CCVI. STUDIES ON REVERSIBLE DEHYDROGENASE SYSTEMS.

II. THE REVERSIBILITY OF THE XANTHINE OXIDASE SYSTEM.

BY DAVID EZRA GREEN.

From the Biochemical Laboratory, Cambridge.

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DIXON [1926] attempted to measure the potential of the xanthine oxidase system (hypoxanthine in presence of the enzyme) by means of the Clark series of indicators and found that all the indicators were reduced, including the most negative, *viz.* indigodisulphonate, which has an E_0' of $-0{\cdot}125$ v. at p_H 7·0. The lack of reversible indicators covering more negative ranges of potential made it impossible to continue this study. In recent years, Michaelis [1931], Michaelis and Eagle [1930] and Michaelis and Hill [1933] have discovered several indicators of very negative potential, in particular rosinduline and the viologens, which cover the range of potential between r_H 0 and 5. The availability of these new indicators offered the possibility of pursuing the work of Dixon, and evidence will be presented in this report to show that the systems hypoxanthine-xanthine and xanthine-uric acid are reversible.

Table I contains a list of indicators which are reduced by the xanthine oxidase from milk in the presence of hypoxanthine and xanthine respectively at p_H 7·0. The results show clearly that the oxidase system lies in the range of methyl- and benzyl-viologens, and near the level of the hydrogen electrode. It is noteworthy that hypoxanthine and xanthine have comparable reducing powers.

Table I.

Indicator	E_0' at p_H 7·0 and 30°	p_H 7·0, 30°	
		Hypoxanthine	Xanthine
Methylene blue	+0·011	Complete reduction	Complete reduction
Nile blue	−0·142	,,	,,
Cresyl violet	−0·167	,,	,,
Phenosafranine	−0·242	,,	,,
Janus green (rose to colourless)	−0·258	,,	,,
Rosinduline	−0·281	,,	,,
Benzylviologen	−0·359	Partial reduction	Partial reduction
Methylviologen	−0·446	,,	,,

The reversibility of the systems hypoxanthine-uric acid and xanthine-uric acid was then demonstrated in the following qualitative fashion. Xanthine oxidase (whey preparation), hypoxanthine and methylviologen were all dissolved in phosphate buffer p_H 7·4 and mixed in a Thunberg tube, and crystals of

uric acid were placed in the side-bulb of the stopper. Within a few minutes after evacuation of the tube a deep blue colour developed, representing approximately 70 % reduction of the methylviologen. The colour persisted unchanged for an hour. The crystals of uric acid were then tipped into the solution. The blue colour gradually faded until about 30 % of the total amount of the indicator remained in the reduced form. No further change occurred even after several hours. The same type of experiment was carried out replacing hypoxanthine with xanthine. Reduction in this case proceeded to only 15 % of completion. Tipping-in of uric acid resulted in the almost complete reoxidation of the indicator. Hardly a trace of blue colour was evident even after several hours. When crystals of xanthine were tipped into a solution containing hypoxanthine, oxidase and partially reduced methylviologen, the change was from 70 to 50 % reduction in contrast to the change from 70 to 30 % in the case of uric acid.

From these results the conclusion can be drawn that methylviologen comes into equilibrium with the systems hypoxanthine-uric acid, hypoxanthine-xanthine and xanthine-uric acid respectively. By alteration of the ratio of oxidant to reductant the potentials of these three systems vary, as shown by the changes in the extent of reduction of the indicator.

Whilst these studies were in progress, Filitti [1933; 1934] published two short notes dealing with the potentials of the xanthine oxidase system. She assigned $E_0' - 0{\cdot}410$ v. to the system hypoxanthine-uric acid at p_H 7·24 and developed the following electrode formula:

$$E_h = E_0' - \frac{RT}{4F} \ln \frac{[HX]}{[Uric]}.$$

No evidence was presented in her notes that the observed potentials agreed with those predicted by the assigned formula. In all the various measurements the ratio of oxidant to reductant was kept constant. From her experimental data therefore she could draw no conclusions concerning the thermodynamic reversibility of the system. Furthermore, as justification for assigning the value of 4 for the number of equivalents n in the electrode formula, Filitti states that both hypoxanthine and xanthine can reduce all the indicators down to and including methylviologen, and that therefore hypoxanthine and xanthine have identical reducing powers. This conclusion of course does not follow. If the equilibrium points of the systems hypoxanthine-xanthine and xanthine-uric acid are close together, the difference in reducing power will show up only with methylviologen or benzylviologen and not with any of the other oxidation-reduction indicators. Filitti has overlooked what is the greatest complication in the study of the potentials of the xanthine oxidase system. If the assumption is made that the oxidation of hypoxanthine to uric acid involves one step of four equivalents, a straightforward analysis of the system can be made with the ordinary electrode equations. But if the oxidation of hypoxanthine to uric acid occurs in two distinct steps, then a unique case develops which conforms to a special type of electrode equation.

Hitherto two-step oxidations each involving two equivalents have been rarely encountered[1] and no adequate theory has been developed to take account of such cases. It is proposed therefore to consider in some detail how two-step oxidations of the type described can be handled theoretically.

[1] Cannan [1926] encountered a two-step oxidation of two equivalents per step in his study of the potentials of hermidin. But the two steps did not overlap sufficiently to interfere with the ordinary formula.

Theory of two-step oxidation.

I. Suppose we start with substances A and C in certain concentrations and assume that the oxidation-reduction systems $A \rightleftharpoons B+2e$, $B \rightleftharpoons C+2e$ come into equilibrium with one another, how will the potentials depend upon the original ratio of A to C?

Originally let $[A]=a$; $[C]=c$.

At equilibrium $[A]=a-x$; $[C]=c-x$; $[B]=2x$; the equilibrium reaction being $A+C \rightleftharpoons 2B$, where x represents the amount of A or of C which has disappeared, and therefore half the amount of B which has been formed by the interaction of A and C. The basic assumption in this proof is that regardless of which pair of substances is taken—A and B, A and C, or B and C—a dismutation, or internal oxido-reduction, occurs as a result of which the third substance is formed to an extent determined by the equilibrium constant for the reaction.

Also at equilibrium

$$E_h = E_{0_1}' - \frac{RT}{2F}\ln\frac{[A]}{[B]} = E_{0_2}' - \frac{RT}{2F}\ln\frac{[B]}{[C]},$$

or

$$\ln\frac{[A]}{[B]} = -\frac{(E_h - E_{0_1}')\,2F}{RT};\quad \ln\frac{[B]}{[C]} = -\frac{(E_h - E_{0_2}')\,2F}{RT}.$$

Therefore

$$\frac{[A]}{[B]} = e^{-\Delta E_1 \alpha} \text{ and } \frac{[B]}{[C]} = e^{-\Delta E_2 \alpha},$$

where $\Delta E = E_h - E_0'$ and $\alpha = \frac{2F}{RT}$.

By substitution

$$\frac{a-x}{2x} = e^{-\Delta E_1 \alpha} \text{ and } \frac{2x}{c-x} = e^{-\Delta E_2 \alpha},$$

whence

$$x = \frac{a}{1+2e^{-\Delta E_1 \alpha}} = \frac{c}{1+2e^{\Delta E_2 \alpha}},$$

and finally

$$\frac{a}{c} = \frac{1+2e^{-\Delta E_1 \alpha}}{1+2e^{\Delta E_2 \alpha}}.$$

II. Suppose we start with a mixture of B and C, how will the potentials depend upon the original ratio of concentrations?

Originally let $[B]=b$; $[C]=c$.

At equilibrium $[B]=b-2x$; $[C]=c+x$; $[A]=x$; the equilibrium reaction being $A+C \rightleftharpoons 2B$, where x represents the amount of A or of C which has been formed, and therefore half the amount of B which has disappeared.

Also as before

$$E_h = E_{0_1}' - \frac{RT}{2F}\ln\frac{[A]}{[B]} = E_{0_2}' - \frac{RT}{2F}\ln\frac{[B]}{[C]}.$$

Therefore

$$\frac{x}{b-2x} = e^{-\Delta E_1 \alpha};\quad \frac{b-2x}{c+x} = e^{-\Delta E_2 \alpha},$$

and

$$x = \frac{be^{-\Delta E_1 \alpha}}{1+2e^{-\Delta E_1 \alpha}}.$$

By substitution

$$\frac{b - \frac{2be^{-\Delta E_1 \alpha}}{1+2e^{-\Delta E_1 \alpha}}}{c + \frac{be^{-\Delta E_1 \alpha}}{1+2e^{-\Delta E_1 \alpha}}} = e^{-\Delta E_2 \alpha},$$

$$b-\frac{2be^{-\Delta E_1\alpha}}{1+2e^{-\Delta E_1\alpha}}=ce^{-\Delta E_2\alpha}+\frac{be^{-(\Delta E_1+\Delta E_2)\alpha}}{1+2e^{-\Delta E_1\alpha}},$$

$$b\left[\frac{1-e^{-(\Delta E_1+\Delta E_2)\alpha}}{1+2e^{-\Delta E_1\alpha}}\right]=ce^{-\Delta E_2\alpha}.$$

Finally

$$\frac{b}{c}=\frac{e^{-\Delta E_2\alpha}+2e^{-(\Delta E_1+\Delta E_2)\alpha}}{1-e^{-(\Delta E_1+\Delta E_2)\alpha}},$$

$$\frac{b}{c}=\frac{1+2e^{-\Delta E_1\alpha}}{e^{\Delta E_2\alpha}-e^{-\Delta E_1\alpha}}.$$

III. If the starting pair of substances are A and B, the equation is derived as follows:

Originally let $[A]=a$; $[B]=b$.

At equilibrium $[A]=a+x$; $[B]=b-2x$; $[C]=x$; the equilibrium reaction being $A+C \rightleftharpoons 2B$, where x has the same meaning as in case II.

Therefore

$$\frac{a+x}{b-2x}=e^{-\Delta E_1\alpha};\quad \frac{b-2x}{x}=e^{-\Delta E_2\alpha},$$

and

$$x=\frac{b}{e^{-\Delta E_2\alpha}+2}.$$

By substitution

$$\frac{a+\frac{b}{e^{-\Delta E_2\alpha}+2}}{b-\frac{2b}{e^{-\Delta E_2\alpha}+2}}=e^{-\Delta E_1\alpha},$$

$$\frac{\frac{a(e^{-\Delta E_2\alpha}+2)+b}{e^{-\Delta E_2\alpha}+2}}{\frac{b(e^{-\Delta E_2\alpha}+2)-2b}{e^{-\Delta E_2\alpha}+2}}=e^{-\Delta E_1\alpha},$$

$$a(e^{-\Delta E_2\alpha}+2)+b=b(e^{-\Delta E_1\alpha})(e^{-\Delta E_1\alpha}).$$

Finally

$$\frac{a}{b}=\frac{e^{-(\Delta E_1+\Delta E_2)\alpha}-1}{e^{-\Delta E_2\alpha}+2},$$

$$\frac{a}{b}=\frac{e^{-\Delta E_1\alpha}-e^{\Delta E_2\alpha}}{1+2e^{\Delta E_2\alpha}}.$$

The final equations for a/b, b/c and a/c have been worked out for the cases where the differences between E_{0_1}' and E_{0_2}' are 0, 5, 10, 20 and 30 mv. respectively. Table II contains the data for calculation purposes and Tables III, IV and V

Table II.

ΔE in volts	$e^{-\Delta E\alpha}$	$e^{\Delta E\alpha}$	ΔE in volts	$e^{-\Delta E\alpha}$	$e^{\Delta E\alpha}$
−0·060	99·5	0·010	−0·015	3·16	0·319
−0·055	68·1	0·012	−0·013	2·71	0·369
−0·050	46·6	0·022	−0·012	2·51	0·398
−0·045	31·9	0·032	−0·010	2·16	0·466
−0·040	21·6	0·047	−0·008	1·85	0·541
−0·035	14·5	0·068	−0·007	1·71	0·584
−0·030	9·97	0·100	−0·005	1·47	0·682
−0·025	6·82	0·146	−0·004	1·36	0·736
−0·020	4·65	0·216	−0·003	1·26	0·795
−0·018	3·98	0·251	−0·002	1·16	0·857
−0·017	3·69	0·271	−0·001	1·08	0·926

α at 30° = 76·8 using logarithms to the base e.

contain the calculated values for the various cases. Calculations were made with an accuracy of one part in the third place. The graphs for Tables III, IV and V are shown in Figs. 1, 2 and 3 respectively. Tables VI and VII offer a convenient summary of all the data.

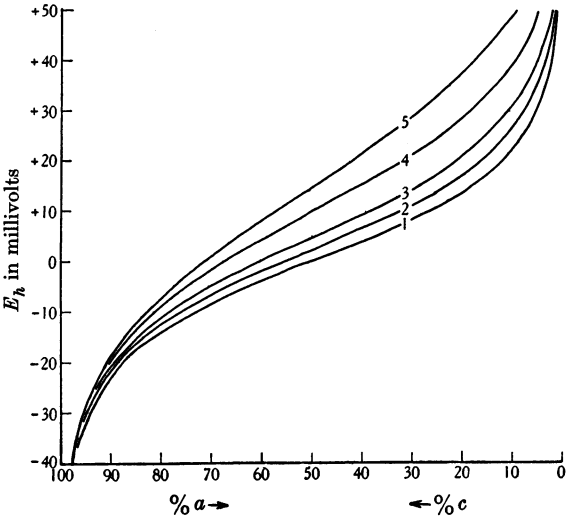

Fig. 1. Variation of E_h with change in the ratio a/c, when $b=0$. The numbers refer to the cases worked out in Table III.

Table III. *Variation of* E_h *with change in the ratio* a/c, *when* $b=0$.

E_h in volts	Case 1 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}000$ v. % a	Case 2 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}005$ v. % a	Case 3 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}010$ v. % a	Case 4 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}020$ v. % a	Case 5 $E_{01}'=0{\cdot}000$ v. $E_{02}'=0{\cdot}030$ v. % a
−0·030	94·6	94·6	95·0	95·0	95·0
−0·025	91·8	92·4	92·4	93·5	93·5
−0·020	88·3	88·8	89·6	90·7	90·8
−0·015	81·8	83·7	85·0	86·5	87·4
−0·010	73·3	76·5	78·8	81·6	82·8
−0·005	62·6	67·1	70·7	75·5	77·6
0·000	50·0	56·0	60·9	67·7	71·4
+0·005	37·4	44·0	50·0	59·1	64·7
+0·010	26·7	32·7	39·2	50·0	57·4
+0·015	18·2	23·5	29·3	40·8	50·0
+0·020	11·7	16·3	21·2	32·3	42·5
+0·025	8·2	11·2	15·0	24·5	35·3
+0·030	5·4	7·6	10·4	18·4	28·6
+0·035	3·5	5·4	7·6	13·5	22·4
+0·040	—	3·5	5·0	9·3	17·2
+0·045	—	—	—	6·5	12·6
+0·050	—	—	—	—	9·1

It is clear that the curves for a/c are S-shaped and symmetrical about the mid-point whereas the curves for b/c and a/b are exponential and hence asymmetrical. Inspection of the latter two sets of curves shows that one is the mirror image of the other.

In Tables IV and V negative values for the ratio of a/b or b/c are obtained for certain values of E_h. That can only mean that it is impossible to start with a finite ratio and reach that potential level. From Fig. 2 it is apparent that the

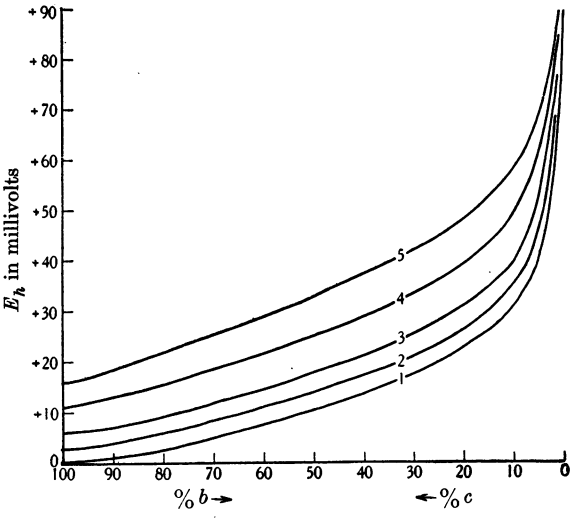

Fig. 2. Variation of E_h with change in the ratio b/c, when $a=0$. The numbers refer to the cases worked out in Table IV.

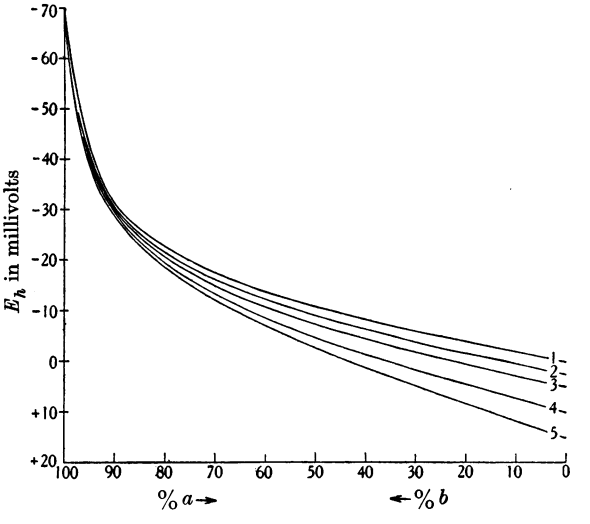

Fig. 3. Variation of E_h with change in the ratio a/b, when $c=0$. The numbers refer to the cases worked out in Table V.

curve describing the variation of E_h with b/c may be extended beyond 0 % to the negative side of the abscissa, but the curve then has no physical significance. Similarly the curve for a/b may be extended beyond 100 % to negative percentages but here again the extension of the curve has no physical meaning.

A change of $\Delta(E_{0_2}'-E_{0_1}')$ from 0 to 30 mv. produces a much smaller change in the potential values of a/b, b/c and a/c for the two cases. In effect, this means

Table IV. *Variation of* E_h *with change in the ratio* b/c, *when* $a=0$.

	Case 1	Case 2	Case 3	Case 4	Case 5
	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.
	$E_{0_2}'=0{\cdot}000$ v.	$E_{0_2}'=0{\cdot}005$ v.	$E_{0_2}'=0{\cdot}010$ v.	$E_{0_2}'=0{\cdot}020$ v.	$E_{0_2}'=0{\cdot}030$ v.
E_h in volts	% b	% b	% b	% b	% b
0·000	∞	Neg.	Neg.	Neg.	Neg.
+0·002	90·0	,,	,,	,,	,,
+0·003	84·9	,,	,,	,,	,,
+0·004	79·9	92·9	,,	,,	,,
+0·005	74·9	88·1	∞	,,	,,
+0·007	—	—	91·2	,,	,,
+0·008	—	—	86·8	,,	,,
+0·010	53·2	65·9	78·1	∞	,,
+0·012	—	—	—	92·8	,,
+0·013	—	—	—	89·2	,,
+0·015	36·6	47·1	58·8	82·0	∞
+0·017	—	—	—	—	93·9
+0·018	—	—	—	—	90·9
+0·020	24·4	32·7	42·4	64·8	85·1
+0·025	16·2	22·3	30·0	49·5	70·5
+0·030	10·8	15·2	20·9	36·8	57·1
+0·035	7·3	10·3	14·4	26·7	44·5
+0·040	4·8	7·0	9·9	19·2	34·6
+0·045	—	4·7	6·8	13·6	25·3
+0·050	—	—	—	9·5	18·4
+0·055	—	—	—	6·6	13·0
+0·060	—	—	—	—	9·3
+0·065	—	—	—	—	6·6

Table V. *Variation of* E_h *with change in the ratio* a/b, *when* $c=0$.

	Case 1	Case 2	Case 3	Case 4	Case 5
	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.	$E_{0_1}'=0{\cdot}000$ v.
	$E_{0_2}'=0{\cdot}000$ v.	$E_{0_2}'=0{\cdot}005$ v.	$E_{0_2}'=0{\cdot}010$ v.	$E_{0_2}'=0{\cdot}020$ v.	$E_{0_2}'=0{\cdot}030$ v.
E_h in volts	% a	% a	% a	% a	% a
−0·035	92·7	93·0	93·2	93·4	93·4
−0·030	89·2	89·7	90·1	90·5	90·7
−0·025	83·8	84·8	85·6	86·4	87·0
−0·020	75·6	77·7	79·1	80·8	81·6
−0·015	63·4	67·3	70·0	73·3	74·7
−0·010	46·8	52·9	57·6	63·2	65·4
−0·005	25·1	34·1	41·2	50·5	55·5
−0·004	20·1	—	—	—	—
−0·003	15·1	—	—	—	—
−0·002	10·0	—	—	—	—
0·000	0	11·9	21·9	35·2	42·9
+0·001	Neg.	7·1	—	—	—
+0·002	,,	—	13·2	—	—
+0·003	,,	Neg.	8·8	—	—
+0·005	,,	,,	0	18·0	29·3
+0·007	,,	,,	Neg.	10·8	—
+0·008	,,	,,	,,	7·2	—
+0·010	,,	,,	,,	0	14·9
+0·012	,,	,,	,,	Neg.	9·1
+0·013	,,	,,	,,	,,	6·1
+0·015	,,	,,	,,	,,	0

that an experimental error of 1–2 mv. will introduce an inaccuracy of 5–10 mv. in the evaluation of E_{0_1}' and E_{0_2}' respectively.

It is now possible to decide between the alternative of a one-step oxidation of hypoxanthine to uric acid involving four equivalents or a two-step oxidation

involving two equivalents for each step. On the assumption of a one-step oxidation, the variation of the potentials of the hypoxanthine-uric acid system between 90 and 10 % oxidation should be 29 mv., whereas for a two-step oxidation the span of potentials should be between 40 and 60 mv., depending upon the difference in magnitudes of E_{0_1}' and E_{0_2}'. Similarly for the xanthine-uric acid system, the span of potentials should be 58 mv. for a one-step oxidation but about 30 mv. for the case of a two-step oxidation.

Experimental methods.

Xanthine oxidase was prepared from whey according to the directions of Dixon and Kodama [1926]. This preparation retains its activity over a period of months when kept in a vacuum-desiccator. If a more or less clear enzyme solution were required, as in the case of colorimetric studies, the whey preparation was dissolved in water and purified by treatment with charcoal.

Twice recrystallised samples of hypoxanthine, xanthine and uric acid were used in this study. Hypoxanthine and xanthine were dissolved in the minimum quantity of NaOH to yield a 0·03 *M* solution, whereas uric acid was dissolved in a solution containing an equivalent amount of lithium carbonate and a slight excess of alkali. Immediately before each experiment, 5 ml. of the standard solution were brought to the desired p_H and diluted with *M*/5 phosphate buffer to make 10 ml. This manipulation must be very rapid indeed since near neutrality the three purines are rather insoluble and in the strengths used precipitate out within a few minutes unless further diluted with the experimental solutions.

The electrode vessels and procedure have been described in detail in the first paper of this series by Green and Stickland [1934]. There is a point connected with the preparation of agar bridges which may be worth mentioning. Borsook and Schott [1931] have outlined a rather tedious and complicated procedure for filling the capillary tube of the electrode vessel with agar which will withstand a vacuum. The experience of the author has been that by adding 1 g. of finely divided kaolin to 100 ml. of clear filtered agar saturated with KCl, bridges can be made without any undue precautions of slow cooling *etc.* The molten agar is sucked into the capillary tube and cooled immediately. The bridge is very resistant to a vacuum over a period of weeks [Lehmann, 1930].

The electrode vessels were filled with the following solutions: 5 ml. of *M*/5 buffer, 0·5 ml. of 0·001 *M* methyl- or benzyl-viologen, 1 ml. of neutralised purine mixture (0·015 *M*), and 1 ml. of xanthine oxidase solution. Benzylviologen was used between p_H 6 and 7, and methylviologen in the more alkaline range. Both indicators were used at p_H 7·0.

Experimental results.

Table VIII contains a summary of the observed and theoretical potentials for various p_H levels. Each of the observed values is the mean of from four to six separate determinations.

The following procedure was employed for obtaining the theoretical potentials. First, it was determined by inspection which case best fitted the potentials of HX/U, X/U and HX/X for a given p_H. From one observed potential, usually the 5/5 ratio of the HX/U series, all the other eight potentials could easily be evaluated from Table VI. It is noteworthy that given a single potential value for any ratio of two of the purines and knowing the E_{0_1}' and E_{0_2}' values at that

p_H, then the potential value for any other ratio, be it HX/X, X/U or HX/U, can easily be determined.

As pointed out previously an experimental accuracy within 1 mv. would be necessary in order to fix the E_0' values with a certainty of 5 mv. But in enzyme systems an accuracy within 2 mv. is difficult to attain. Hence a considerable inaccuracy must be expected in the evaluation of the characteristic constants.

The reasonable agreement between the observed and theoretical values in Table VIII is convincing proof that in the xanthine oxidase system we are dealing

Table VI. *Calculated millivolt changes for various changes in ratio.*

	HX/U			X/U			HX/X		
	9/1 – 5/5	8/2 – 5/5	5/5 – 2/8	9/1 – 5/5	8/2 – 5/5	5/5 – 2/8	9/1 – 5/5	8/2 – 5/5	5/5 – 2/8
Case 1	23	14	14	9	8	12	21	12	8
Case 2	25	15	15	10	8	13	22	13	8
Case 3	26	16	16	11	9	13	22	13	9
Case 4	29	19	19	12	10	15	24	15	10
Case 5	34	23	23	15	11	16	27	16	11

Table VII. *Calculated* E_h *for 5/5 ratio.*

	E_{01}'	E_{02}'	HX/U	X/U	HX/X
Case 1	0·000	0·000	0·000	+0·011	−0·012
Case 2	,,	+0·005	+0·0025	+0·014	−0·009
Case 3	,,	+0·010	+0·005	+0·018	−0·007
Case 4	,,	+0·020	+0·010	+0·025	−0·005
Case 5	,,	+0·030	+0·015	+0·033	−0·003

Table VIII.

p_H	E_{01}'	E_{02}'	System		9/1	8/2	5/5	2/8
6·93	−0·371	−0·361	HX/U	Calc.	—	−0·382	−0·366	—
				Obs.	—	−0·382	−0·366	—
			X/U	Calc.	—	−0·362	−0·353	−0·340
				Obs.	—	−0·362	−0·349	−0·335
			HX/X	Calc.	—	−0·391	−0·378	−0·369
				Obs.	—	−0·393	−0·381	−0·372
7·32	−0·410	−0·390	HX/U	Calc.	—	−0·419	−0·400	−0·381
				Obs.	—	−0·422	−0·400	−0·380
			X/U	Calc.	—	−0·395	−0·385	−0·371
				Obs.	—	−0·390	−0·380	−0·370
			HX/X	Calc.	—	−0·430	−0·415	−0·405
				Obs.	—	−0·425	−0·413	−0·404
7·65	−0·435	−0·415	HX/U	Calc.	−0·454	—	−0·425	−0·406
				Obs.	−0·454	—	−0·425	−0·404
			X/U	Calc.	—	—	−0·410	−0·395
				Obs.	—	—	−0·415	−0·400
8·07	−0·471	−0·441	HX/U	Calc.	−0·490	—	−0·456	−0·433
				Obs.	−0·484	—	−0·456	−0·432
			X/U	Calc.	—	−0·455	−0·444	—
				Obs.	—	−0·452	−0·440	—
8·43	−0·484	−0·454	HX/U	Calc.	−0·503	—	−0·469	−0·446
				Obs.	−0·497	—	−0·469	−0·450
			X/U	Calc.	—	−0·462	−0·451	−0·435
				Obs.	—	−0·465	−0·454	−0·437
9·10	−0·512	−0·482	HX/U	Calc.	—	—	−0·497	—
				Obs.	—	—	−0·497	—
			X/U	Calc.	−0·494	—	−0·479	—
				Obs.	−0·495	—	−0·476	—

with a two-step oxidation. It is noteworthy that the observed span of potentials for HX/U is approximately twice as much as the ordinary equation predicts, whereas the span of potentials for X/U and HX/X is approximately one-half of the theoretical span for a 2 equivalent system.

In the derivation of the equation for a/b, b/c and a/c, it was assumed that the following reaction is possible:

$$A + C \rightleftharpoons 2B.$$

If the xanthine oxidase system is to be considered as a case of a two-step oxidation, then the formation of uric acid and hypoxanthine from xanthine and the reverse process must be shown to take place anaerobically in the presence of the enzyme, to an extent determined by the equilibrium constants. The following experiments definitely prove that this dismutation does occur. Xanthine oxidase solution (3 ml.) was incubated with xanthine (3 mg.) anaerobically for 3 hours at 37°. After deproteinisation of the enzyme solution with uranium acetate, the filtrate was tested for uric acid with Benedict's reagent. An intense blue colour developed representing about 1 mg. of uric acid. With boiled enzyme, the test was completely negative. Thus the anaerobic production of uric acid from xanthine is obviously enzymic in nature. When hypoxanthine was incubated with the enzyme under similar conditions, the test was likewise negative. Thunberg tubes were then set up containing different ratios of HX/X and constant amounts of enzyme solution. The results showed that the larger the ratio of HX/X the smaller the amount of uric acid produced and conversely. These observations are in complete accordance with the mathematical theory. Furthermore it was demonstrated that when hypoxanthine and uric acid are incubated anaerobically with the oxidase, a portion of the hypoxanthine and uric acid disappears to form xanthine. The quantitative aspect of this dismutation will be dealt with in a future communication.

Bach and Michlin [1927] claimed that both hypoxanthine and xanthine in presence of the oxidase formed uric acid anaerobically. Wieland [Wieland and Rosenfeld, 1929] was unable to confirm these findings. The fact that hypoxanthine as well as xanthine yielded uric acid indicates that Bach and Michlin were not studying a dismutation but an oxidation—due to leakage of oxygen. The short duration of Wieland's experiments (*ca.* 100 mins.) may explain why the dismutation of xanthine to hypoxanthine and uric acid was not observed. The reaction is rather slow and, unless very active enzyme preparations are employed, at least 6 hours are required for a measurable quantity of uric acid to be formed.

Summary

The reversibility of the xanthine oxidase system has been demonstrated by colorimetric and potentiometric methods. The mathematical theory of two-step oxidations involving two equivalents for each step has been developed and evidence presented that the xanthine oxidase system is an example of a two-step oxidation system. The E_0' of the hypoxanthine-xanthine at p_H 7·0 is −0·371 v., and the E_0' of the xanthine-uric acid is −0·361 v. The prediction by the theory of the partial anaerobic formation of uric acid and hypoxanthine from xanthine and the partial anaerobic formation of xanthine from hypoxanthine and uric acid have been confirmed experimentally

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