CXIV. ELECTRODE POTENTIALS OF HERMIDIN, THE CHROMOGEN OF *MERCURIALIS PERENNIS*.

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(Received July 21st, 1926.)

HAAS and HILL [1925, 1] have recently directed attention to a chromogen of considerable interest which escapes from the plasmolysed cells of *Mercurialis perennis*. If the coarsely divided tissue be steeped, anaerobically, in chloroform water for a short time, there results an extract which, on exposure to oxygen, assumes first a rich blue and then a yellow-brown colour. These investigators [1925, 2] have further shown that, within the experimental limits imposed by the difficulty of arresting the oxidation exactly at the stage of maximum intensity of blue colour, the two successive reactions are attended by an equal absorption of oxygen. The oxidation to the blue substance (cyanohermidin) was demonstrated to be readily reversible and Haas and Hill [1926] were further persuaded that the second stage of oxidation is also, at least partially, reversible, although they were unable quantitatively or consistently to effect the reduction of the yellow compound (chrysohermidin) through the blue to the chromogen. The data presented in the papers cited suggested that the systems involved might be amenable to electrometric study and the present paper reports the measure of success attained.

The application of electrometric methods to organic systems has been extensively discussed in its theoretical aspects by Clark and Cohen [1923, 1]. The general relations may be summarised in a form of the Nernst electrode equation

$$E_h = E'_0 - \frac{RT}{nF} \log \frac{[S_r]}{[S_o]}$$

where E_h represents the observed potential compared with that of the normal hydrogen electrode, E'_0 is a constant, n is the number of equivalents of hydrogen involved in the reaction and $[S_r]$ and $[S_o]$ are the respective concentrations of the reductant and the oxidant which are in equilibrium at the observed potential. If, by reason of acid-base equilibria, the concentrations of the *actual* oxidant and reductant are not identical with the total concentrations of the reactants, the simple equation must be modified to embrace terms involving the $[H']$ and the various dissociation constants effective over the range of p_H investigated. E'_0 then becomes a function of p_H and the

equilibrium constants. In order to establish fully the equilibrium states in a reversible oxidation-reduction it is, therefore, necessary to determine first the equilibrium potentials at constant $[H']$ and then to examine the variation of these with p_H .

No attempt has been made to prepare the pigment in the pure state. The orderly character of the results obtained with crude extracts of the plant would seem to indicate that the impurities present were not such as to interfere seriously with the equilibria established at the electrode by hermidin and its oxidation products. When the pure substance becomes available it may be found that there is a displacement of the systems on the oxidation-reduction potential scale, but it is improbable that the main implications of the present work will thereby require modification. On the other hand, the full interpretation of the electrometric data must, perforce, await the elucidation of the structure of the pigment.

Titration curve at constant $[H']$.

The green shoots or, in some of the work, the colourless rhizomes were cut into small pieces and extracted under nitrogen with enough water to cover them, together with a little ether or chloroform. After an hour the extract was removed, filtered and stored under nitrogen. The nitrogen for this and all operations was purified of all trace of oxygen by passage over heated copper and conducted through copper or glass tubing, rubber connections being avoided. The electrode vessel was of the type employed by Clark and Cohen [1923, 2] carrying two gold-plated platinum electrodes to allow duplicate readings of potential. Contact was made with a saturated calomel electrode by means of a saturated potassium chloride bridge in the usual way and the whole cell was held in a thermostat at $30 \pm 0.1^\circ$. 40 cc. of $M/20$ buffer solution (Clark) of the desired p_H were introduced into the vessel and de-aerated with a stream of nitrogen, and 10 cc. of the plant extract were then added from a burette without contact with the air. The gas stream was continued throughout the titration and gave efficient stirring. Preliminary work indicated that the extracts were of the order of $M/100$ with respect to the chromogen and, in consequence, the titrating solutions were made of similar strength. In order that the $[H']$ should be maintained as constant as possible during the progress of the titration, the titrating solutions were made up by diluting a $M/20$ solution in water with four volumes of the buffer solution to which the plant extract had been added. These solutions were de-aerated before use and held in a protected burette under nitrogen. Experience indicated that, despite all precautions, the hermidin was 5 to 10 % in the oxidised form by the time the extracts had been introduced into the electrode vessel. It was therefore customary, before titrating with an oxidising agent, to make a preliminary titration with sodium hydrosulphite until the electrode indicated complete reduction. This permitted the complete curve to be traced by the subsequent oxidation.

THE SYSTEM HERMIDIN-CYANOHERMIDIN.

Studies of the first stage in the oxidation of hermidin gave very satisfactory results. On any one plant extract the curves traced by titrating the chromogen with benzoquinone, potassium ferricyanide or oxygen, or by reducing the blue compound with sodium hydrosulphite were, within the experimental error, identical.

Independent electrodes came rapidly into agreement at each step in the titration and the equilibrium potentials were remarkably constant. With extracts prepared on different days the value of E'_0 was found to vary, the whole curve being correspondingly displaced. In the worst case this discrepancy reached a value of 0.01v. For the explanation of this disconcerting fact we may have to await the preparation of the pure pigment.

The curve in Fig. 1 has been constructed from data abbreviated in Table I and represents a typical titration curve. Having made a graphic approximation of the end-point, the value of E'_0 is disclosed since this is equal to the potential at the mid-point of the curve, and it remains to calculate n and thence the theoretical potentials corresponding to the experimental values of $\frac{[S_r]}{[S_0]}$. The electrode equation may be written in the form

$$n = \frac{0.0601}{E'_0 - E_h} \log \frac{[S_r]}{[S_0]}$$

in which the value of RT/F at 30° has been substituted. In Table II this equation has been solved for a number of observed potentials on the flat portion of the curve. Since n is a whole number it is obvious that its value is 2. This reaction, in common with the majority of reversible organic oxidation-reductions is thus seen to be one involving the loss or gain of an electron pair, *i.e.* two equivalents of hydrogen.

A comparison of the observed potentials with those calculated from the electrode equation is made in Table I. It will be seen that the deviations exceed a few tenths of a millivolt only towards the end of the titration where we have reason to suspect interference in the equilibria due to the production of chrysohermidin (the yellow oxidation product).

Table I. *Titration of hermidin with M/150 benzoquinone.*

p_H 3.99	Temp. 30°	$E_0' + 0.1266$		
		E_h volts		
Quinone cc.	Percentage oxidation	found	calculated	Deviation volts
0.80	8.25	+ 0.0956	+ 0.0952	+ 0.0004
1.40	14.43	0.1030	0.1034	- 0.0004
2.40	24.74	0.1121	0.1121	—
3.40	35.05	0.1187	0.1186	+ 0.0001
4.40	45.36	0.1241	0.1242	- 0.0001
5.40	55.67	0.1298	0.1296	+ 0.0002
6.90	71.12	0.1386	0.1384	+ 0.0002
7.90	81.43	0.1459	0.1459	—
8.90	91.75	0.1571	0.1580	- 0.0009
9.10	93.80	0.1585	0.1610	- 0.0025
9.47	97.61	0.1656	0.1750	- 0.0094
9.70 (end-point)

Fig. 1 (cf. Table I). Titration of hermidin with $M/150$ benzoquinone at p_H 3.99.

Table II (cf. Table I). *The value of n .*

$E'_0 - E_h$	Percentage oxidation	$0.0601 \log \frac{[S_r]}{[S_o]}$	n
+0.0236	14.43	+0.0465	1.97
+0.0079	35.05	+0.0161	2.04
+0.0025	45.36	+0.0049	1.94
-0.0032	55.67	-0.0059	1.86
-0.0120	71.12	-0.0235	1.96
-0.0305	91.75	-0.0629	2.06
			Mean 1.97

The relation of E'_0 to p_H .

This relation was investigated by determining the equilibrium potentials of 5 cc. of a mixture of hermidin and cyanohermidin—approximately equimolecular—added to 20 cc. of a series of $M/20$ buffer solutions with the usual precautions against intrusion of oxygen. One of the buffer solutions chosen was that employed in determining the titration curve. In this way a constant correction was derived to bring the potential of the mixture, for which the exact value of the ratio $\frac{[S_r]}{[S_o]}$ was unknown, to that of a mixture whose ratio was unity. This is, of course, the value E'_0 . Owing to the unknown buffering power of the plant extract the p_H of the buffer-pigment mixture might differ appreciably from that of a buffer diluted with an amount of water equal to the volume of extract used. To overcome this difficulty advantage was taken of the irreversibility of the oxidation to chrysohermidin in acid solution (see later). A sample of the extract (which has an acid reaction) was allowed to oxidise in the air and was warmed to complete the formation of chrysohermidin. 5 cc. of this solution were added to 20 cc. of each buffer solution and the $[H^+]$ determined by the hydrogen electrode. The results are assembled in Table III and Fig. 2. The reader is again reminded that the value of E'_0

at a given $[H^+]$ was reproducible only with a single sample of plant extract. For this reason the actual values reported may be taken to be accurate only for the material actually used in the experiments quoted. The data of Tables I-IV refer to a single extract.

Table III. *Hermidin-cyanohermidin. Relation of E_0' to p_H .*

The calculated values of E_0' have been derived from the equation

$$E_0' = E_0 + 0.03006 \log [K_2 (H^+)^2 + (H^+)^3] - 0.03006 \log [K_0 + (H^+)].$$

E_0 has been given the value $+0.3665$ and the hypothetical constants K_0 and K_2 the values $K_0 = 1.0 \times 10^{-8}$ and $K_2 = 5.0 \times 10^{-7}$.

p_H	Buffer	E_0'		Deviation
		found	calc.	
2.0	Phthalate	+0.2460	+0.2462	-0.0002
2.45	"	0.2185	0.2192	-0.0007
3.08	"	0.1817	0.1814	+0.0003
3.47	"	0.1579	0.1579	—
3.99	"	0.1266	0.1266	—
4.54	"	0.0931	0.0939	-0.0008
5.30	"	0.0490	0.0490	—
5.64	Phosphate	0.0295	0.0298	-0.0003
6.08	"	+0.0071	+0.0067	+0.0004
6.57	"	-0.0156	-0.0153	-0.0003
6.89	"	-0.0286	-0.0279	-0.0007
7.33	"	-0.0446	-0.0445	-0.0001
7.61	"	-0.0555	-0.0555	—
7.91	"	-0.0679	-0.0681	+0.0002
8.16	Borate	-0.0788	-0.0797	+0.0009
8.86	"	-0.1158	-0.1169	+0.0011

Fig. 2 (cf. Table III). $E_0' : p_H$ curves. Potentials of 50 % mixtures of oxidant and reductant of hermidin and certain other systems [Clark, 1925] at various $[H^+]$. A, hermidin-cyanohermidin; I, indigo white—indigo carmine; M, methylene white—methylene blue; Q, quinhydrone; H, hydrogen electrode.

The interpretation of the $E'_0 : p_H$ curve is seriously hampered by our ignorance of the structures participating in the equilibria. From p_H 2 to about 6 the slope of the curve indicates an increasing negative potential of 0.06v. per unit of p_H . The curve here changes towards a "0.03 slope" but reverts, about p_H 8, to the original direction. Probably the values in the alkaline range are uncertain as borate buffers alone were available and these have been found to give anomalous results in other cases. Alkaline to p_H 9 the potentials became subject to uncertain drifts which made precarious the continuation of the curve beyond p_H 9. The intersections of the two slopes are indicative of acid-base dissociations at the corresponding $[H']$, but nothing can be said of their nature. A variety of electrode equations may be derived, involving a variety of equilibrium constants, and would equally well satisfy the observed results. One has been chosen in Table III solely to provide a means of indicating the general symmetry of the results, and it is not intended that the assumptions upon which the equation is based should be taken to indicate correctly the acid-base character of the molecule. It is assumed that the inflexion in the more acid region is due to the destruction of an anion by the oxidation (K_2). The second inflexion is attributed to an acid group common to both oxidant and reductant but differing in strength in the two reactants. In the oxidant its constant is K_0 whilst in the reductant it is too weak to have influence within the experimental range of $[H']$. These assumptions lead to the equation in Table III. The agreement between observed and calculated values of E'_0 is within the accuracy of the $[H']$ determinations.

THE SYSTEM HERMIDIN-CYANOHERMIDIN-CHRYSOHERMIDIN.

When the titration was continued beyond the end-point indicated in Fig. 1, a further region of poised potentials was encountered corresponding to the second stage in the oxidation of hermidin. In general, however, no equilibrium potentials could be determined as persistent and rapid drifts were found. However, in the region of p_H 7 to 8 the drifts were slow and it was possible to trace very satisfactory curves by reading the potential as soon as the electrode had made its initial rapid adjustment to each addition of titrating agent and so pursuing the titration as rapidly as possible. The lower curve in Fig. 3 and the data of Table IV record such a titration.

The complete curve should be the resultant of the curves given by

$$E_n = E'_0{}_1 + 0.03006 \log \frac{\alpha_1}{1-\alpha_1} = E'_0{}_2 + 0.03006 \log \frac{\alpha_2}{1-\alpha_2}$$

where $_1$ and $_2$ refer respectively to the first and the second stages in the oxidation and α is the fraction of the system which is present in the oxidised form. Having made a graphic approximation to the values of the end-point of the whole reaction and to $E'_0{}_1$ and $E'_0{}_2$, the equations may be solved for α_1 and α_2 . The sum $100 \left(\frac{\alpha_1 + \alpha_2}{2} \right)$ is the percentage oxidation at any point in the titration and this value may be compared with that given by the titre and the end-

point. In Table IV the values of $E_{0,1}'$ and $E_{0,2}'$ have been subjected to minor adjustment to improve the agreement between the equations and the observed results.

Table IV (cf. Fig. 3). *Titration of hermidin with M/100 benzoquinone at p_H 7.61.*

Quinone cc.	E_h volts	α_1	α_2	Percentage total oxidation		Deviation
				calc.	found	
0.70	-0.0814	0.1225	0.0006	6.16	6.08	-0.08
1.00	-0.0763	0.1712	0.0009	8.61	8.69	+0.08
1.40	-0.0704	0.2448	0.0014	12.31	12.17	-0.14
1.80	-0.0659	0.3140	0.0019	15.80	15.45	-0.35
2.40	-0.0600	0.4183	0.0030	21.07	20.86	-0.21
3.00	-0.0548	0.5171	0.0043	26.07	26.08	+0.01
4.20	-0.0442	0.7068	0.0099	35.84	36.52	+0.68
4.60	-0.0392	0.7798	0.0145	39.72	40.00	+0.28
5.10	-0.0330	0.8506	0.0233	43.70	44.35	+0.65
5.45	-0.0263	0.9047	0.0382	47.15	47.39	+0.24
5.65	-0.0220	0.9299	0.0525	49.12	49.13	+0.01
5.90	-0.0138	0.9620	0.0939	52.80	51.30	-1.50
6.30	-0.0092	0.973	0.1285	55.08	54.78	-0.30
6.80	-0.0019	0.984	0.2046	59.43	59.13	-0.30
7.30	+0.0040	0.989	0.2883	63.85	63.48	-0.37
7.90	-0.0092	0.993	0.3763	68.46	68.70	+0.24
8.60	-0.0159	0.996	0.4904	74.89	74.78	-0.11
9.10	-0.0197	0.998	0.5741	78.60	79.13	+0.53
9.55	-0.0239	0.999	0.6504	82.47	83.04	+0.57
10.40	-0.0339	1.000	0.8000	90.00	90.43	+0.43
11.05	-0.0480	1.000	0.9212	96.06	96.10	+0.04
11.20	-0.0515	1.000	0.9380	96.90	97.40	+0.50
11.50 (end-point)

Fig. 3 (cf. Table IV). Lower curve. Titration curve of hermidin with M/100 benzoquinone at p_H 7.61. Upper curve. Change of potential with time in hermidin solution buffered at p_H 7.00 when submitted to stream of oxygen diluted with nitrogen to 2 %.

The drifts in potentials corresponding to the second stage in the oxidation of hermidin became accelerated with increasing $[H^+]$. From a survey of the electrode behaviour of this system and the fact that these drifts were consistently towards more negative values such as would result from the progressive increase in the ratio $\frac{[S_r]}{[S_0]}$ by loss of oxidant in a secondary irreversible change, the suggestion is made tentatively that the true oxidant of cyanohermidin is subject to such an irreversible change whose velocity is a function of $[H^+]$. This change does not appear to be oxidative, but there is nothing further to indicate its character. The series of reactions may, therefore, be summarised in the form

The curve expressing the relation of the ratio of the reactants in a reversible oxidation-reduction to the electrode potential has been compared with an acid-base titration curve. In hermidin under restricted conditions of $[H^+]$ we have the first recorded example of an oxidation-reduction comparable with a system exhibiting two acid-base dissociations.

The rate of oxidation by molecular oxygen.

The writer has made a number of observations on the changes in potential of an inert electrode in solutions of the leuco-compounds of electromotively active dyes—*e.g.* methylene blue, phenol-indophenols—when submitted to a controlled stream of oxygen. If the potentials observed be plotted against time from the initiation of the stream of oxygen there results a curve which, over the significant portion of its course, is a replica of the titration curve at the same p_H . The possibility of the electrode behaving as an oxygen electrode does not, therefore, appear to arise and the conclusion must be that, under the particular experimental conditions, the rate of oxidation of the reduced dye is constant throughout the reaction. If this be general, the method offers a simple form of exploratory titration under conditions—*e.g.* presence of oxidisable impurities—where the employment of a more “active” oxidising agent would give uncertain results. The method is, however, limited to those systems and to those ranges of $[H^+]$ within which the rate of oxidation of the given compound proceeds at a measurable rate. If the rate be too slow the procedure is inconvenient, and if too rapid the inherent lag in the response of the electrode to the constantly changing equilibrium becomes a serious factor which, moreover, will be reflected unevenly at different stages in the reaction. Results by the oxygen method must, however, be suspect unless controlled by other more conventional methods. In the present case this control is not wanting and it would seem, therefore, that the ordered character of the deductions respecting the rates of oxidation is significant.

The upper curve in Fig. 3 represents the results obtained in an experiment

differing from the quinone titration only in that oxidation was effected by a rapid stream of nitrogen containing about 2 % of oxygen. The curve makes sufficiently evident what a detailed analysis of the data fully confirms, that the amount of reductant oxidised per minute is constant throughout the reaction. In Table V are summarised a series of such "oxygen titrations." The first point of interest is that the rates of oxidation of the two stages of oxidation are almost identical in every case. Now the experimental conditions are highly limited and this result might be held to have no general significance had it not been found that other pigments studied under identical conditions were oxidised at widely differing rates. It is suggested that the results observed indicate that the two stages in the oxidation involve very similar chemical groups. In the second place it seems from a comparison of the rates of oxidation at different $[H']$ that the former vary inversely as the square root of the $[H']$.

Table V. *Effect of $[H']$ on the rate of oxidation of hermidin and of cyanohermidin by a stream of oxygen diluted with nitrogen to 2 %.*

From the oxygen "titration curve" the value of the end-point in minutes and of $E_0'_1$ and $E_0'_2$ were determined graphically. With these the theoretical curves were superimposed upon the composite curve.

p_H	c_H	Span of curve		Rate % per min.		$k = v\sqrt{[H+]}$	
		1	2	1	2	1	2
6.59	2.55×10^{-8}	80 min.	75 min.	1.25 %	1.33 %	6.3×10^{-4}	6.7×10^{-4}
7.00	1.0×10^{-8}	51 "	49 "	1.96	2.04	6.2×10^{-4}	6.5×10^{-4}
7.42	0.38×10^{-8}	30 "	29 "	3.33	3.45	6.5×10^{-4}	6.7×10^{-4}
7.81	0.16×10^{-8}	20 "	20 "	5.00	5.00	6.2×10^{-4}	6.2×10^{-4}

DISCUSSION.

Haas and Hill have suggested that hermidin may properly be classed with the respiratory pigments of Palladin. The present paper makes no immediate contribution to this question but rather provides more of the quantitative data upon which must be made the final assessment of the respiratory rôle of hermidin. One thing is clear. The reversible oxidation of the chromogen is not a change involving a specific dissociable compound with oxygen but is an oxidation-reduction to be formulated in electrochemical terms. Cyanohermidin is a hydrogen acceptor of the same type as methylene blue and quinone. Were methylene white to be found as a natural constituent of a living cell we should find it difficult to refuse to it a function in respiration. We must not, however, be over-persuaded by the traditional importance to tissue respiration which an experimental convenience has conferred upon this dye, since, apart from glutathione—and the electrode behaviour of this system is not typical—no reversible system of the type here considered has yet been shown to have a fundamental rôle in the respiration of any cell. Yet the lack of evidence is no denial, for it can scarcely be doubted that, under conditions which are not improbable within the living cell, hermidin must function not

only as an efficient hydrogen "transferrer" but, as its ease of oxidation by molecular oxygen implies, as an active oxygen carrier.

From the present work it may be concluded that hermidin exists within the plant in the reduced form to the extent of not less than 95 %. *Mercurialis* is an example of a plant with an oxidation-reduction indicator occurring naturally within it. There is no question, therefore, of the imposition of artificial conditions by the injection of an alien dye. Now the reduction potential of hermidin which is 95 % reduced corresponds with a reduction intensity represented by r_H 10. By the micro-injection of indicators Needham and Needham [1925, 1926] have found that the majority of unicellular organisms which have been studied have given a reduction intensity corresponding to a system in equilibrium with a hypothetical pressure of hydrogen of 10^{-18} atmospheres (*i.e.* a r_H of 18). Only organisms having pronounced anaerobic characteristics, and these only in the absence of oxygen, have shown reduction intensities approaching r_H 10. We hesitate to classify *Mercurialis* with the facultative anaerobes. Moreover, since *Mercurialis* is a green plant there remains to be explained the occurrence within the same cell of a photosynthetic production of oxygen under the pressure of the atmosphere and a state of reduction of hermidin which, it may be calculated, can be in *equilibrium* with a pressure of oxygen of only 10^{-60} atmospheres. It is obvious that the cell sap containing the pigment is not in simple equilibrium with the chloroplast. The theoretical potential of the oxygen electrode is 1.23v. and that of the hermidin electrode 0.35v. more positive than that of the hydrogen electrode: so that a potential difference of almost a volt would thereby exist within the cell. This extravagant figure is mitigated by the fact that oxygen does not appear to exert anything like its full theoretical potential in biological systems. The direct observations of Cannan, Cohen and Clark [1926] and the micro-injection studies referred to agree in giving to "biological oxygen" an oxidation intensity of about r_H 18 corresponding to a potential about 0.54v. positive to that of the hydrogen electrode. This would still leave a potential difference of 0.2v. within the cell containing reduced hermidin. The importance of such an intracellular gradient of reduction intensity cannot be denied, but our knowledge of the conditions pertaining in protoplasm is far too scanty to permit this point to be laboured at present. The determination of the r_H of the whole cell, like the determination of the p_H , may well provide results of much interest but, at the best, would seem to be a precarious undertaking. Protoplasm is a polyphasic system and we shall not expect homogeneous equilibria to prevail.

SUMMARY.

1. The electrode potentials of the system hermidin-cyanohermidin have been determined over the range p_H 2.0 to 8.0 and of the system cyanohermidin-oxidation product over the range p_H 7.0 to 8.0.
2. It is suggested that the second system is reversible only in the neigh-

bourhood of neutrality because the true oxidant is subject to an irreversible non-oxidative change whose velocity is a function of the $[H^+]$.

3. Some electrometric studies of the rates of oxidation of hermidin and cyanohermidin in relation to $[H^+]$ are recorded.

4. The significance of these results to the suggested respiratory rôle of hermidin, and to the question of oxidation-reduction equilibria within the cell, is discussed.

The writer gratefully acknowledges the valuable criticism of Prof. W. M. Clark and the continued interest of Dr P. Haas and Dr T. G. Hill, to whom, further, he is indebted for the supply of *Mercurialis*.

Acknowledgment is made to the Government Grants Committee of the Royal Society for a grant from which the expenses of the present work have been defrayed.

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