0.5 cc. 1 % *p*-phenylenediamine + 4.3 cc. buffer solution ($p_H = 7.5$) took 73 minutes to become decolorised. Both experiments were performed at 37°. No such effect was observed in aerobic and anaerobic experiments with catechol, quinol, *p*-cresol, or adrenaline.

In order to determine whether the effect of the *p*-phenylenediamine was due to traces of iron contained in it accelerating the reaction [see Toda, 1926], we investigated the effect of cyanide as follows:

Contents of tubes						Reduction time min.
0.5 cc. meth. blue	+ 0.2 cc. GSH (1 %)	+ 1.3 cc. buffer ($p_H = 7.4-7.6$)	...	...	...	68
0.5 "	" + 0.2 "	" + 0.9 "	+ 0.4 cc. <i>M</i> /100 KCN	...	...	300
0.5 "	" + 0.2 "	" + 0.4 "	+ 0.5 cc. <i>p</i> -phenylenediamine (1 %)	...	...	28
0.5 "	" + 0.2 "	" + 0.4 cc. <i>M</i> /100 KCN	+ 0.5 cc. <i>p</i> -phenylenediam. (1 %)	...	...	58

GSH = glutathione 40 % reduced. All solutions were carefully neutralised before use.

It will be seen that the cyanide has decreased the velocity of reduction by the GSH to less than one quarter of the normal, in agreement with the results of Toda. On the addition of *p*-phenylenediamine the reducing power is again restored, and there is even a slight acceleration in spite of the presence of cyanide. It is quite clear, therefore, that *p*-phenylenediamine is able to activate the SH group quite apart from any traces of iron which it may contain. *p*-Phenylenediamine even acts like iron in this case.

We further observed that the reduction potential (measured with a gold electrode) of mixtures of GSH and *p*-phenylenediamine was much higher than that of either substance alone; *e.g.* reduction potential against saturated calomel electrode (20°) of .00003 *M* of our preparation of GSH (= .0000075 *M* GSH):—55 millivolts; 0.16 % *p*-phenylenediamine:—80 millivolts; mixture of both:—121 millivolts; all substances were dissolved in phosphate buffer of p_H 7.4.

In connection with these observations it is of interest to note that McCance [1925] found that catechol, which is unable to reduce methylene blue, forms compounds with certain amines and these compounds rapidly reduce methylene blue.

III.

For the promotion of chemical reactions by living tissues two factors must be taken into consideration: (1) chemical factors involving the influence of certain substances or groups; (2) physical factors, *i.e.* possibilities of orientation of these molecules: but how such an orientation of molecules, *e.g.*, on surfaces, may modify the possibilities of chemical reaction ("Aktivierung") is not yet well understood.

Table VII and Figs. 4 and 5 show the effect of different surfaces on the oxidation of phenols, etc. The oxidation of *p*-phenylenediamine, which is slightly autoxidisable in the concentrations used by us, is catalysed by washed muscle and charcoal considerably, and by the first not very much more than by the second. It is not affected by coagulated muscle-powder, which it also

fails to colour. *p*-Phenylenediamine, moreover, has no influence on the muscle-powder + glutathione system (Table VI). The oxidation of 0.1 % quinol under the same circumstances is hardly catalysed by charcoal, but by washed muscle it is catalysed two and a half times as much as is *p*-phenylenediamine, whilst 0.1 % catechol is catalysed by both charcoal and muscle to the same extent, namely, about half as much as *p*-phenylenediamine.

Fig. 4.

I.	Washed muscle	+ 1 % <i>p</i> -phenylenediamine
II.	" "	(60°) + 1 % " "
III.	" "	+ 1 % quinol "
IV.	" "	(46°) + 1 % " "
V.	" "	(60°) + 1 % " "

Fig. 5.

 Table VII. 38°, $p_H = 7.4$; 5 hours.

	Without addition	+ Muscle-powder	+ Charcoal	+ Washed muscle
		Oxygen uptake mm. ³		
0.1 % <i>p</i> -phenylenediamine	38	69	398	550
0.1 % quinol	192	200	235	1056
0.1 % catechol	100	182	284	258

If the muscle is washed to excess (ten times), it has more effect on the oxidation of *p*-phenylenediamine; thus 0.5 g. twice washed muscle + 0.1 % *p*-phenylenediamine takes up 555 mm.³ (cf. Table V), but 0.5 g. ten times

washed muscle + 0.1 % *p*-phenylenediamine took up in one experiment 727 mm.³, in another 850 mm.³; washing therefore seems to increase this catalytic activity of muscle. If this muscle be warmed to 60° for 1 hour, the catalytic effect is diminished without lessening the final uptake; the delay of the uptake is still greater in the case of quinol. This can perhaps be explained by the alteration of the tissue by loss of water (cf. Table VIII). Heating to 80°, on the other hand, nearly completely destroys the activity.

Table VIII. 5 hours, 38°.

Material						Oxygen uptake mm. ³	Remarks
0.1 % <i>p</i> -phenylenediamine	...	...	...	...	...	39	
+ 1 g. muscle washed 10 times	...	...	...	...	...	727*	
+ 0.75 g. " " warmed to 46° for 1 hour	...	...	...	...	...	780*	Loss of weight 28 %; slow oxidation
+ 0.5 " " warmed to 60° for 1 hour	...	...	...	...	...	730*	Loss of weight 48 %; slow oxidation (cf. Fig. 4)
+ 0.4 " " coagulated at 80°	...	...	...	...	...	100*	Loss of weight 58 %
+ 0.2 " " " " " and dried by alcohol	...	...	...	...	...	68*	" " 80 %
0.1 % quinol	...	...	...	...	...	192	
+ 1 g. muscle washed 10 times	...	...	...	...	...	1056*	
+ 0.75 g. " " warmed to 46° for 1 hour	...	...	...	...	...	979	Slow oxidation
+ 0.5 " " " to 60° for 1 hour	...	...	...	...	...	857	" "
+ 0.2 " " coagulated at 80°, dried by alcohol	...	...	...	...	...	216*	

In the experiments * the oxygen uptake was complete.

From these experiments the following conclusions concerning the biological oxidation of these phenols seem justified. The oxidation of *p*-phenylenediamine is accelerated on surfaces; washed muscle and charcoal show little difference in this respect. Consequently, for this catalysis only an adsorbing surface is necessary, it is therefore for the most part physical. For oxidation of quinol a chemical activation given by the non-coagulated muscle is the essential process, and charcoal and muscle-powder have no effect; also for oxidation of catechol there seems to be necessary such a chemical activation, which however does not occur in muscle.

Table IX. $p_H = 7.4$; 38°.

Washed muscle g.	Final concentration of lactic acid	Final concentration of <i>p</i> -phenylenediamine %	Charcoal g.	Oxygen uptake (mm. ³)			
				5 hrs.	4 hrs.	4 hrs.	4 hrs.
1	—	—	—	50	50	50	—
1	0.2 <i>M</i>	—	—	230	—	—	—
1	0.175 <i>M</i>	—	—	—	158	—	—
1	0.2 <i>M</i>	—	0.2	30	—	—	—
1	—	0.1	—	520	549	—	—
1	0.2 <i>M</i>	0.1	—	720	—	—	—
1	—	0.1	0.2	440	—	416	—
1	0.2 <i>M</i>	0.1	0.2	554	—	—	—
1	0.175 <i>M</i>	—	0.2	—	65	—	30
1	0.175 <i>M</i>	0.1	—	—	—	—	684
1	0.175 <i>M</i>	0.1	0.2	—	496	510	484

IV.

In order to make my results more comparable with those of Szent-Györgyi I made certain further investigations, in which muscle and an adsorbing surface were rivals for the phenol. In these experiments I put in one vessel of the manometer 1 g. washed muscle + 0.2 g. charcoal + the substances to be tested, and in the other the same amount of charcoal. Table IX gives the details of some results on these lines which showed a fairly good agreement.

The oxygen uptake of the system muscle + charcoal + *p*-phenylenediamine appears to be increased by adding lactic acid to a greater extent than additive effects would account for, but lactic acid oxidation by muscle is greatly hindered by charcoal, just as we have already seen it to be hindered by glass-powder. Therefore, the most probable explanation of Szent-Györgyi's inconstant results, showing that the oxidation of lactic acid by muscle is increased by *p*-phenylenediamine, seems to be that added lactic acid, otherwise adsorbed by the glass-powder, is displaced from the glass-powder surface by *p*-phenylenediamine and so may be oxidised by the muscle. Thus, in his experiments there was a rearrangement of the surfaces involved in his oxidising systems which disturbed the true physiological relations and obscured the real factors involved in the oxidation of lactic acid. That no more lactic acid is oxidised by muscle as the result of the presence of Szent-Györgyi's ice-water extract has been shown above. That the presence or absence of *p*-phenylenediamine is immaterial is evident from Table X. In two manometers hanging side by side we investigated two identical mixtures; in one manometer was added, as usual, 20 % KOH to absorb CO₂, but none in the other; in this manner we could measure roughly the CO₂ formed by the oxidation; as by the oxidation of *p*-phenylenediamine no CO₂ is formed, the whole measured amount of CO₂ (apart from that produced by the muscle alone, which will be the same in all experiments) is produced by the oxidation of lactic acid.

Table X. 38°; $p_H = 7.4-7.6$.

min.	Washed muscle + lactic acid			Washed muscle + lactic acid + <i>p</i> -phenylenediamine			Washed muscle + lactic acid + <i>p</i> -phenylenediamine + charcoal		
	mm. ³ O ₂	O ₂ -CO ₂	CO ₂	mm. ³ O ₂	O ₂ -CO ₂	CO ₂	mm. ³ O ₂	O ₂ -CO ₂	CO ₂
17	31	8.5	22.5	188	129	59	166	104	62
28	53	20	33	296	222	74	232	154	78
55	103	48	55	498	406	92	332	208	124
70	—	—	—	572	474	98	368	236	132
131	185	85	100	674	549	125	448	304	144
255	240	114	126	710	591	119	526	366	160

In these experiments were used washed muscle, 1 g.; charcoal, 0.2 g. The final concentration of lactic acid was 0.2 *M*, of *p*-phenylenediamine, 0.1 %.

Consequently we see that the oxidation of lactic acid is not altered by adding *p*-phenylenediamine, but there is an increase if charcoal is added.

SUMMARY.

1. Szent-Györgyi's results on the activation of the oxidation of lactic acid by muscle by addition of ice-water extract of the muscle and of *p*-phenylenediamine could not be confirmed.

2. The effects observed by Szent-Györgyi are shown to be due to a displacement of lactic acid from the surface of the glass-powder present in his muscle preparations by *p*-phenylenediamine, thus enabling it to be oxidised by the tissue.

3. Haemoglobin and methaemoglobin catalyse the oxygen uptake of washed muscle but do not affect lactic acid oxidation.

4. *p*-Phenylenediamine is oxidised by washed muscle and by charcoal, but not by coagulated muscle. Heating washed muscle to 60° for an hour diminishes the activity, heating to 80° entirely destroys it. *p*-Phenylenediamine accelerates aerobically and anaerobically the oxidation of reduced glutathione, probably by forming a compound with it. The oxidation of *p*-phenylenediamine is essentially a surface catalysis; the oxidation of quinol and catechol, on the other hand, is not accelerated by surfaces such as charcoal.

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