XXVI. NATURAL REVERSIBLE OXIDATION-REDUCTION SYSTEMS AS ACCESSORY CATALYSTS IN RESPIRATION: JUGLON AND LAWSON.

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(Received December 23rd, 1933.)

LAWSON, the colouring material contained in the leaves of the henna plant (*Lawsonia inermis*), and juglon, the colouring material contained in the husks of walnuts, both form leuco-compounds when exposed to the influence of various reducing agents. The leuco-compounds at once regenerate the pigment in contact with molecular oxygen. Thus they fulfil one of the main conditions which might enable them to play a part in cell-respiration by acting as autoxidisable acceptors of hydrogen.

This paper deals with the effect of juglon and lawson on cell-respiration, the establishment of the oxidation-reduction potentials of both substances and the correlation of the two series of results.

The comparison between the physicochemical and biological properties of the two substances appears all the more attractive in view of the fact that they are isomeric compounds, their formulae being 2-hydroxynaphthoquinone [Tommasi, 1920] for lawson, and 5-hydroxynaphthoquinone for juglon [Bernthsen and Semper, 1885].

A. Juglon.

Preparation. Juglon was obtained by extraction of the husks of walnuts by the method of Bernthsen and Semper and also synthetically by oxidation of 1:5-dihydroxynaphthalene with chromic-sulphuric acid mixture and subsequent crystallisation from light petroleum. The natural and synthetic products gave identical results in the physicochemical and biological experiments described below.

Potentiometric titration. In order to determine the oxidation-reduction potentials, 10 ml. of *M*/1000 solution in various mixtures of buffer substances were first reduced by hydrogen in the presence of colloidal palladium and subsequently titrated against benzoquinone in a current of purified nitrogen at a temperature of $20^{\circ} \pm 0.1^{\circ}$. The potentials were read against two polished platinum electrodes, using a saturated half-cell of calomel as standard. The potentials adjusted themselves rapidly and remained stable, and the readings for both electrodes differed by not more than 0.05 mv. during the middle phase of titration.

The potentials thus determined correspond to the formula of Nernst:

$$E = E_0 + \frac{RT}{n} \log \frac{(OX)}{(RED)}.$$

The system juglon-hydrojuglon can therefore be regarded as thermodynamically reversible (see Table I).

Table I. *Juglon*.

$p_H = 5.13$ (phosphate) reduced with $Pd-H_2$ titrated against quinone at $20.0^\circ \pm 0.05^\circ$.

Potentials calculated according to the formula $E = 0.1332 + 0.029 \log \frac{\% \text{ oxidation}}{100 - \% \text{ oxidation}}$.

All potentials referred to the standard hydrogen electrode at 20° .

Quinone ml.	Oxidation	Potential		Discrepancy
		Found	Calculated	
0.00	0.0	—	—	—
0.35	15.8	0.1062	0.1121	- 0.0059
0.50	22.6	0.1152	0.1177	- 0.0025
0.64	28.8	0.1206	0.1218	- 0.0012
0.80	36.1	0.1254	0.1260	- 0.0006
1.02	45.9	0.1308	0.1311	- 0.0003
1.26	56.7	0.1371	0.1366	+ 0.0005
1.58	71.2	0.1457	0.1446	+ 0.0011
1.76	79.3	0.1520	0.1501	+ 0.0019
2.02	91.0	0.1670*	0.1622	+ 0.0048
2.17	97.6	0.1866*	0.1799	+ 0.0067
2.22	100.0	0.2672*	—	—

* Potentials drifting.

The determination of n , the number of electrons which distinguish the reduced from the oxidised form. According to the chemical constitution of the substance, n must be 2. This statement is corroborated by the oxidation equivalent found by titration, as well as by the slope of the titration curve.

(a) Hydrojuglon uses up equimolecular amounts of benzoquinone during its oxidation.

Exp. Juglon mol. wt.: 174.

Standard solution: 43.557 mg. in 50 ml. ethyl alcohol.

1 ml. corresponds to $0.5 \cdot 10^{-5} M$.

Benzoquinone mol. wt.: 108.

Titration solution: 44.16 mg. in 100 ml. dist. H_2O .

1 ml. corresponds to $4.083 \cdot 10^{-5} M$.

2 ml. of the standard solution of juglon reduced with $Pd-H_2$ in 8 ml. of acetate buffer solution of $p_H = 4.62$, require 2.44 ml. of titrating solution for oxidation in a current of nitrogen (the complete oxidation being indicated by a jump of potential).

$1.0 \cdot 10^{-5} M$ juglon is equivalent to $\frac{2.44 \times 4.083}{100} = 0.996 \cdot 10^{-5} M$ benzoquinone.

(b) At acid reactions, the titration curves clearly show a 30 mv. slope, corresponding to $n=2$. When the p_H reaches 7, the curves become steeper, without reaching however a slope of 60 mv. This irregularity, which points towards a formation of semiquinone or meroquinone, will not be further discussed here, considering the instability of juglon in alkaline media.

The standard potential E_0 (determined in a mixture of equal parts of the oxidised and reduced forms of the substance) is shown in Fig. 1 and Table II as a function of the p_H . According to Clark and Gibbs, the turning-points of the E_0/p_H curve can be interpreted as representing constants of dissociation (see Table V).

B. *Lawson*.

The pure substance was obtained by extraction from henna leaves by the method of Tommasi; the calcium salt was crystallised and the free hydroxyquinone recrystallised from ether. Electrometric titration by means of the method

Table II.

The normal potential of juglon (mixture of equal parts of the oxidised and reduced form) at varying p_H . Potentials referred to the standard hydrogen electrode at 20°.

p_H	Buffer	E_0	Titrated against
1.994	Citrate	+0.311	Quinone
3.263	"	+0.239	K_3FeCy_6
4.746	Acetate	+0.154	Quinone
5.133	Phosphate I and II	+0.133	"
5.707	"	+0.103	"
6.263	"	+0.071	"
6.799	"	+0.041	"
7.316	"	+0.023	"
8.001	"	0.000	"
8.389	"	-0.023	"
9.401	II Phosphate and NaOH	-0.071	"
10.785	"	-0.131	"
11.41	"	-0.160	$K_2S_2O_8$
11.929	"	-0.175	Quinone
12.10	"	-0.185	$K_2S_2O_8$

Table III. *Lawson*.

$p_H = 2.24$ (citrate + HCl) reduced with $Pd-H_2$ titrated against quinone at $20.0^\circ \pm 0.05^\circ$.

Potentials calculated according to the formula $E = 0.2198 + 0.029 \log \frac{\% \text{ oxidation}}{100 - \% \text{ oxidation}}$.

All potentials referred to the standard hydrogen electrode at 20°.

Quinone ml.	Oxidation %	Potential		Discrepancy
		Found	Calculated	
0.00	0.0	—	—	—
0.14	7.2	0.1828*	0.1870	-0.0042
0.24	12.4	0.1929	0.1947	-0.0018
0.44	22.9	0.2036	0.2040	-0.0004
0.66	33.8	0.2107	0.2108	-0.0001
0.86	44.3	0.2162	0.2163	-0.0001
1.08	55.6	0.2222	0.2221	+0.0001
1.34	69.0	0.2295	0.2293	+0.0002
1.56	80.6	0.2379	0.2372	+0.0007
1.73	89.0	0.2470	0.2455	+0.0015
1.88	96.8	0.2692*	0.2615	—
1.91	98.0	0.2912*	0.2682	—
1.94	100.0	0.3500*	—	—

* Potentials drifting.

described in the case of juglon shows that we are dealing again with a true reversible system. All the titration curves show a slope of 30 mv., corresponding to $n=2$. An example is recorded in Table III. The dependence of the standard potential E_0 is shown in Table IV and Fig. 1, and the values for the dissociation constants which can be derived from the former are shown in Table V.

C. A comparison between the various constants of juglon and lawson.

The 2-hydroxynaphthoquinone, lawson, is more acid and more "negative" than juglon. As compared with juglon the acid character of lawson is enhanced, not only in the phenolic hydroxyl of the oxidised form, but also in the hydroxyl of the reduced form. In the case of lawson, the first dissociation constant of the reduced form is so close to the constant of the oxidised form that the

Table IV.

The normal potential of lawson (mixture of equal parts of the oxidised and reduced form) at varying p_H . Potentials referred to the standard hydrogen electrode at 20°.

p_H	Buffer	E_0	Titrated against
0.360	N HCl + N/10 KCl	+0.337	K_2FeCy_6
1.322	Citrate	+0.2757	Quinone
1.423	"	+0.270	"
2.245	"	+0.220	"
2.460	"	+0.2087	"
3.248	"	+0.1635	"
3.680	"	+0.1298	"
3.881	"	+0.1205	"
4.280	"	+0.0862	"
4.620	Acetate	+0.061	"
4.945	"	+0.032	"
6.194	Phosphate I and II	-0.071	K_2FeCy_6
6.641	"	-0.1105	Quinone
7.180	"	-0.154	"
7.560	"	-0.186	K_2FeCy_6
8.718	Veronal	-0.281	Quinone
9.180	"	-0.318	$K_2S_2O_8$
10.075	II Phosphate and NaOH	-0.3648	Quinone
10.510	"	-0.395	$K_2S_2O_8$
11.34	"	-0.428	"
12.23	"	-0.461	"

Fig. 1. Normal potentials of juglon and lawson at varied p_H . Potentials referred to the standard hydrogen electrode at 20°.

Table V.

	Juglon	Lawson
Dissociation constant of the oxidised form (of the phenolic hydroxyl)	K 10^{-8}	K $1.4 \cdot 10^{-4}$
1st dissociation constant of the reduced form	$2.5 \cdot 10^{-7}$	About $1.4 \cdot 10^{-4}$
2nd dissociation constant of the reduced form	$2.5 \cdot 10^{-11}$	$2.4 \cdot 10^{-10}$

Temperature: 20°.

two cannot be sharply separated. The flattening effect of the pk_r combines with the steepening effect of pk_0 to give to the E_0/p_H curve a slope of 84 mv.

Conant and Fieser [1924] have found that the introduction of the hydroxyl group into the quinoid nucleus of α -naphthoquinone lowers the oxidation-reduction potential much more markedly than its introduction into the adjoining benzene ring. Their results support our findings with regard to the increased negativity of lawson as compared with juglon. These authors have determined the oxidation-reduction potentials of the two substances exclusively in strong alcoholic-hydrochloric acid solution. On account of the difference of the solvents the values obtained by them cannot be directly compared with those recorded in this paper.

The relative positions of juglon and lawson in the scale of oxidation-reduction indicators can be seen in Table VI.

Table VI. *Potentials of several oxidation-reduction systems in semi-reduced condition at $p_H = 7.0$.*

(Potentials referred to the normal hydrogen electrode.)

Haemoglobin-	+0.092	Conant und Fieser [1925]
methaemoglobin	± 0.022	
Pigment of <i>Penicillium phoeniceum</i>	+0.047	Friedheim [1933, 3]
Juglon	+0.033	
Pigment of <i>Arion rufus</i>	+0.025	Friedheim [1933, 1]
Hallachrome	+0.022	Friedheim [1933, 2]
Methylene blue	+0.011	
Pyocyanine	-0.033	Friedheim and Michaelis [1931]
Indigotetrasulphonate	-0.046	Clark, cited from Michaelis [1933]
Indigodisulphonate	-0.125	
Lawson	-0.139	
Echinochrome	-0.220	Cannan [1927]
β -Anthraquinon-sulphonate	-0.260	

D. *Juglon and lawson as natural oxidation-reduction indicators.*

In the husks of walnuts, juglon is generally found in the reduced form, lawson on the other hand exists in henna leaves in the oxidised state. If we consider the two pigments as natural intracellular oxidation-reduction indicators, we can infer that the oxidation-reduction potential of the plant-cells under consideration is more negative than the standard potential of juglon and more positive than the standard potential of lawson.

At $p_H = 7.0$ this statement may be expressed as:

$$+0.033 > E_h > -0.139$$

juglon cell lawson

or in terms of r_H

$$15.1 > r_H \text{ cell} > 9.2^1.$$

¹ Haas and Hill [1925] have described a plant pigment, hermidin, which has been shown by Cannan [1926] to behave like a reversible oxidation-reduction system. Calculated from the data given by Cannan, the standard potential would assume at $p_H 7$ a value of $E_0 - 0.033$ and $r_H 12.7$. These figures fall well within the limits given above. It may be noted that the standard potential of hermidin coincides exactly with that of pyocyanine, the blue pigment of *Bacillus pyocyaneus*.

These limits, although far apart, are important because they hold good under physiological conditions, *e.g.* in air. They refer to the actual equilibrium between processes of oxidation and reduction in the living cell. This equilibrium is the result not only of thermodynamic but also of kinetic conditions, *i.e.* of diffusion and reaction velocities.

We owe our present knowledge of intracellular oxidation-reduction potentials up to date to the works of Needham and Needham [1925], Cohen *et al.* [1928], Wurmser and Rapkin [1931], *etc.* who have used the method of direct introduction of indicators into the living cell. It is important to supplement this method by means of direct observation of natural intracellular oxidation-reduction indicators, as the latter method eliminates possible sources of error such as toxicity of the indicator, trauma of injection *etc.*¹

E. *The influence of juglon and lawson on the respiration of red corpuscles (rabbits).*

The work of Barron and Harrop [1928], as well as that of Michaelis and Salomon [1929–30], goes to prove that reversible oxidation-reduction systems are capable of increasing the respiration of red corpuscles, if non-toxic, able to permeate and possessing a normal potential more positive than that of indigo-disulphonate. Juglon and lawson, which fulfil these conditions, both behave in accordance with the above-mentioned rule: under their influence the respiration of the erythrocytes of rabbits is several times increased (see Fig. 2).

Fig. 2. The action of juglon and lawson on the respiration of red blood cells (rabbit).

Phosphate-Ringer p_H 7.2.
 Phosphate concentration $M/75$.
 0.2 % glucose.
 Absorption of CO_2 by 5 % NaOH.
 37° air.

At ↓ the dye is added from the side-bulb. Final dye concentration $M/1000$.

In our experiments juglon increases the respiration 5 times, and lawson 5.8 times. The effect of both these isomeric bodies is of the same numerical order, which however is difficult to reproduce exactly. It is independent of the nature of the medium (0.9 % NaCl, carbonate- or phosphate-Ringer) provided glucose is present.

¹ The conception of "intracellular" is as undefined as an address "London". In the various regions of the cell can be found different oxidation-reduction potentials (*e.g.* granula of oxidase and the "Sauerstofforte" of *Unna*). As fresh material is not available at present a more precise localisation of juglon and lawson will be attempted later on.

F. *The mechanism of increase of respiration in red corpuscles by reversible oxidation-reduction systems.*

Warburg *et al.* [1930] and Wendel [1929-30] have discovered the following mechanism for the increase of respiration of red corpuscles in the presence of methylene blue:

1. Methylene blue + haemoglobin + H_2O = methaemoglobin + leuco-methylene blue.
2. Methaemoglobin + carbohydrate = haemoglobin + oxidation products of carbohydrate.
3. Leuco-methylene blue + $1/2 O_2$ = methylene blue + H_2O .

Michaelis and Salomon [1929-30] have further confirmed this theory for a number of reversible dyes and have brought it into correlation with the normal potentials of these dyes. The potential interval between indigodisulphonate and indigotetrasulphonate was defined as the critical level, on the positive side of which the increase in respiration runs approximately parallel with the formation of methaemoglobin. On the negative side, dyes are known to exist which can form methaemoglobin (indigodisulphonate, β -anthraquinonesulphonate, safranine) without exercising any influence on the respiration. This latter circumstance strengthens the view according to which the formation of methaemoglobin need not necessarily represent the crucial process in the increase of respiration by quinoid dyes.

Let us consider the conditions prevailing in the case of our hydroxynaphthoquinones: lawson and juglon both increase the respiration of the red corpuscles to considerable and approximately equal extents. But whereas the positive juglon, the normal potential of which is almost identical with that of methylene blue, rapidly oxidises haemoglobin to methaemoglobin, lawson, which is only a few mv. more negative than indigodisulphonate (at p_H 7) forms no methaemoglobin under conditions where an increase of respiration takes place. This has been ascertained from spectroscopic observation as well as from the determination of the oxygen capacity.

Table VII. *Oxygen capacity of one ml. of a suspension of red blood corpuscles (rabbits)*.*

Temperature		mm. ³ O ₂
I. 25°	Control	61.5
	40 mins. after addition of $M/1000$ lawson (duplicate experiment)	(a) 61.7
	Spectroscopically: no methaemoglobin	(b) 62.0
II. 37°	Control	91.9
	40 mins. after addition of $M/1000$ lawson	91.6
	Spectroscopically: no methaemoglobin	
	40 mins. after addition of $M/1000$ juglon	25.5
	Typical methaemoglobin bands at 635, shifted towards 610 under the influence of NaF	=28 %

* Method of Warburg *et al.* [1931].

Certain quinoid dyestuffs produce methaemoglobin without provoking any increase of respiration, whereas others, such as lawson, increase respiration without producing methaemoglobin. Thus the catalytic action of quinoid dyes on the respiration of red corpuscles is not indissolubly linked up with the action of the haemoglobin-iron.

The limits of potential of the juglon-hydrojuglon system overlap widely with those of the haemoglobin-methaemoglobin system. It is therefore possible for juglon to oxidise haemoglobin. At p_H 7.0 juglon, oxidised to approx. 99 % ($E_h = +0.093$), forms a thermodynamical equilibrium with haemoglobin oxidised to about 50 % ($E_h = +0.092 \pm 0.022$). On the other hand, lawson, oxidised to the extent of 99 % ($E_h = -0.079$) does not come within the range of the potential of a haemoglobin-methaemoglobin system reduced to the extent of 99 % ($E_h = -0.028 \pm 0.022$). The formation of methaemoglobin in the presence of lawson appears therefore rather improbable.

With regard to the formation of methaemoglobin by means of quinoid dyes, one should remember that the thermodynamical possibility, as expressed in terms of the standard potentials, is no guarantee that the reaction actually takes place.

β -Antraquinonesulphonate oxidises haemoglobin: the more positive lawson ought to have at least a similar effect. But "kinetic factors outweigh thermodynamic factors in all reactions concerning haemoglobin" (Michaelis). Even after ten hours of exposure of red corpuscles to the action of lawson, no formation of methaemoglobin could be observed. Neither would a belated appearance of methaemoglobin be of any significance with regard to the mechanism of increase of respiration, as our experiments have shown that the latter itself appears immediately when lawson has been brought into contact with red corpuscles¹.

¹ I have described a number of natural reversible oxidation-reduction systems as "accessory respiratory enzymes." Stern [1933] questions the advisability of applying the term "enzyme" to the cases where methaemoglobin appears during the reaction. Whether or not these systems should be called enzymes depends entirely on the accepted definition.

Kuhn's definition in the work on ferments by Oppenheimer that "ferments are catalysts manufactured by the cell," holds good for the chemical bodies with which we are concerned, including juglon and lawson. If one accepts on the other hand Haldane's restriction of the definition to the effect that "ferments are colloidal catalysts," then these crystalline substances cannot be regarded as ferments.

By no means can we consider the nature of intermediate products, e.g. the possible formation of methaemoglobin, as an argument either for or against the enzymic character of the reaction.

As in this paper the formation of methaemoglobin has been shown to be irrelevant for the enhancing effect of reversible oxidation-reduction systems on respiration of red blood cells, Stern's discussion appears all the more beside the point.

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