LVI. THE REDUCTION OF DINITROPHENOLS BY REDOX INDICATORS AND ENZYMES.

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Two types of reagent are known to cause an increase in the respiration of surviving tissues and yeasts when added to the medium in which these cells are respiring: firstly certain organic dyestuffs which form reversible and electro-active systems with their readily autoxidisable leuco-forms [Barron, 1930; Chang and Gerard, 1931; Gerard, 1932; Friedheim, 1932; 1934; Fleischmann and Kann, 1933; Dickens, 1934]; and secondly the dinitrophenols [Dodds and Greville, 1933; 1934; Ehrenfest and Ronzoni, 1933; Field *et al.*, 1933; 1934].

As in many ways there is a striking resemblance between the actions of the two types of agent, which even extends to their effects on the anaerobic glycolysis of frog muscle [Gerard, 1931; Ehrenfest and Ronzoni, 1933], it seemed of interest to inquire whether the dinitrophenols can be found in any way to form a reversible system under physiological conditions. A nitrophenol might conceivably form an oxidation-reduction system in two ways: it might be the oxidant of a system the reductant of which is formed by reduction of a nitro-group, or it might be the reductant of a system the oxidant of which is formed by oxidation of the phenolic hydroxyl group. From the data of Fieser [1930, 1, 2] it would appear that the oxidation of a nitrophenol would require an oxidation intensity far in excess of that possessed by living cells or by any system so far isolated from them (about 1 v.). Hence for the present problem the second alternative may be neglected. On the other hand it is known that dinitrophenol is reduced by the living animal to nitroaminophenols [Guerbet and Mayer, 1932]. We have therefore concentrated on the reduction of nitrophenols by various agents. An attempt was made to see whether this would lead to the formation of a reversible system, and whether the reduction could be performed by enzymes extractable from living cells.

EXPERIMENTAL.

Tests for reversibility.

Potentiometric experiments. We tested for reversibility at p_H 7 by the methods commonly employed in potentiometric analysis, namely the methods of reductive and oxidative titration and the method of mixtures.

Titration of $10^{-3}M$ 4:6-dinitro-*o*-cresol with hydrosulphite did not yield typically S-shaped curves. The potentials measured on the two electrodes employed were not stable and differed widely. Flattening of the curves in different experiments occurred in different E_H ranges. The colour changes observed during the course of the titration were: lemon-yellow (before addition of the titrant) $\rightarrow$ orange $\rightarrow$ red $\rightarrow$ colourless.

The same sequence of colour changes could be observed on reduction with hydrogen in the presence of colloidal palladium. Since reversibility of the system

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was the more likely to be found the less extensive the reduction, the dinitro-cresol was reduced by palladium-hydrogen under photo-electric control to maximum red colour production, presumably to the nitroaminocresol stage. The hydrogen was then replaced by nitrogen and the system was titrated back with ferricyanide. Each addition of a small amount of the titrant caused an initial rise of the potential followed by a drifting back to approximately the original value, until finally a point was reached where the potential suddenly rose into the potential range of the ferricyanide system and remained there. During the titration the colour changed from dark red to dark brown.

Finally the method of mixtures was tried. An equimolar amount of 4-nitro-2-aminophenol was added to a de-aerated buffered solution of 2:4-dinitrophenol. No steady potential was reached within 40 mins. and the two electrodes disagreed. A small quantity of methylene blue was added without any stabilising effect being observed. In order to see whether the presence of living cells would cause the system to attain a stable potential, a small quantity of washed baker's yeast was added to an equimolar mixture of dinitrophenol and nitroaminophenol. This caused the potential to become somewhat more negative. When methylene blue was added to the system, a slow potential decrease was observed with a flattening of the time-potential curve as the poisoning range of the indicator was approached. It follows that the presence of the nitrophenols did not alter the course of events which normally occurs with de-aerated suspensions of yeast cells in the presence of methylene blue [Cannan *et al.*, 1926].

Experiments with mammalian erythrocytes.

It has been found that reversible oxidation-reduction systems, the normal potentials of which may vary within very wide limits, cause an increase in the oxygen uptake of non-nucleated red cells, the respiration of which is normally very low [Harrop and Barron, 1928; Michaelis and Salomon, 1931; De Meio *et al.*, 1934]. Hence if nitrophenols were found to increase catalytically the oxygen uptake of such cells, this would provide evidence for their conversion into a reversible system when in contact with living matter. Experiments with human and rabbit erythrocytes, however, failed to demonstrate any such effect. The results of a typical experiment are given below.

Material: Washed rabbit erythrocytes made up to blood volume with phosphate-saline mixture, containing 0.25 % lithium *D*-lactate, p_H 7.3.

Each Warburg vessel contained 2 ml. of this suspension, oxygen capacity 142 μ l.

Temperature: 38°. Observation time: 60 mins.

Added agent	O ₂ uptake in μ l.
None	2.5
10 ⁻⁴ <i>M</i> methylene blue	112
10 ⁻⁴ <i>M</i> thionine	115
3.10 ⁻⁶ <i>M</i> thionine	35
10 ⁻³ <i>M</i> dinitro- <i>o</i> -cresol	2
10 ⁻⁴ <i>M</i> dinitro- <i>o</i> -cresol	3
10 ⁻⁵ <i>M</i> dinitro- <i>o</i> -cresol	1
3.10 ⁻⁶ <i>M</i> dinitro- <i>o</i> -cresol	3.5
10 ⁻⁶ <i>M</i> dinitro- <i>o</i> -cresol	0

In contrast to these findings the respiration of avian (nucleated) erythrocytes, which normally is already considerable, is greatly increased by dinitrophenol [De Meio and Barron, 1934]. Dinitrophenols have so far been shown to exert their effect only on those cells which already possess a complete respiratory system.

REDUCTION EXPERIMENTS.

Reaction of dinitro-o-cresol with redox indicators.

We first sought to determine the level of reduction intensity at which, in the absence of bio-catalysts, the rate of reduction of the nitro-groups in dinitro-*o*-cresol becomes appreciable. A scale of reduction intensities is provided by a series of partially reduced redox indicators. The method employed was that of "potentiometric indicators" [Conant and Lutz, 1923], which consists in determining electrometrically the rate of change of potential following the addition of the substance under investigation to equilibrated mixtures of redox indicators and their leuco forms. For discussion of the theory of the method the reader is referred to Fieser [1930, 2].

Seven indicator systems were employed, covering the E_h range between +0.040 and -0.270 v. All experiments were carried out at p_H 7.3 and at a temperature of 30°. The apparatus used is described below. 40 ml. of a buffered solution of the selected indicator were titrated with chromous acetate to a definite potential well inside the poisoning range. In most cases a value near the normal potential (E_0') of the indicator was selected. When the electrode potential had become perfectly steady, an equimolar amount of dinitro-*o*-cresol dissolved in 1 ml. of carefully de-aerated buffer was added. The total concentration of the indicator was $10^{-4} M$. In addition one experiment with gallophenin was carried out with a five times higher concentration of dyestuff and dinitrocresol. The results are shown in Table I, and some curves are given in Fig. 1.

Table I.

Redox indicator	E_0' at p_H 7.3 v.	Increase of potential in mv. after				E_h		% oxidation		Increase %
		15 mins.	30 mins.	60 mins.	90 mins.	$t=0$ mins.	$t=30$ mins.	$t=0$ mins.	$t=30$ mins.	
Thionine	+0.040	0	0	0	0	+0.041	+0.040	50	50	0
Indigotetrasulphonate	-0.059	0	0	0	0	-0.056	-0.055	55	55	0
Indigotrisulphonate	-0.094	4	3	3	3	-0.0875	-0.0845	62	67.5	5.5
Indigodisulphonate	-0.138	4	11	—	—	-0.118	-0.107	84	91.5	7.5
Indigodisulphonate	-0.138	12.5	21	36	—	-0.130	-0.109	65	91	26
Gallophenin	-0.157	1	1	2	4	-0.157	-0.156	50	50	0
		4	7	14	16	-0.158	-0.1505	50	62	12*
1:5-Anthraquinone- disulphonate	-0.220	26	35	—	—	-0.197	-0.162	85.5	94	8.5
Anthraquinone- β - sulphonate	-0.270	114	136	—	—	-0.267	-0.1309	55.5	100	44.5

* Five times higher concentrations.

Fig. 1.

It is obvious that the rate of reduction of dinitrocresol is not determined solely by the potential of the indicator system, which finding is in agreement with the observations of Conant and Lutz [1923]. It may, however, be said that

the rate of reduction is negligible above -0.05 v., small between -0.05 and -0.20 v., and considerable below -0.25 v. It is clear that this statement holds only for the experimental conditions chosen.

Reduction of dinitrophenols by enzyme systems.

It is now known that the activation of different substrates, *e.g.* formate or lactate, by the appropriate dehydrogenase systems gives rise to different levels of reduction intensity. Certain dehydrogenases (succinic, lactic and xanthine dehydrogenases) indeed are found to set up with the oxidised and reduced forms of their substrates reversible systems whose potentials have been measured by indirect methods [for literature see Stern, 1934, 2]. We have studied the reduction of dinitrophenols by four different dehydrogenase-substrate systems whose reduction intensities cover a wide range.

(1) *Formic dehydrogenase*. Though its reversibility has not yet been established, the formic dehydrogenase-formate system reduces very negative indicators so easily that a very high reduction intensity is indicated. One might therefore expect that dinitro-*o*-cresol would be easily reduced by this system. It was nevertheless found that formic dehydrogenase in the presence of formate caused no visible reduction of dinitro-*o*-cresol in the Thunberg tube. If, however, a small quantity of benzyl viologen ($E_0' - 0.359$ v.) was added, a brown colour rapidly appeared. The protocol of a typical experiment is given below.

Formic dehydrogenase prepared by tryptic digestion of a washed suspension of *Bact. coli commune* Escherich according to the method of Stickland [1929]. 0.1 ml. of this preparation reduced 1 ml. of 1/5000 methylene blue in the presence of formate in 3 mins. at 21°. In the absence of substrate the reduction time exceeded 16 hours.

Benzyl viologen was prepared by the method of Michaelis and Hill [1933].

The experiment was carried out in vacuum tubes at 38° in the manner described below.

Solutions: phosphate buffer, 0.1 *M*, p_H 7.3; dinitro-*o*-cresol, 10^{-2} *M*; benzyl viologen, $4 \cdot 10^{-3}$ *M*; sodium formate, 0.2 *M*.

Tube	...	...	1	2	3	4
			ml.	ml.	ml.	ml.
Buffer			2.0	2.5	2.0	2.0
Enzyme			0.5	0.5	0.5	0.5*
Substrate			—	0.2	0.2	0.2
Dinitrocresol			0.4	0.4	0.4	0.4
Viologen			0.5	—	0.5	0.5
Water			0.6	0.4	0.4	0.4
At beginning:			Lemon-yellow	Lemon-yellow	Lemon-yellow	Lemon-yellow
After 5 mins.:			No change	No change	Brown-yellow	No change
After 45 mins.:			No change	No change	Red-brown	No change

* Heated to 100°.

Passing oxygen through the solution in tube 3 at the end of the experiment for 30 mins. resulted in no change of colour.

Experiments with 2:4-dinitrophenol led to similar results. The coloured reaction product was identified in this case as 4-nitro-2-aminophenol by following the procedures described by Guerbet [1932], who has worked out methods for the separation and detection of small quantities of the reduction products of 2:4-dinitrophenol. 30 ml. of reaction mixture were incubated at 30° for 5.5 hours in a stream of pure nitrogen. At the end of the experiment diaminophenol was tested for with dichromate with a negative result. This test was found to be sensitive to 1/50,000 diaminophenol added to the reaction mixture. 4-Nitro-2-aminophenol was separated in aqueous solution from the remainder of the

reaction mixture by Guerbet's procedure and identified by Derrien's reaction (diazotisation and coupling with β -naphthol). The characteristic two-banded spectrum was observed after the addition of zinc acetate in methyl alcohol to the ethereal solution of the azo- β -naphthol. The amount of 4-nitro-2-amino-phenol formed was roughly estimated by comparison in Nessler glasses with a standard solution of this compound (solvent: 5 % ammonia). From this comparison the degree of conversion was seen to be at least 45 %. 2-Nitro-4-amino-phenol could not be detected.

In order to obtain insight into the mechanism of the coupled reaction observed in the Thunberg tube experiments, the system was subjected to potentiometric and photometric analysis in the apparatus described below. The phenomena observed in these experiments are represented in the composite diagrams of Fig. 2. In the first experiment (upper diagram), after the formic dehydro-

Fig. 2. Formic dehydrogenase and benzyl viologen. *A*, Formate added. *B*, 1 ml. dinitrocresol added. *C*, 0.1 ml. dinitrocresol added. *D*, 0.3 ml. dinitrocresol added. ——— Electrode potentials. - - - - Photo-electric potentials.

genase-formate system had been allowed to reduce the benzyl viologen, the addition of a relatively large amount of dinitro-*o*-cresol caused a sudden rise in potential to a level which was maintained for over 3 hours. The violet colour of the reduced indicator was discharged immediately after addition of the dinitrocresol, and during the subsequent observation time the solution became

increasingly red. At the steady potential level attained no appreciable quantities of the reduced viologen indicator could exist. It was concluded therefore that the enzyme-formate system reduces the viologen, and the reduced form of the indicator reacts with the dinitrocresol (when the latter is in excess) as fast as it is formed. This view was confirmed by the experiment shown in the lower part of Fig. 2, in which successive small quantities of dinitro-*o*-cresol were added. Here the potential fell and the purple colour of reduced viologen appeared each time that the whole of the dinitrocresol present had been used up.

In addition to benzyl viologen the following indicators were found to bring about the coupled reduction of dinitro-*o*-cresol: brilliant cresyl blue ($E_0' + 0.045$ v. (p_H 7)), Nile blue ($E_0' - 0.116$ v. (p_H 7)), Janus green ($E_0' - 0.256$ (p_H 7)). In order to judge the degree of reduction of the dinitrocresol at the end of the experiments the dyestuffs were removed after admission of air by shaking with kieselguhr and filtering.

(2) *Xanthine oxidase*. The redox potential of the hypoxanthine-enzyme-uric acid system is -0.44 v. at p_H 7 [Filitti, 1933]. It was found that this system, in contrast to the formic system, was able to reduce dinitrocresol in the absence of any indicator. The rate of reduction, however, was increased by the addition of benzyl viologen.

Enzyme: whey preparation of Dixon and Kodama [1926]. 1 ml. of the solution used reduced 1 ml. of 1/5000 methylene blue in the presence of 0.2 mg. of hypoxanthine in 1 min. 50 secs. at 37° .
Solutions: phosphate buffer, 0.1 *M*, p_H 7.3; hypoxanthine, 1 mg. per ml.; benzyl viologen, $2 \cdot 10^{-3}$ *M*; dinitro-*o*-cresol, 10^{-2} *M*.

Temperature: 37° .

Tube	...	...	1	2	3	4
			ml.	ml.	ml.	ml.
Buffer			1.0	1.0	1.0	1.0
Enzyme			1.0	1.0	1.0	1.0
Substrate			—	0.2	0.2	0.2
Dinitrocresol			0.3	0.3	0.3	—
Viologen			—	—	0.5	0.5
Water			0.7	0.5	—	0.3
At beginning:			Lemon-yellow	Lemon-yellow	Lemon-yellow	Colourless
After 15 mins.:			No change	Slightly orange	Deep orange	Purple
After 1 hour:			No change	Orange	Brown	Purple

2:4-, 2:5-, and 2:6-dinitrophenols are also reduced by this enzyme system. An attempt to identify the brown reduction product of 2:4-dinitrophenol failed.

Since the xanthine oxidase system showed such an interesting difference from the formate system in that no extraneous carrier was required for the reduction of dinitrocresol, we subjected this reduction process to potentiometric and photo-electric analysis (Fig. 3). Whilst reduction of the dinitrocresol was proceeding in the absence of benzyl viologen, as seen from the light transmission curve, the potential remained fairly steady at a comparatively positive level. Addition of benzyl viologen caused the potential to fall. Another experiment conducted in a manner similar to that of the second experiment with formic dehydrogenase (Fig. 2, lower part) gave results similar to that experiment; in both the reduction of dinitrocresol proceeded at approximately the same potential level.

(3) *Lactic dehydrogenase*. The normal potential of the lactate-enzyme-pyruvate system has been found by various workers to be near -0.200 v. at p_H 7 [see Stern, 1934, 2]. It was found that yeast lactic dehydrogenase in the presence of lactate would not reduce dinitrocresol to a brown compound, but in the

presence of the following indicators it was capable of doing so: pyocyanin ($E_0' - 0.034$ v. (p_H 7)), toxoflavin ($E_0' - 0.049$ v. (p_H 7); [Stern, 1935], β -anthraquinonesulphonate ($E_0' - 0.250$ v. (p_H 7)).

Fig. 3. Xanthine oxidase + dinitro-*o*-cresol, p_H 7.3, 30°. A. Hypoxanthine added. B. Benzyl viologen added. ——— Electrode potentials. - - - - Photo-electric potentials.

Enzyme: extracted from acetone-yeast by the method of Bernheim [1928]. The extract was dialysed against 0.15 *M* phosphate buffer p_H 7.3 to minimise volume increase. 0.5 ml. enzyme solution reduced 1 ml. 1/5000 methylene blue in presence of lactate in 14 mins. at 38°.

Solutions: phosphate buffer, p_H 7.3, containing enzyme; dinitro-*o*-cresol, 2.10^{-2} *M*; lithium *d*-lactate, *M*; pyocyanin (from *Ps. pyocyanea*), 2.10^{-3} *M*; toxoflavin (from *Bact. bongkokrek*), 2.10^{-3} *M*.

Temperature: 38°.

Tube	...	...	1	2	3	4	5
			ml.	ml.	ml.	ml.	ml.
Enzyme			2.0	2.0	2.0	2.0	2.0
Substrate			0.5	0.5	0.5	0.5	0.5
Dinitrocresol			0.2	—	0.2	—	0.2
Pyocyanin			—	0.3	0.3	—	—
Toxoflavin			—	—	—	0.5	0.5
Water			0.3	0.2	—	1.0	0.8
At beginning:			Lemon-yellow	Blue	Green	Yellow	Yellow
After 1 hour:			No change	Colourless	Orange	Colourless	Yellow
After 3 hours:			No change	Colourless	Brown-orange	Colourless	Orange

The reaction product in the case of toxoflavin as indicator gave a positive reaction for a 4-nitro-2-aminophenol.

We have found that yeast lactic dehydrogenase + lactate will not take up oxygen in the presence of varying concentrations of dinitro-*o*-cresol in the Warburg apparatus. De Meio and Barron [1934] have just reported similar results with the α -hydroxy-oxidase of gonococci. This is readily explained by the fact that nitrophenols cannot be reduced by the lactate system in the absence of carriers, apart from any considerations of reversibility.

(4) *Succinic dehydrogenase*. The normal potential of the succinate-enzyme-fumarate system has been found to be near 0 v. at p_H 7 [Lehmann, 1929; Borsook and Schott, 1931]. It could hardly be expected that such a relatively positive system would be able to reduce nitrophenols readily even in presence of indicators. It was indeed found that a strongly active succinic dehydrogenase solution prepared according to Lehmann's directions, 0.5 ml. of which decolorised 0.3 ml. 1/2000 methylene blue in 15 mins. at 38° , failed to reduce dinitro-*o*-cresol either alone or in presence of the dyestuffs brilliant cresyl blue or thionine, although these dyes were completely decolorised.

As with the lactic dehydrogenase system, we tested the capability of dinitro-cresol to provoke an oxygen uptake in the succinic dehydrogenase-succinate system. Washed chopped guinea-pig muscle, in the presence of cyanide to inhibit the normal oxygen utilisation, failed to take up oxygen in the presence of succinate when concentrations of dinitro-*o*-cresol varying between $2 \cdot 10^{-3}$ and $5 \cdot 10^{-7} M$ were added (Fig. 4).

Fig. 4. Washed chopped guinea-pig muscle.

1. Succinate.
2. Succinate + $5 \times 10^{-3} M$ KCN + $2 \times 10^{-3} M$ methylene blue.
3. Succinate + $5 \times 10^{-3} M$ KCN + $2 \times 10^{-4} M$ methylene blue
4. Succinate + $5 \times 10^{-3} M$ KCN + $10^{-4} M$ dinitrocresol.
5. Succinate + $5 \times 10^{-3} M$ KCN + $10^{-5} M$ dinitrocresol.
6. Succinate + $5 \times 10^{-3} M$ KCN.
7. Succinate + $5 \times 10^{-3} M$ KCN + $2 \times 10^{-3} M$ dinitrocresol.
8. Succinate + $5 \times 10^{-3} M$ KCN + $5 \times 10^{-7} M$ dinitrocresol.

(5) *Reduction by muscle*. Unwashed chopped rat muscle contains systems which reduce benzyl viologen. Dinitro-*o*-cresol is likewise reduced, and the rate

of reduction is increased in the presence of benzyl viologen. Washing the muscle decreases its capability of reducing dinitrocresol, but the rate of reduction is still increased by the addition of benzyl viologen. We have not yet ascertained what systems are responsible for these reductions.

TECHNIQUE.

Experiments with Thunberg tubes. The vacuum tubes used in these experiments were mostly of the type shown in Fig. 5. They incorporate Keilin's hollow stopper, and in addition they are provided with glass collars which, together with the side arms, are filled with liquid paraffin. Fig. 5 also shows the arrange-

Fig. 5.

ment used for alternately evacuating and refilling the tubes with nitrogen from a cylinder. The three-way tap *T* is first turned so that the Thunberg tube is connected with the water pump. When the liquid in the tube is boiling, the valve of the nitrogen cylinder is opened and the tap *T* is so turned that nitrogen passes into the Thunberg tube. The capillary constriction *C* prevents the rushing in of water from the safety device *S*. Evacuation and refilling with nitrogen are then repeated, and the tube is finally evacuated by leaving it in connection with the pump for 1 or 2 minutes.

Fig. 6. 1. Pre-heating tower. 2. Photo-electric cell. 3. Light filters. 4. Light source. 5. Siphon system. 6. Calomel cell. 7. Diaphragm.

Potentiometric and photometric measurements. The apparatus illustrated in Fig. 6 allows simultaneous measurements of potential changes and of changes

in light transmission to be made. The electrode vessel was fitted up in the manner described by Clark and Cohen [1923]. Two electrodes were used, one being of blank platinum foil and the other of the Westhaver type (platinum film on glass). Connection with the saturated calomel electrode was made by means of a syphon system [*cf.* Elema, 1931]. Before entering the titration vessel the gases passed through the pre-heating tower. Gas purification, temperature control and potential measurements were carried out as previously described [Stern, 1934, 1]. The electrode vessel was of square section with polished surfaces, the breadth being 4 cm. It was mounted on an optical bench between a 100-watt opal metal filament lamp and a photo-electric cell of the selenium type (Weston Photronic Cell). The light was filtered by means of a suitable combination of Schott coloured glass filters before it entered the titration vessel. The three filters used, each 1 mm. thick, were BG 17, 13, and 10. The photo-electric potentials, which varied between 1 and 14 mv., were measured with the same potentiometer and galvanometer as were used for the electrode potential measurements, the higher sensitivity range of the potentiometer being selected. The light source was switched on about 20 secs. before each reading. Stray light was eliminated by suitable housings for light source and photo-cells and by the provision of diaphragms.

DISCUSSION.

It is proposed first to discuss the question of the reversibility of the reduction of dinitrophenols. The reason for taking nitroaminophenol as the reductant in our potentiometric experiments was that the orange-red colour due to this compound begins to appear right from the beginning of reductive titrations. We have controlled this point by cautious titration of dinitrocresol with leucobenzyl viologen. The brownish colour appeared at once. Furthermore nitroaminophenol has been isolated as a product of the reduction of dinitrophenol by mild agents. Attainment of this stage, however, involves six hydrogen equivalents. No reversible system is known to be concerned with the exchange of more than two hydrogen equivalents in a single step. Since no typical reduction curves could be traced with dinitrocresol, no conclusions can be drawn from their slope as to the number of hydrogen equivalents involved.

Very probably all so-called irreversible systems possess a reversible phase in their oxidation or reduction as the case may be. The possibility of its detection is dependent upon the life time of the primary reaction product, which may usually be depicted as a radical liable to decomposition or secondary condensation. The refinement of methods in recent years has led to the demonstration of such reversible phases in systems hitherto regarded as irreversible. The rate of the disappearance of the radical determines the method to be chosen. Though the simple methods applied by us have failed, it may well be that by the application of such methods as the flow method [Ball *et al.*, 1933] or the method of discontinuous titration [Fieser, 1930, 1] a reversible stage may still be demonstrated in the reduction of dinitrophenols. From our data the possibility that dinitrophenol together with nitroaminophenol forms a sluggishly reversible system, comparable with the haemoglobin-methaemoglobin system, would appear to be ruled out.

For the time being Fieser [1930, 2] has recommended the method of potentiometric indicators for systems in which reversibility cannot yet be demonstrated. The critical oxidation potential introduced by this author is a unique function of the normal potential, by analogy with systems where both constants could be measured. Our experiments with potentiometric indicators suggest that the

critical reduction potential of dinitroresol is in the neighbourhood of -0.1 v. If it be assumed that there exists a reversible phase in the reduction of dinitroresol involving two hydrogen equivalents, then following the deductions of Fieser one would arrive at a normal potential near -0.2 v., which would be in the region of the so-called anaerobic reduction potential of cells as determined by various methods (for literature see Stern [1934, 2]).

The experiments with the enzyme systems tried show that dinitrophenols can be reduced by enzyme systems which cover a wide range of reduction intensity provided a suitable carrier be present. It is not impossible that the cell contains such a carrier [see however Green, Stickland and Tarr, 1934]. With xanthine oxidase no extraneous carrier was necessary. We do not know whether this system contains already an appropriate carrier or whether it does not need one [cf. Green and Dixon, 1934]. The reduction of dinitrophenols in this respect parallels that of nitrate which is reduced by xanthine oxidase in the absence of a carrier, whereas its reduction by Stickland's preparation demands the presence of an added indicator [Green, Stickland and Tarr, 1934].

Our experiments show that dinitrophenols are not available to the dehydrogenases tried, with the exception of the xanthine oxidase system. An apparent inconsistency in our results concerning the coupled reduction of dinitroresol demands comment. This compound is reduced in the presence of formic dehydrogenase-formate by an indicator, brilliant cresyl blue, which even in the 99 % reduced state does not possess a potential level low enough to cause an appreciable rate of reduction, as judged from our potentiometric indicator results. We would remark, however, that (1) the conditions in the potentiometric indicator and in the Thunberg tube experiments are not entirely comparable; (2) the potential of a fully reduced indicator is theoretically $-\infty$; (3) a leuco-dye formed by reduction on an enzyme surface is possibly in an "activated" state which may be very different from that of a dye reduced by non-enzymic agents.

We would emphasise the importance of arriving at an explanation of the action of nitrophenols on the living cell. In the stimulation which they produce, there is an overcoming of the processes which normally limit the respiration, and in some cases the glycolysis, of normal and tumour tissues. If the effect of the nitrophenols could be referred to their behaving as reversible redox systems, their action would be brought into line with that of reversible dyestuffs such as methylene blue, pyocyanin and thionine. If, on the other hand, the mechanism of their action were quite different, the possibility would arise that the reversible dyestuffs which stimulate the metabolism of tissue do so in virtue of some property not necessarily dependent on the reversibility of their reduction or the autoxidisability of their leuco-forms.

Whilst the work described in this paper was being completed, two communications appeared which give hypotheses for the mode of action of nitrophenols. De Meio and Barron [1934] state: "...the increase in respiration produced by dinitrophenol is not due to direct oxidation of the oxidisable substrates. It is suggested that dinitrophenol acts by combining with some of the substances acting as agents for the control of the speed of cellular oxidations, thus increasing the activity of the oxidising enzymes". On the other hand, according to Krahle and Clowes [1934], it is likely that dinitroresol "stimulates cellular respiration by accelerating the oxidation by cytochrome of some substrate previously or simultaneously acted upon by the anaerobic dehydrogenases of the cell. It is too early to say whether DNC acts as a diffusible oxygen carrier between cytochrome and the substances normally oxidised or as an artificial

substitute for a co-enzyme in the activation of substrates which do not normally play an important rôle in respiration”.

We do not propose to comment upon these views, save to remark that De Meio and Barron's most weighty evidence for their views, namely that dinitrophenol is without effect when the respiration of cells and tissues has been inhibited by cyanide or carbon monoxide, does not exclude the possibility that the nitrophenols are converted into reversible systems in the presence of actively functioning oxidases or other cyanide-sensitive systems of the cell.

This paper does not attempt to explain the mechanism of nitrophenol action. It can, however, be said that, if the action of the nitrophenols depends on their reduction, a way in which such reduction may occur in the cell has been indicated.

SUMMARY.

1. The reduction of nitrophenols by enzymic and non-enzymic means has been studied, in the hope of obtaining an insight into the action of these compounds on the cell.

2. No evidence has been obtained that the reduction of dinitrophenols by agents such as hydrosulphite or palladium-hydrogen is reversible. The possibility of detecting a reversibility by more refined means than those employed is discussed.

3. The approximate potential level at which the reduction of dinitro-*o*-cresol becomes apparent has been determined by the method of potentiometric indicators.

4. Dinitrophenols are reduced by xanthine oxidase, formic dehydrogenase and lactic dehydrogenase in the presence of the appropriate substrates. With the last two enzymes, a small quantity of a reversible redox system must be added before reduction occurs. The product of reduction of 2:4-dinitrophenol by formic dehydrogenase-formate is 4-nitro-2-aminophenol.

5. No theory for the action of nitrophenols on cellular respiration is advanced; but the significance of the results is discussed.

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