LVII. OXIDATION-REDUCTION POTENTIALS OF TOXOFLAVIN.

By KURT GUENTER STERN.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London.

(*Received December 21st, 1934.*)

TOXOFLAVIN is the name given by van Veen and Mertens [1934] to the prosthetic group of the yellow pigment which is formed in cultures of *Bacterium bongkrek* under aerobic conditions. The native pigment complex together with a colourless compound is thought by these authors to be responsible for the occurrence of a number of cases of foodstuff poisoning among the natives of Java. Whereas the native pigment is highly toxic when administered perorally or intraperitoneally to monkeys, pigeons and rats, toxoflavin in crystallised state has only retained its toxicity for rats when injected intraperitoneally, 5–25γ representing the lethal dose. The decrease in toxicity is ascribed by the authors to the detachment of the prosthetic group from some colloidal carrier substance in the course of purification.

The empirical formula assigned to toxoflavin is $C_6H_6O_2N_4$. The constitutional formula is unknown. Of the chemical properties reported in van Veen and Mertens's publication only the following may be mentioned here in view of their bearing on the present work. The compound (M.P. 171°) is readily soluble in water and alcohol, less soluble in ethyl acetate and chloroform, hardly soluble or insoluble in ether, benzene, and light petroleum. In water the yellow colour is only stable between p_H 3 and 7. Slow decoloration takes place at p_H 1 and 9. Addition of mineral acids or strong alkali causes immediate decoloration. Toxoflavin is stable towards oxidising agents (nitric acid, hydrogen peroxide, bromine water). In weakly acid or alkaline solution the yellow colour is discharged instantaneously by sulphurous acid and it returns on shaking with air or addition of hydrogen peroxide or bromine water. On catalytic hydrogenation according to the selected conditions either approximately one or three hydrogen molecules are taken up by one molecule. One methylimide group was found. The murexide test is only positive if potassium chlorate and hydrochloric acid are used instead of nitric acid. As oxidation products alloxan (or methylalloxan), methylamine and ammonia were found. In concentrated aqueous solution toxoflavin forms a brick-red, sparingly soluble addition compound with bisulphite. The solutions of toxoflavin, especially in amyl alcohol, are stated to exhibit a green fluorescence.

The idea of carrying out the experiments which are subsequently described arose from the conclusion drawn by van Veen and Mertens from their findings that toxoflavin was to be regarded as a member of the series of flavin or lyochrome pigments recently studied by Warburg, Ellinger, Kuhn, Karrer, von Euler and by the present author.

This investigation was made possible by the generous gift of about 20 mg. of pure recrystallised toxoflavin by Dr A. G. van Veen to whom sincere thanks are due. He consented to the potentiometric and spectrographic examination

of the pigment[1], on lines along which detailed information in respect to flavin pigments was available from this laboratory [Holiday and Stern, 1934; Stern, 1934, 2].

Experimental.

Material. The sample of toxoflavin which was used for the following experiments was sent from Batavia-Centrum in a sealed ampoule. The colour of the crystals, which is much like that of picric acid, is stated to change somewhat to a brownish tint in a tropical climate when exposed to the air, but the crystals did not show any detectable change during their 4 months' storage in a stoppered tube in this laboratory.

The crystals were found to be free from moisture (3·04 mg. when subjected to a slow current of air, previously dried over calcium chloride, for 3 hours at 75° in a platinum boat in Pregl's micro-desiccator (suction applied by high pressure water-pump) did not show any loss of weight detectable with a Sartorius microbalance).

The properties were identical with those described by van Veen and Mertens with the exception of the fluorescence which was found to be hardly perceptible even in a strong beam of Wood's light when the substance was dissolved in water, chloroform or amyl alcohol. The weakness of the fluorescence became especially obvious when as controls similar solutions of photo-hepatoflavin or of 9-methylalloxazin were used.

The aqueous solution of toxoflavin reacts weakly acid. A $10^{-4}\,M$ solution showed a p_H of 6·5 when measured with the glass electrode.

Toxoflavin does not couple with diazobenzenesulphonic acid. No free NH_2-group is detectable with 1: 2-naphthoquinone-4-sulphonic acid.

Methods. The arrangement and method of potentiometric measurements were the same as described in a study dealing with photo-flavin [Stern, 1934, 2]. The potassium ferricyanide preparation used for oxidative titrations of leuco-toxoflavin was an analytically pure sample. It was free from ferrocyanide, and on titration with permanganate after reduction with sodium peroxide 100 % of the calculated iron content was found. For the purpose of reductive titrations the technique described by Clark and Perkins [1932] was employed. The stock solution of chromous acetate was stored under hydrogen and delivered from a Bang microburette. To the tip of this burette a small vessel containing the necessary amount of buffer solution was attached. After thorough de-aeration with hydrogen or nitrogen the calculated amount of the stock solution was run into the buffer mixture. The receiving vessel was detached from the Bang burette and the diluted titrant was quickly covered with liquid paraffin. It was then drawn up into the actual microburette serving for the electrometric titrations protected by a paraffin seal. The strength of the chromous acetate stock solution was determined before and after the preparation of the dilution by means of a standardised ferric alum solution with ammonium thiocyanate as internal indicator.

The spectrographic measurements were made with the aid of a medium-size Hilger quartz spectrograph with attached Spekker quartz photometer. The condensed spark discharge between tungsten steel electrodes served as light source. The photographs were evaluated by the match point method.

For the manometric experiments the usual Barcroft-Warburg technique was employed.

[1] A short account of this work was presented jointly with Holiday at the Meeting of the Biochemical Society on November 16th, 1934 [Stern and Holiday, 1934, 2].

Results.

Oxidation-reduction potentials of toxoflavin.

Nineteen titration experiments were performed covering the p_H range from 2·68 to 8·49. In accordance with the qualitative findings of van Veen and Mertens the stability of the pigment was sufficient for reproducible measurements only between p_H 4·6 and 8·3. At p_H 2·68 the oxidised (yellow) form of the system became unstable. At p_H 4·12 and 8·49 the reduced form was not sufficiently stable to allow the tracing of a definite titration curve. Between p_H 4·6 and 8·3 toxoflavin represents the oxidant of a stable, reversible and electro-active redox system. Titrations of the leuco-form with ferricyanide and of the oxidant with chromous acetate yield substantially consistent results. In the poising range of the system equilibrium is almost instantaneously reached, the potentials are stable in time, and individual electrodes differ seldom by more than 1 mv. Generally they agree in the range from 20 to 80 % oxidation within 0·1 mv.

Potential range. The normal potentials of toxoflavin as obtained by graphical interpolation from the individual titration curves at varying hydrogen-ion concentrations are given in Table I in column "E_0'". The potentials were

Table I. *Results of titrations of 10^{-4}M leuco-toxoflavin with $2 \cdot 10^{-3}$M potassium ferricyanide (30°).*

(Potential values referring to the normal H_2 electrode.)

p_H	Buffer	$E_{\frac{1}{4}}$	$E_0' = E_{\frac{1}{2}}$	$E_{\frac{3}{4}}$	E_1	$0 \cdot 03 \log K$ $E_M - E_1 =$ $E_2 - E_M$	(after Michaelis) log K	(after Michaelis) K	K (after Elema)
4·65	Acetate	+0·036	+0·058	+0·081	0·0225				
5·22	Veronal-acetate-HCl	+0·0015	+0·0245	+0·0495	0·024				
5·41	,,	−0·007	+0·014	+0·037	0·022				
5·69	,,	−0·019	+0·001	+0·023	0·021	Mean ~0	±0	±1	—
6·07	,,	−0·0365	−0·0185	+0·0015	0·019				
6·36	,,	−0·043	−0·024	−0·005	0·019				
6·82	,,	−0·064	−0·040	−0·015	0·0245	+0·0085	+0·283	1·91	1·39
7·37	Phosphate	−0·090	−0·059	−0·029	0·0305	+0·021	+0·7	5·01	4·56
8·09	Veronal-acetate-HCl	−0·1085	−0·0735	−0·0395	0·0345	+0·028	+0·93	8·51	10·1
8·30	,,	−0·110	−0·077	−0·042	0·034	+0·0275	+0·917	8·26	10·82

measured against the saturated calomel half cell and the values with reference to the normal hydrogen electrode were then calculated therefrom. The curve combining these E_0' values is plotted in the right-hand part of the composite diagram in Fig. 1. It will be seen from this curve that the toxoflavin system is much more positive than the flavin systems studied by the writer. For instance, whereas E_0' for toxoflavin at p_H 7 is −0·049 v., the corresponding value for photo-hepatoflavin is −0·227 v. [Stern, 1934, 2] and for hepato-, malto- and uro-flavin the mean value is −0·215 v. [Stern, 1934, 1]. On the other hand the toxoflavin potential at p_H 7 is very near to the pyocyanin potential (−0·034 v. [Friedheim and Michaelis, 1931; Elema, 1931]).

The shape of the E_0'/p_H curve for toxoflavin reveals the existence of two dissociation constants in the range of stability of both components of the system: p_{K_1} about 5·8 and p_{K_2} about 7·2.

The slope of the curve from p_H 4·6 to 5·7 is $\frac{-\Delta E}{\Delta p_H} = 60$ mv., from p_H 6·1 to 7 = 35 mv., and from p_H 7·4 to 8·3 = 19 mv. The changing of the slope of the curve, due to p_{K_1}, by approximately 30 mv. suggests that the nature of the

system is a "two electron-system" ($n=2$). p_{K_2} however changes the slope in an atypical manner. This may possibly be explained by the assumption that the part of the curve extending from p_H 7·4 to 8·3 is eventually tending to a

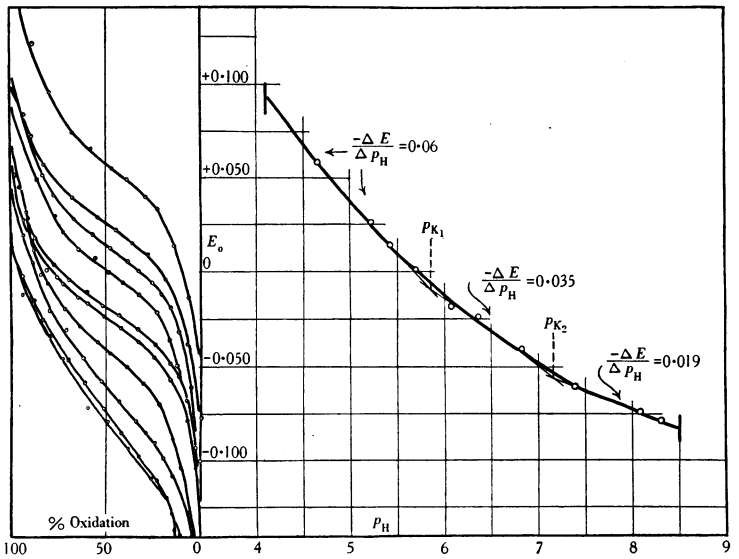

Fig. 1. Toxoflavin: Relation of E_0' to p_H.

zero mv. slope. This hypothesis however cannot be tested experimentally in view of the instability of the system beyond p_H 8·3.

The experiments which served for the compilation of Table I as well as for the diagram (Fig. 1) were all done by titrating leuco-toxoflavin with potassium ferricyanide.

Electron number. As will be seen by inspection of the individual titration curves plotted in the left-hand side of Fig. 1 as well as from the column "E_1" of Table I, the slope of these curves is throughout an atypical one. This is best demonstrated by the value of the so-called index potential (E_1), *i.e.* the potential difference between 50 and 25 % (or 75 % respectively) oxidation. Whereas E_1 for a system losing two electrons in a single step on oxidation, which is the most common type of organic redox system, would amount to 14 mv., E_1 for a one-electron system like cytochrome would be 28 mv.

With the present system E_1 averages 21 mv. between p_H 4·6 and 6·4, and then increases with p_H, to become 34 mv. at p_H 8·3. A variation of E_1 with p_H is also shown by the two step reduction systems recently studied by Michaelis [1933] and by Elema [1931; 1933], *e.g.* pyocyanin and chlororaphin. The flavins have been shown to belong to the same class of systems [Stern, 1934, 2]. A fundamental difference between the above-mentioned systems and toxoflavin however is the fact that the steepening of the titration curves and simultaneous visible semiquinone formation in the case of pyocyanin etc. is observed in the acid range, whereas with toxoflavin the change occurs in the alkaline range without any visible semiquinone formation[1].

[1] The only similar case of which the writer is aware is that of the pigment of *Halla parthenopea*—hallachrome—which was recently studied by Friedheim [1933]. Here E_1 is about 20 mv. from p_H 0·19 to 9·8. Further towards the alkaline range E_1 increases and becomes 34 mv. at p_H 11·47.

It follows therefore that the number of electrons involved in the process of oxidation-reduction of toxoflavin cannot be derived from the slope of individual titration curves. If the view be accepted that we are dealing here with a two-step reduction system, though no formation of an intermediary form may be detected by a colour change on reduction and though no complete separation of the two steps involved could be realised owing to the overlapping of the steps within the p_H range of stability, the complete process of reduction would consist in the uptake of two hydrogen equivalents or electrons. This conclusion is supported by the above-mentioned change in the slope of the E_0'/p_H curve of approximately 30 mv. by the first dissociation constant of the reduced form, and also by the observation of van Veen and Mertens that either one or three molecules of hydrogen are taken up by toxoflavin according to the conditions chosen. The uptake of one H_2 molecule would correspond to the reversible process amenable to electrometric study.

Treatment of toxoflavin as a two-step reduction system.

Assuming that toxoflavin is reduced in two steps increasingly overlapping towards the acid range and each involving the uptake of one electron, the process may tentatively be subjected to theoretical treatment along the lines developed by Michaelis and independently by Elema. These authors have shown how the distance of the normal potentials of the two single steps of such a system from the midpoint of the whole titration curve may easily be determined even in the range of considerable overlapping. Furthermore they have made available short cuts for obtaining the value of the characteristic "effective formation constant of the intermediary form (semiquinone)" at any given p_H value. The magnitude of this constant, K, is an adequate representation of the probability of free radical (semiquinone) formation and also of its stability. Michaelis [1933] for this purpose has compiled a table correlating the experimentally observed E_1 values with the distance of the separate normal potentials ($E_M - E_1 = E_2 - E_M$) from the midpoint of the titration curves and with $\log K$. Elema [1933] on the other hand has shown that a simple relation holds between K and the tangent to the titration curve at the midpoint. The height of ordinate defined by the midpoint of the curve and by the point of intersection of the tangent with the ordinate is called S. Then $K = (76{\cdot}63 . S - 2)^2$. In Table I both methods have been applied to the data obtained for toxoflavin. Since the intervals in the table of Michaelis are somewhat too large for simple interpolation ($E_M - E_1 = 0{\cdot}03 \log K$ being no linear function of E_1) a diagram was first constructed from the table of Michaelis and the values given in Table I were obtained by graphical interpolation. As will be seen from Table I, there is a general agreement between the K values derived by the methods of Michaelis and of Elema. Theoretically the agreement between the results of the two methods should be closer, which implies the conclusion that the differences actually found are to be ascribed to experimental errors.

Molecular weight of toxoflavin.

On the ground of molecular weight determinations by the cryoscopic method (solvent, water) and by Rast's method (solvent, camphor) van Veen and Mertens assign to toxoflavin the formula $C_6H_6O_2N_4$. The Rast determination is however invalidated by decomposition of the pigment in melting camphor. It was therefore thought to be desirable to obtain information about the molecular weight of toxoflavin by an independent method. In the case of pyocyanin Friedheim and Michaelis [1931] were able by the mere slope of the titration

curves to establish the true molecular weight as half the value given by Wrede and Strack. In the case of toxoflavin where the slope of the titration curves is atypical throughout the whole p_H range the stoichiometric relations had to be taken into consideration. The basic assumption to be made was that two hydrogen equivalents are concerned in the reversible process of oxidation-reduction. No decision could be reached by oxidative titration experiments with potassium ferricyanide. In these experiments 10 ml. toxoflavin solution containing 0·166 to 0·167 mg. were reduced with palladium-hydrogen and then titrated with a $2 \cdot 10^{-3}$ *M* ferricyanide solution. For $n=2$ there should have been required 0·5 ml. ferricyanide if the molecular weight was 332, and 1·0 ml. for a molecular weight of 166. Actually the end-points observed on electrometric titration fell invariably near 0·7 ml. ferricyanide. The same irregularity has previously been observed in the case of photo-hepatoflavin [Stern, 1934, 2]. Better results were obtained by means of reductive titrations with chromous acetate. 10 ml. toxoflavin solution containing 0·166 mg. were titrated at p_H 5·67 with $5 \cdot 35 . 10^{-3}$ *M* chromous acetate solution. If the molecular weight of toxoflavin were 332, 0·187 ml. of the titrant, and if it were 166, 0·374 ml. should be required. As determined from the titration graph 0·32 *M* chromous acetate solution was necessary. In a similar experiment at p_H 4·6 the quantity of titrant used was 0·325 ml. of a $5 \cdot 16 . 10^{-3}$ *M* chromous acetate solution (calculated for mol. wt. 166:0·387 ml., for mol. wt. 332:0·193 ml.).

These experiments are therefore corroborative evidence in favour of the formula of toxoflavin proposed by the discoverers.

Results of spectrographic study.

The measurements of light absorption have been carried out by Dr Holiday at the Laboratories of the Medical Unit of the London Hospital Medical School [*cf.* Stern and Holiday, 1934, 2]. Their result was that a general resemblance

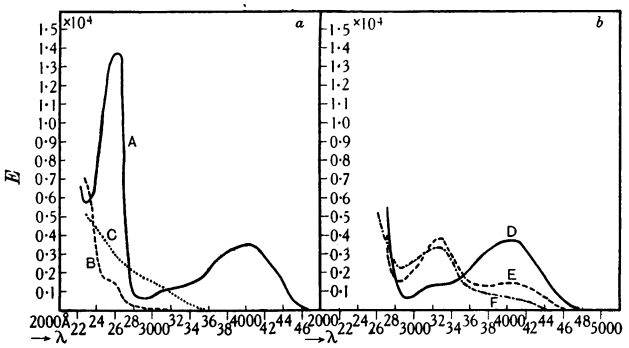

Fig. 2. Spectral behaviour of toxoflavin. Ordinates: mol. extinct. coeff. Abscissae: wave-lengths in Å.

A. ——— p_H 6·5.
B. – – – – p_H 3·0 after standing.
C. ------ p_H 11·0 after standing.
D. ——— p_H 6·5.
E. – – – – p_H 9·0 immediately after dissolving.
F. – · – · – p_H 11·0 immediately after dissolving.

exists between the patterns of the flavin and of the toxoflavin spectra. In aqueous solution (p_H 6·5) a steep main absorption band at 260$m\mu$ is accompanied by an inflection at 310$m\mu$ and by a lower and rather broad band centred at 405$m\mu$. The molecular extinction coefficients calculated for a mol. wt. of 166 amount

to about one-half of the corresponding values for flavins. After standing for a short time at p_H 3 or p_H 11 the fading of the yellow colour is accompanied by an almost complete disappearance of specific light absorption. These results are illustrated in Fig. 2. At p_H 9 the height of the long wave band decreases and a distinct band is formed instead at $320 m\mu$. This band also slowly disappears with time.

The disappearance of specific absorption in acid and in alkaline solution seems to indicate an opening of a ring structure in the molecule.

Effect of toxoflavin on cell respiration.

Judged from the value of its normal potential toxoflavin should increase the oxygen uptake of non-nucleated red blood corpuscles to a similar extent to *e.g.* methylene blue or pyocyanin, assuming of course that the effect is not obscured by secondary factors such as toxicity or diffusibility.

Manometric experiments have been carried out by Mr Greville with human and rabbit erythrocytes. The result was that toxoflavin stimulates their respiration to about the same extent as thionine ($E_0' = +0{\cdot}062$ at p_H 7). The course of an experiment with rabbit blood corpuscles is illustrated in Fig. 3. In view of current discussion on the mechanism of stimulation of respiration in erythrocytes

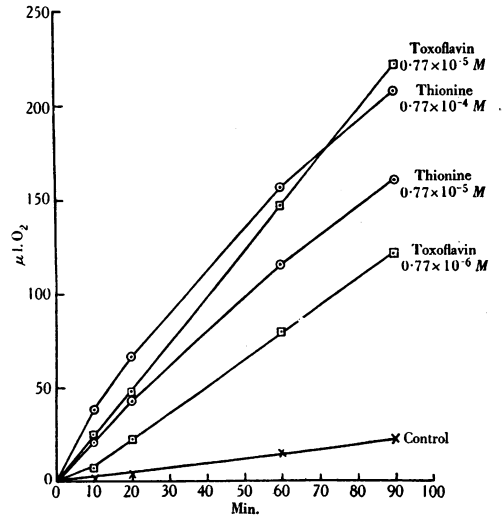

Fig. 3. Effect of toxoflavin and of thionine on respiration of rabbit erythrocytes in 0·2 % glucose.

by oxidation-reduction systems it may not be without interest to mention that toxoflavin is capable of transforming oxyhaemoglobin with fair velocity into methaemoglobin.

The effect of toxoflavin on the respiration of Jensen rat sarcoma and on normal tissues (*e.g.* brain) is under investigation. Evidence so far available indicates that the respiration of Jensen rat sarcoma in glucose is also increased by toxoflavin (140 % increase by 2.10^{-5} mol. toxoflavin). The effect however decreases rapidly, probably because of a toxic action of the pigment which is shown better by brain slices, where a concentration of 10^{-5} mol. already strongly inhibits the respiration.

Discussion.

It is proposed to consider first the bearing of the results of this investigation on the question of a structural relationship between toxoflavin and the group of flavin (lyochrome) pigments. Van Veen and Mertens were led to assume such a relationship on the ground of similarities of a more qualitative nature, *e.g.* colour, fluorescence, stability towards oxidising agents and reversible reducibility. Though at the time of the completion of their paper (December, 1933) the flavin chemistry was still in its early stages, the authors were well aware of certain fundamental differences between toxoflavin and the other flavin pigments, *e.g.* in respect to behaviour towards radiation, formation of sparingly soluble silver salts and nitrogen content. In spite of the fact that the exact constitution of toxoflavin itself is still unknown, the rapid progress of flavin chemistry since the publication of Van Veen and Mertens's paper together with the data made available by the present experiments should furnish a definite answer to that question. If at the outset there existed a link of comparison between toxoflavin and the class of flavin pigments, such a link could only be seen in the photoflavins (or lumi-flavins), *i.e.* in the photo-decomposition products of the native flavins, but not in the native pigments themselves. Toxoflavin shares with a photo-flavin the solubility both in water and in chloroform, the stability towards oxidants, the reversibility of reduction and the presence of a methylimide group. There exists also a similarity in the general pattern of the absorption spectrum. On a closer examination of the data obtained on photo-flavins [Stern, 1934, 2] and on toxoflavin (this paper) with the same methods, these similarities turn out to be of a rather superficial nature. The major points are brought out in Table II.

Table II.

	Toxoflavin	Photoflavins
Range of stability	p_H 4 to 8	Conc. H_2SO_4—cold *N* NaOH
Normal potential at p_H 7	$E_0' = -0{\cdot}049$ v.	$E_0' = -0{\cdot}22$ v.
Slope of titration curves	Atypical throughout, increasing steepness towards alkaline range	Typical for $n=2$ in physiological range, increasing steepness toward acid range
Semiquinone formation	Not detectable; if any, then in alkaline range	Stable, red semiquinone in acid solution
Dissociation constants:		
p_K of reduced form	5·8 and 7·2	7·7
p_K of oxidised form	—	0·4 and 10
Maxima of light absorption	260; 310; 405 $m\mu$ (p_H 6·5)	267; 364; 440 $m\mu$ (p_H 7) (photo-hepatoflavin)

The data given in the column "Photoflavins" apply, with the noted exception of absorption bands, to the whole group of photoflavins, including those formed on irradiation of native flavins (discovered by Warburg and Christian [1932]) and the synthetic members of this group [Stern and Holiday, 1934, 1]. For further material for comparison in respect to the spectral behaviour of toxoflavin and the photoflavins, the reader is referred to the paper by Holiday and Stern [1934].

There are also several points of biological interest which deserve discussion. Whereas it is shown in this paper that toxoflavin in accordance with its potential may act as a stimulant after respiration of mammalian red blood cells and may convert oxyhaemoglobin into methaemoglobin, flavins do not possess such properties [Wagner-Jauregg *et al.*, 1933; 1934].

According to the description given by van Veen, toxoflavin seems to be present also outside the bacterium in the culture medium, though linked up to a high molecular complex. On the other hand bacterial flavin pigments have been found exclusively inside the cell in a non-diffusible form. Their concentration seems to be highest in strictly anaerobic organisms [Warburg and Christian, 1933], whereas toxoflavin is said not to be formed under anaerobic conditions. Finally, the flavin pigments so far investigated have all been found to be non-toxic.

It appears therefore that whatever similarity may have brought about the tentative classification of toxoflavin among the flavin series it is at present so much outweighed by fundamental differences between toxoflavin and all the known flavins, that this assumption were better abandoned. To our mind toxoflavin appears to be a new and interesting bacterial product of yellow colour without structural or functional relationship to the flavin pigments. The neighbourhood of its normal potential to that of pyocyanin suggests a similar function, probably that of an accessory respiratory catalyst [*cf.* Friedheim, 1931]. It is not unlikely that *Bacterium bongkrek* apart from toxoflavin will be found to contain a real flavin pigment concerned with a different phase of metabolism.

SUMMARY.

Toxoflavin, the prosthetic group of a yellow pigment formed by *Bacterium bongkrek* (van Veen and Mertens) represents the oxidant of an oxidation-reduction system which is fully reversible and electro-active between p_H 4 and p_H 8. The normal potential, as referred to the normal hydrogen electrode, at p_H 7·0 is −0·049 v. The slope of the individual titration curves is atypical throughout the p_H range investigated and shows an increasing steepness towards the alkaline range. Though no colour change indicating semiquinone formation was observed, the experimental data have tentatively been subjected to theoretical interpretation in the light of the theory of Michaelis and Elema of two-step reduction. The relation of toxoflavin to the group of flavin pigments is discussed.

The author wishes to thank Prof. E. C. Dodds for his interest and encouragement. Furthermore he wishes to thank him and the Council of the Middlesex Medical School for the hospitality afforded.

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