tryptophan peroxidase, superimposed upon the slower removal of kynurenine by kynureninase lacking an adequate concentration of its coenzyme.

SUMMARY

1. Under optimal conditions, the average kynureninase activity of normal rat liver was 11·1 μmoles L-kynurenine removed/g. dry wt./hr. The activity in pyridoxine-deficient rats was decreased by lack of the coenzyme to 5·9 μmoles/g. dry wt./hr. The kynureninase was not increased adaptively in the animals by administration of kynurenine or tryptophan.

2. For comparison, kynurenine can be formed by the tryptophan peroxidase in normal rat liver at an average rate of 9·3 μmoles/g. dry wt./hr., and this rate can be adaptively increased tenfold by tryptophan administration to the animal.

3. The abnormal accumulation of tryptophan metabolites can therefore be attributed to a relative inadequacy of kynurenine removal, due to a decrease of kynureninase through lack of its coenzyme, or to an adaptive increase of the kynurenine-forming enzyme, tryptophan peroxidase, or to both. These mechanisms can account for the marked accumulation of metabolites in even normal animals given supplemental tryptophan, as well as in pyridoxine-deficient animals.

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Studies on the Polarography of Proteins

1. THE RELATION OF WAVE HEIGHTS TO PROTEIN CONCENTRATION AND THE ORIGIN OF WAVE III

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When a protein is added to a polarographic cell containing a buffer, the polarogram consists of a single large wave which begins to rise at about −1·8 V. with reference to the saturated calomel electrode (S.C.E.). This wave was identified as a protein wave and its characteristics were described by Heyrovský & Babička (1930); it was called the prenatrium wave by Herles & Vančura (1932). Brdička (1933*a*) accidentally discovered a second protein effect. If the electrolyte contained ammonia and either bivalent or trivalent cobalt in addition to the protein, the reduction wave of bivalent cobalt was followed by two waves (frequently referred to as a double wave) which were due to the protein (Fig. 1). These last waves will be referred to as waves I and II respectively. He found also that, of a series of amino-acids tested, only cystine or cysteine produced a similar effect, for in an ammoniacal

cobaltous chloride solution each of these compounds gave rise to a wave which was in the same position as protein wave II. For a given molarity, cystine produced a wave that was twice the height of the corresponding cysteine wave (Brdička, 1933*b*). Brdička concluded that the disulphide cystine is reduced electrolytically to yield two cysteine molecules and that the protein current is due to the reduction of hydrogen ions which are supplied by the sulphydryl groups. The amino-acid or protein acts as a hydrogen-ion carrier.

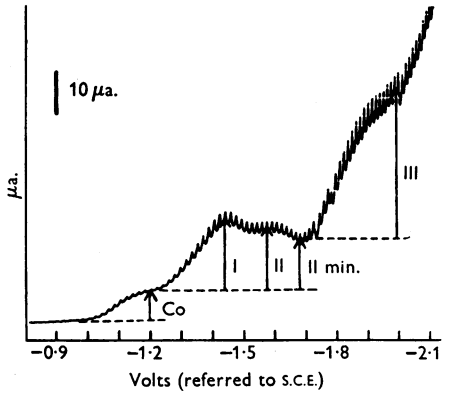

Fig. 1. A protein polarogram. Electrolyte contained bovine plasma albumin, 0·025 mg./ml., 0·5 M-NH_4Cl, 0·001 M-$CoCl_2$ and sufficient NH_3 to raise pH to 8·97. Arrows illustrate method of measurement of the components. Co is the reduction wave of $Co(H_2O)_6^{2+}$ and I, II and III indicate the three protein waves. $II_{min.}$ indicates the current at the minimum following wave II.

Some years later, Jurka (1939) pointed out that if the electrolysis was observed up to sufficiently high applied potentials, waves I and II were followed by a third wave (wave III) as shown in Fig. 1. By comparing the height of wave III with the height of the 'prenatrium' wave obtained in cobalt-free media, she showed that wave III of the complete polarogram in the presence of cobalt is the same as the 'prenatrium' wave.

The work described in the present series of papers arose from an independent discovery of wave III of insulin during an investigation of the effect of toxic metals on the polarographic protein waves. The author was not familiar with Jurka's paper at the time, and a series of experiments was initiated in order to establish that wave III was actually a protein wave and to study its characteristics. Brdička (1934) had shown previously that the relationship between the concentration of cystine and the height of the amino-acid wave followed a Langmuir adsorption curve. It was considered that if wave III gave a similar response then it was probably a protein wave also.

TERMINOLOGY

All ions do not produce a polarogram of ideal shape. The record of most metallic ions is distorted by a 'maximum' which is usually a spike-like formation superimposed on the polarographic wave. It may have a rounded, hump-like shape. In practice, a maximum is undesirable since it interferes with the measurement of the wave height, but it can usually be eliminated by the addition of surface-active compounds to the solution. Thus the cobalt polarogram exhibits a maximum which is suppressed when protein is added to the cell. Depending upon the protein concentration and the pH and concentration of the buffer, the individual three protein waves may or may not show a humped appearance as though there were one or more maxima. It has always been assumed that the humps that appear on the protein waves are maxima in the sense in which this word is used in polarographic terminology. The author submits that we do not know enough about the cause of polarographic maxima or of the origin of the protein waves to accept this view without question and that it is advisable to speak of protein wave 'peaks' rather than 'maxima' until the question is settled. Well known maximum suppressors such as methyl red and other dyes, methylcellulose or gelatin, will eliminate the maxima that appear on the polarograms of metallic ions, but these substances fail to suppress the peaks or 'maxima' of the protein waves. The whole wave may be depressed but the peak remains.

Protein waves I and II are usually measured from the level of the cobalt limiting current up to the peaks of the waves. The magnitude of the current, measured from the cobalt limiting current, at the minimum which follows wave II may be the diffusion current of wave II; in one of his early papers, Brdička (1933*b*) used this terminology for the comparable point on the cystine wave. According to custom, the attainment of a diffusion current denotes a state in which the concentration of reactive ion has been decreased almost to zero in the layer of solution surrounding the drop, so that the current is limited by the rate of diffusion of reactive ion to the electrode and by the concentration gradient between the bulk of the solution and the electrode surface. It seems preferable to refer to the minimum that follows wave II as a minimum rather than a diffusion current. The protein current may be limited by the diffusion rates of cobalt and, under some circumstances, by the rate of diffusion of ammonium ion, but it is a matter of opinion as to whether the rate of diffusion of protein is of significance. Cobalt and probably ammonium ion are removed at the electrode surface but the protein is probably involved in an adsorption equilibrium at the electrode-solution interface.

MATERIALS AND METHODS

Crystalline proteins commercially available were used for the study. Insulin was supplied by Dr Gordon Romans of the Connaught Medical Research Laboratories, Toronto. Pepsin, bovine plasma albumin, chymotrypsin and trypsin were obtained from Armour and Co., Chicago. Acetylated insulin was prepared by the method of Stern & White (1938) by passing keten into insulin suspensions (1 mg./ml.) in M-sodium acetate buffer (pH 5·7). The product was centrifuged, washed with acetone and ether, then dried in a desiccator over H_2SO_4. The proteins were dissolved in weak acid (about pH 3·5) and portions of this solution were added to the polarographic cell.

The borate buffer (pH 8) contained 0·05 M-H_3BO_3, 0·05 M-KCl and 0·004 N-NaOH.

The polarographic apparatus was assembled by the author and was of conventional design. A 'Speedomax' recorder (Leeds and Northrop Co., Philadelphia) was adapted for the measurement of current and a motor-driven Helipot potentiometer of 40 turns (Helipot Corp., S. Pasadena, Calif.) was used as a voltage divider. When the capillary was immersed in distilled water with a mercury pressure head of 50 cm., m (flow of mercury) = 1·73 mg./sec., t (droptime) = 4·07 sec. and the radius of the orifice was calculated as 23·7 μ. according to Müller (1944). The electrolyte was maintained at a temperature of 25·0° ± 0·2° and N_2 gas was used to displace dissolved O_2. All pH determinations were made with a model G Beckman pH meter.

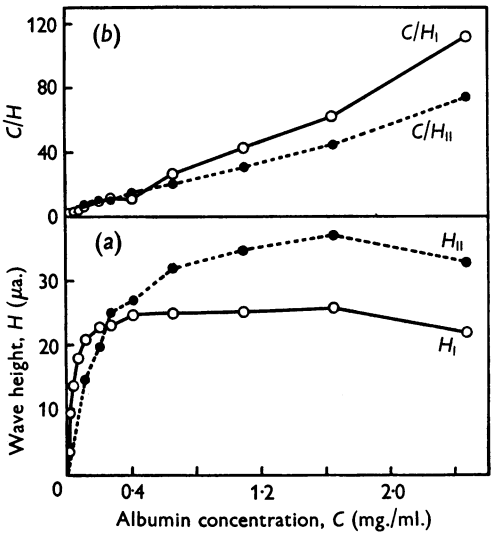

Fig. 2. (*a*) Relation between the heights of waves I and II (H_I and H_{II}) and the concentration of bovine plasma albumin. The electrolyte contained 0·001 M-$CoCl_2$, 0·1 M-NH_4Cl and sufficient NH_3 to give pH 9·6. (*b*) Relation between C/H and C to test for conformity to the Langmuir adsorption isotherm.

The method of measuring wave heights is illustrated in Fig. 1. In this paper and in those that follow, H_I and H_{II} refer to the magnitudes of waves I and II respectively, measured from the level of the cobalt limiting current up to the peaks of the individual waves. This is customary practice. H_{II} min. refers to the magnitude of the current at the minimum which follows the peak of wave II. H_{II} min. is also measured from the cobalt-limiting current. H_{III} refers to the magnitude of wave III which is measured from the minimum following wave II to the peak of wave III.

RESULTS

Relation between wave heights and protein concentration

The height of each of the three waves for a series of protein concentrations has been determined with crystalline insulin, pepsin, bovine plasma albumin, chymotrypsin and trypsin. The protein concentration was altered by progressive dilution in the polarographic cell. The solution initially contained a relatively high concentration of protein (of the order of 2·5 mg./ml.), 0·001 M-cobaltous chloride, 0·1 M-ammonium chloride and sufficient ammonia to raise the pH to 9·6. The diluting solution lacked protein but had the same concentrations of cobalt and ammonia buffer as the initial solution. The values for the wave heights obtained with one protein, bovine plasma albumin, are reported in

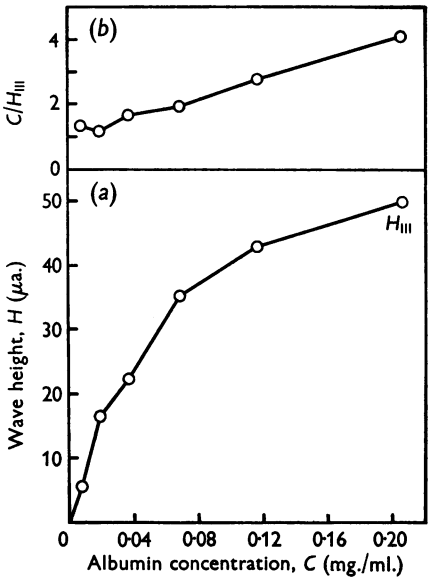

Fig. 3. (*a*) Relation between the height of wave III (H_{III}) and the concentration of bovine plasma albumin. The electrolyte contained 0·001 M-$CoCl_2$, 0·1 M-NH_4Cl and sufficient NH_3 to give pH 9·6. (*b*) Relation between C/H_{III} and C to test for conformity to the Langmuir adsorption isotherm.

Figs. 2 (*a*) and 3 (*a*). It is evident that the magnitudes of waves I and II (Fig. 2) increased to a maximum value with increasing concentration of protein, but that these waves were depressed when the concentration exceeded a value of about 1·65 mg./ml. At protein concentrations greater than about 0·20 mg./ml., the shape of wave III made its measurement very difficult, so that the concentration scale in Fig. 3 is limited and the curve does not show a maximal value. Other proteins yielded curves similar in form but with individual differences with respect both to the rate of rise to the peak value and to the protein concentration at which the peak occurred. Under the conditions of these experiments wave I of all proteins attained a maximal value but wave II did so only in the case of bovine plasma albumin and chymotrypsin.

If the relationship between protein concentration and wave height resembles a Langmuir adsorption isotherm it should have the following general equation

$$H=\frac{aBC}{1+aC}, \quad (1)$$

where H is the wave height (μa.), C is the concentration of protein (mg./ml.) and a and B are constants. A curve of this type approaches a limiting value $H_{max.}$ which is equal to B when C is large. The rate at which H approaches B is proportional to a. For practical purposes it is useful to rearrange the equation into the form

$$\frac{C}{H}=\frac{1}{aB}+\frac{C}{B}. \quad (2)$$

If a straight line results from a plot of C/H against C, then the data conform to the Langmuir type of equation and B and a can be calculated from the slope and intercept of the linear plot.

The concentration against wave-height curves obtained with the five proteins have been tested for conformity to equation (1). For illustration, the values of C/H against C for bovine albumin are given in the upper portions of Figs. 2 (*b*) and 3 (*b*). The points for waves I and II are reasonably linear up to a concentration of about 1·6 mg./ml. and wave III seems to follow equation 1 over the range in which this wave could be measured. Some proteins, such as insulin, give measurable waves at very low concentrations of protein and show a deviation from the Langmuir relationship in the low-protein range. Brdička (1933*b*) has observed this phenomenon with cystine and showed that it was due to adsorption of cystine on the walls of the electrolysis vessel.

The Langmuir constants B and a have been calculated for each curve. The constants vary considerably from one protein to another. Only in one instance did the actual wave height approach the theoretical maximum value, B. At the highest concentration of pepsin that was used (2 mg./ml.) the height of wave I was 22·2 μa. and B for this curve was 22·3. None of the other curves attained the maximum value.

Depression of the wave height at high protein concentration was observed by Tropp, Jühling & Geiger (1939) and these investigators suggested that dehydration of the protein might be offered as an explanation. Another possibility is that the diminished wave is due to the adsorption of more than one layer of protein at the electrode-solution interface, the second layer attaching itself in such a way as to interfere with diffusion processes. That this is not a valid explanation is evident from the calculation of B values. As the Langmuir equation has been applied, B is equivalent to $H_{max.}$ which is proportional to the mass of protein adsorbed/unit area of electrode surface when the protein concentration is high and the surface is saturated. Since the observed wave heights approached $H_{max.}$ only in one case (pepsin wave I), there is no evidence of the formation of a second layer. The failure of other curves to approach $H_{max.}$ values is not to be construed as an indication that the surface was unsaturated at high protein concentration. The wave height would be depressed even though the surface were saturated if the high concentration of protein interfered with the rate of one or more electrode reactions. The following relationship supports this possibility.

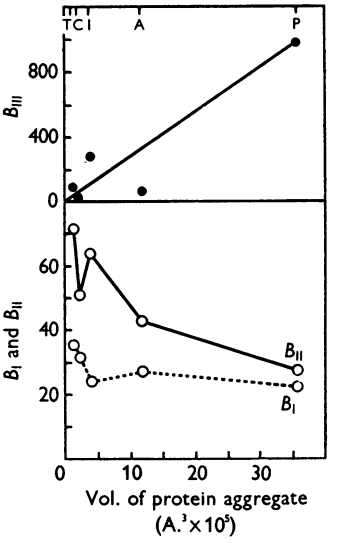

Fig. 4. The relationship between the volume of the protein aggregate and constant B of the Langmuir adsorption isotherm. Subscripts I, II and III refer to waves I, II and III respectively. T, C, I, A and P refer to trypsin, chymotrypsin, insulin, bovine plasma albumin and pepsin respectively.

When the adsorbed molecules are large we might expect to find that the molecular size or the aggregate size would determine B since the size would influence the number of molecules which could be crowded into a given area. In Fig. 4 the values obtained for B_I and B_{II} are reported and plotted against the volume of the protein aggregate as determined by X-ray diffraction (Fankuchen, 1945). The volume determined for horse serum albumin was used for the bovine plasma albumin studied. The volume of the trypsin aggregate was taken from Northrop, Kunitz & Herriott (1948).

B_I and B_{II} are small for proteins of large aggregate volume, as might be expected, since fewer large molecules can be accommodated on unit area of surface. However, comparison with the plot of

B_{III} against aggregate volume (Fig. 4) suggests another interpretation. Wave III does not require the presence of cobalt or ammonia for its development and B_{III} appears to become larger with increasing protein aggregate volume. The difference between the first two curves and that for B_{III} may be due to the fact that large aggregates cover the electrode surface more efficiently and thus exclude cobalt-ammonia complexes from the electrode, or it may mean that the larger molecules interfere with the diffusion of complexes in the bulk of the solution.

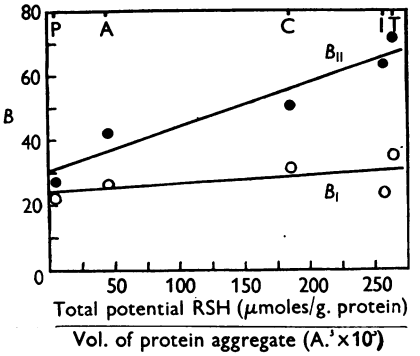

Fig. 5. The relation of the constant B of waves I and II to the ratio: total potential RSH/protein aggregate volume. P, A, C, I and T refer to pepsin, bovine plasma albumin, chymotrypsin, insulin and trypsin respectively.

On the basis of work reported in a following paper (Millar, 1953) the author is convinced that other protein groups in addition to sulphydryl groups are involved in the production of the protein current, but that the sulphydryl groups probably have the greatest influence in determining the magnitudes of waves I and II and thus must be determinants of B_I and B_{II}. In order to take this factor into account the various values of B have been plotted in Fig. 5 against the ratio (total potential RSH/vol. of aggregate), where 'total potential RSH' is the sum of the reported cysteine and half-cystine residues in a given protein (Tristram, 1949). The term represents the total number of sulphydryl groups that would appear if all the cystine residues were reduced and is expressed as μmoles RSH/g. protein.

In Fig. 5, the straight lines are 'best' fits calculated by the method of least squares. B_I and B_{II} seem to be related to the ratio, but whereas the coefficient of correlation between B_{II} and the ratio is 0·95 ($P = 0·02$), the coefficient between B_I and the ratio is only 0·57 ($P = 0·5$) so that a relationship is not proved as far as B_I is concerned. The relationship between B_{III} and the ratio is not apparent. Indeed, the plotted points are widely scattered and suggest that groups other than sulphydryl groups are important determinants of the magnitude of wave III.

The origin of wave III

During the early stages of this investigation it was assumed, as a working hypothesis, that each of the three waves represented the release of hydrogen from three different protein side chains. If this assumption is valid, then it is reasonable to expect that the form of the polarogram could be altered if certain reactive protein groups were conjugated.

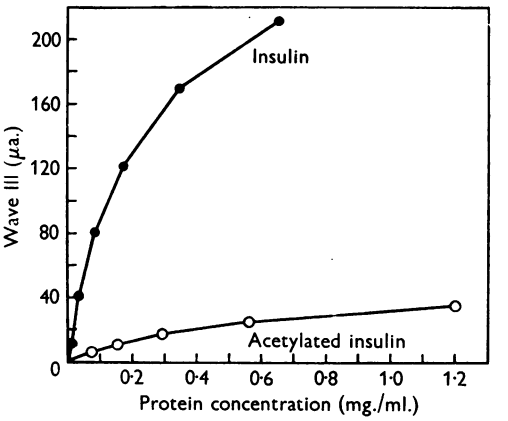

Fig. 6. The effect of acetylation of the amino groups of insulin on the height of wave III. The electrolyte contained insulin or acetylated insulin at the concentrations indicated, 0·001 M-$CoCl_2$ and ammonia buffer at pH 9·25 (0·1 M-NH_4Cl and 0·1 M-NH_3).

Stern & White (1938) reported that insulin can be treated under mild conditions with keten gas in such a way as to acetylate free amino and phenolic hydroxyl groups. Amino groups are conjugated easily by treatment for 5 min. After 45 min. phenolic groups are affected, but they are not completely blocked until acetylation has proceeded for 30 hr. Polarograms of acetylated insulin prepared in this way were obtained for different concentrations of the protein. When the heights of the three waves were plotted against the protein concentration, the curves for acetylated insulin lay below those for insulin. The effect of acetylation on wave III is shown in Fig. 6. Acetylation of the protein for 5, 10 or 60 min. gave essentially the same curves and the data for these acetylated products were combined in order to calculate Langmuir equations to be compared with the equations for untreated insulin.

Exposure to keten diminished the value of the constant B by 1·1, 4·5 and 80·3 % for waves I, II and III respectively, and the values of a were diminished by 86·2, 62·2 and 71·9 %.

Since no wave was entirely abolished by acetylation another experiment was designed to determine whether the residual waves were due to hydrolytic removal of acetyl groups in the ammoniacal

electrolyte of the polarographic cell. Acetylated insulin at a concentration of 0·097 mg./ml. was allowed to stand in cobalt-ammonia buffer. Three polarograms of this solution were recorded 0, 160 and 385 min. after addition of the protein to the cell. During the period at alkaline pH the heights of waves I and II decreased 1 and 5·5 % respectively, while wave III increased 22·5 %. Thus, after 6 hr., wave III of the acetylated product was still only about one-quarter as large as the corresponding wave of insulin would have been.

These results indicate that free amino groups are involved to a considerable degree in the development of wave III, and confirmation of this evidence was sought in the repetition of an experiment described by Brdička (1936). Successive 0·1 ml. portions of 0·01 M-cystine in 0·1 N-sodium hydroxide were added to the polarographic cell, which contained 10 ml. of boric acid-sodium hydroxide buffer of pH 8·0. A polarogram was recorded after each addition. Since the system lacked both cobalt and ammonia, the catalytic sulphydryl wave which corresponds to protein wave II would not be anticipated. The polarogram of the buffer alone was flat until the discharge of sodium at about $-1·9$ V., at which point the current rose sharply. The second record, obtained after the addition of 0·1 ml. of cystine solution to the cell (final cystine concentration 10^{-4} M) was flat until about $-1·7$ V., then it too rose sharply. After five further additions of cystine, the current began to rise at about $-1·6$ V. It was evident that 10^{-4} M-cystine caused the final discharge curve to shift about 0·2 V. to a more positive potential. Brdička obtained the same result and attributed the shift to the sulphydryl groups of cysteine which had been formed by the electrolytic reduction of cystine. Cystine did not produce a well defined 'prenatrium' wave, but Brdička interpreted the shift of the sodium current as being equivalent to a 'prenatrium' wave and concluded that the 'prenatrium' wave is another sulphydryl wave. However, there is no positive evidence to show that the shift was not caused by the amino or carboxyl groups of the amino-acid. Accordingly, the amino groups of cystine were blocked by preparing *NN'*-bis(2:4-dinitrophenyl)cystine according to the method of Abderhalden & Blumberg (1910). Since the disulphide of this conjugated product would be reduced to sulphydryl groups at the electrode, the latter groups could exert their effect in producing a shift of the sodium current but the action of the amino groups would be prevented. When the derivative was added to the boric acid-sodium hydroxide buffer a wave due to the nitro groups appeared with a half-wave potential of about $-0·5$ V. At a concentration of 10^{-4} M, no shift of the sodium current could be detected. However, when the concentration was doubled a typical 'prenatrium' wave could be discerned which became more distinct and larger at higher concentrations. When ammonia gas was bubbled into the solution to pH 8·7 and cobaltous chloride added to a final concentration of $6·8 \times 10^{-4}$ M, the 'prenatrium' wave was preceded by a large catalytic sulphydryl wave. Subsequent tests in which 1-fluoro-2:4-dinitrobenzene was added to the buffer explained these results. The reduction of the nitro groups on the benzene rings of the conjugate yielded amino groups (Astle & McConnell, 1943) and the 'prenatrium' wave was caused by the liberation of hydrogen from these newly formed groups.

A group of nitrogenous substances has been tested to determine whether this property is characteristic of these compounds. The results are summarized in Table 1.

Table 1. *The effect of some nitrogenous compounds on the potential at which sodium ion is discharged*

(0·1 ml. of 0·01 M solution of each compound was added to 10 ml. of borate buffer (0·05 M-H_3BO_3, 0·05 M-KCl, 0·004 N-NaOH; pH 8).)

L-Lysine monohydrochloride	No effect
Methylamine hydrochloride	No effect
1-Aminopropan-2-ol	No effect
Aniline	No effect
L-Histidine monohydrochloride	No effect
DL-Alanine	No effect
m-Aminoacetanilide	No effect
p-Bromoaniline*	No effect. 0·2 V. shift at pH 6·5
L-Cystine†	0·2 V. shift
Gelatin	0·2 V. shift
2:5-Diaminotoluene dihydrochloride	Catalytic wave
NN'-Bis(2:4-dinitrophenyl)cystine‡	Catalytic wave
1-Fluoro-2:4-dinitrobenzene	Catalytic wave
Pyridoxamine hydrochloride	Reduction wave at $-1·45$ V. plus catalytic wave

* Saturated solution (?) at final concentration.
† 0·0025 % (w/v) final concentration.
‡ 2×10^{-4} M final concentration.

Under the conditions of the test, some had no influence, some shifted the sodium discharge to more positive potentials while others exhibited a true catalytic wave which resembled a protein 'prenatrium' wave. When a catalytic wave appeared, the pH of the electrolyte was altered by the addition of hydrochloric acid or sodium hydroxide. Enhancement of the wave by addition of acid and depression by alkali addition was used as a criterion of its catalytic nature. The electrolyte containing 2:5-diaminotoluene became brown so that the effect may be due to the oxidized form. Brdička stated that gelatin would produce no shift because it contains no cystine, but the author found that it did so when the gelatin concentration was 0·0025 % (w/v).

If wave III or the 'prenatrium' wave depends upon amino groups then the addition of formaldehyde to the protein-buffer system should depress the wave, since formaldehyde combines reversibly with amino groups. Formaldehyde itself is reduced at the dropping mercury electrode and the formaldehyde wave appears at voltages which interfere with the protein waves, so that the formaldehyde concentration must be minimal. The following experimental conditions were arranged to test the effect of formaldehyde. The 'prenatrium' wave of bovine plasma albumin (0·070 mg./ml.) was recorded in a solution of 0·1M-boric acid and 0·1M-sodium chloride.

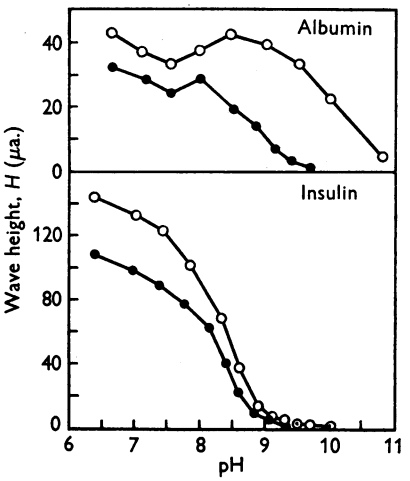

Fig. 7. The effect of formaldehyde on the 'prenatrium' waves of bovine plasma albumin and insulin at various values of electrolyte pH. O—O, electrolyte contained initially 0·1M-H_3BO_3, 0·1M-NaCl and 0·070 mg. protein/ml.; ●—●, same plus $3·7 \times 10^{-4}$M-formaldehyde. pH was altered by adding 4N-NaOH to the initial electrolyte.

Successive additions of 4N-sodium hydroxide altered the pH and a recording was made after each addition. A second experiment was similar to the first except that the solution contained, in addition, formaldehyde at a concentration of $3·7 \times 10^{-4}$M. The results are shown in the upper portion of Fig. 7. In the presence of formaldehyde the 'prenatrium' wave is depressed over the entire pH range investigated. Below pH 8 it was evident that the wave was made up of more than one component and the shape of the polarogram made measurement of the wave difficult. For this reason it is impossible to determine whether the dip in the curves at pH 7·5 has any significance, although it occurred in both experiments.

Fig. 7 also shows the results of two experiments using insulin rather than plasma albumin. The insulin waves could be measured very easily. Again the wave was depressed in the presence of formaldehyde.

DISCUSSION

Brdička (1947) has pointed out that conformity of the wave height-protein concentration curve to the Langmuir adsorption isotherm does not necessarily prove that the protein is adsorbed at the electrode surface since formal fulfilment of the equation might be the result of the rate of an electrode reaction which determines the height of the wave. This possibility must be acknowledged, but, until more is known of the electrode reactions involved in the catalytic protein waves, the implications of the Langmuir equation have been accepted at face value.

The typical polarographic protein response first shown by Brdička (1933*a*) is obtained only in the presence of cobalt and ammonia, and it is probable that it is the presence of the cobaltous hexammine ion, $Co(NH_3)_6^{2+}$, that is responsible for the effect. Brdička (1933*b*) has suggested that co-ordinate linkage between cobalt and the sulphur of sulphydryl groups allows the splitting of hydrogen from the sulphur so that it may be reduced at the electrode. It is probable, therefore, that proteins of large aggregate size depress the protein current by interfering with the free diffusion of cobalt-ammonia complexes.

It was gratifying to find a direct relationship between B and total potential RSH/vol. of aggregate, and yet this result is surprising for it suggests that all the potential sulphydryl groups of the protein are available polarographically. The same conclusion is drawn from other data presented in a subsequent paper (Millar, 1953).

The three values of a for acetylated insulin were small compared with those for the untreated protein. In the original development of the equation (Langmuir, 1918) a is proportional to the fraction of molecules of adsorbate striking the surface which remain adsorbed, so we may assume that the free amino groups of the protein enhance the tendency of the protein to be adsorbed at the electrode surface. For this reason the wave height-protein concentration curves of waves I and II were depressed following acetylation, but the constants B_I and B_{II} were only slightly affected, and we may conclude that the influence of amino groups on waves I and II is indirect through their influence on the adsorption process. In contrast to these results, B_{III} was depressed by 80% following acetylation and we may conclude that amino groups affect wave III through their influence on the adsorption process and also because they are involved in the electrode reactions which produce the wave III current. It is suggested, tentatively, that wave III depends on the following reactions:

$$RNH_3^+ + e \rightarrow RNH_2 + H, \quad (1)$$

$$RNH_2 + H_3O^+ \rightleftarrows RNH_3^+ + HOH. \quad (2)$$

These reactions are analogous to those proposed by Brdička (1933*b*) to explain the catalytic waves of cystine, cysteine and protein waves I and II. Reaction (1), the electrolytic reduction of hydrogen supplied by charged amino groups, provides the current of wave III. By reaction (2) the buffer regenerates charged amino groups which may participate once more in reaction (1). This interpretation is compatible with the known effect of altered buffer pH on this wave. Brdička (1936) found that the 'prenatrium' wave was enhanced by lowering the pH of the buffer.

The appearance of 'prenatrium' waves in the polarograms of many organic compounds confirms the interpretation presented above and shows that the 'prenatrium' wave is not a specific protein effect but that it is associated with nitrogenous groups. This hypothesis is supported by the work of Kirkpatrick (1947), although his data did not lead him to this particular conclusion. Kirkpatrick investigated the polarographic response of a series of alkaloids and he showed that many of these compounds produce catalytic waves which resemble protein 'prenatrium' waves.

Not all nitrogenous compounds yield 'prenatrium' waves and some do no more than shift the discharge curve of the alkali to more positive potentials, so that it is probable that the proximity of neighbouring groups and the presence of conjugated bonds are factors which determine the response. A point of particular interest, however, is that the presence of a sulphydryl group is not a prerequisite for the formation of a wave with the characteristics of a protein 'prenatrium' wave.

It will be recalled that acetylation of insulin did not completely eliminate wave III. The residual wave cannot be explained at present. Exposure of the acetylated product to the alkaline electrolyte for 6 hr. produced a slow enhancement of wave III, but the simultaneous gradual depression of waves I and II suggests that these changes were due to a small change in the pH of the electrolyte during the exposure period. There is no evidence that the residual wave III is due to the liberation of amino groups by hydrolysis unless acetate groups are removed very readily from a certain proportion of the amino groups during the recording of the first polarograms. It is possible that wave III depends on the presence of two different protein groups, one of which is blocked by short exposure to keten, perhaps an amino group, and another which cannot be blocked under these conditions and which is responsible for the residual wave of the acetylated product.

It is of interest to note that the insulin curves of Fig. 7 are shifted to the left with respect to the curves of plasma albumin. So far, it has not been possible to correlate the position of the curves with the amino-acid content of the proteins.

SUMMARY

1. Polarograms have been recorded in 0·001 M-cobaltous chloride and ammonia buffer at pH 9·6 for the following proteins: insulin, pepsin, bovine plasma albumin, chymotrypsin and trypsin. The heights of all waves are reported.

2. The Langmuir adsorption isotherm equations have been calculated for the curves relating the wave height to the protein concentration for the three waves I, II and III.

3. The relation between the Langmuir constant B and the volume of the protein aggregate suggests that the protein interferes with the diffusion of cobalt-ammonia complexes when the protein concentration is high and that the magnitudes of waves I and II are limited by the size of the protein aggregate.

4. B_{II} is linearly related to the ratio (total potential sulphydryl groups/vol. of protein aggregate) which implies that all the potential sulphydryl groups of the protein are available polarographically.

5. Acetylation of insulin amino groups by ketene depresses the apparent adsorption of the protein on the electrode surface.

6. The depression of B_{III} by acetylation of the protein or by the addition of formaldehyde to the electrolyte and the appearance of 'prenatrium' waves in the polarograms of certain organic compounds suggest that wave III depends upon the presence of nitrogenous groups rather than sulphydryl groups.

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Studies on the Polarography of Proteins

2. THE INFLUENCE OF pH AND CONCENTRATIONS OF AMMONIA AND AMMONIUM ION UPON THE POLAROGRAPHIC WAVES

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If a protein is dissolved in a simple buffer, the polarogram of the system consists of a single large catalytic wave known as the 'prenatrium' wave (Heyrovský & Babička, 1930). Brdička (1933*a*) reported that if the protein was in an ammoniacal cobalt solution, the protein produced two waves which followed the cobalt reduction step. Later, Jurka (1939) found that the complete polarogram in ammoniacal cobalt solutions consisted of three waves which are referred to here as waves I, II and III respectively. Wave III has the same characteristics as the 'prenatrium' wave. It does not require the presence of cobalt.

Brdička (1933*a*) tested the effect of increasing the concentration of either the ammonia or ammonium chloride of the buffer in order to throw some light on the controlling factors of the system. When the concentration of either of these buffer components was increased, he observed enhanced protein waves. Cystine or cysteine in the same electrolyte produced a single wave at the same voltage as the second component (wave II) of the protein record, and he observed that alteration of the concentrations of the individual buffer constituents had an enhancing effect upon the amino-acid wave also (Brdička, 1933*b*). It is evident that the changes which Brdička observed had a complicated origin since his procedure altered the pH of the medium as well as the concentrations of the buffer components. The present author's interest in the effect of the concentration of the buffer components arose from a desire to test the effect of the electrolyte pH on the polarograms of proteins. It is obvious that the two questions are linked. Brdička (1936) studied the effect of altering the electrolyte pH upon the 'prenatrium' wave, but its influence upon waves I and II has not been reported.

METHODS

The polarographic instrument and proteins used are described in the previous paper (Millar, 1953). Electrolyte pH values were measured with a Beckman Model G pH meter which was provided with a type 1190 E glass electrode for the major portion of the work. In the early experiments, portions of the solution were removed from the electrolytic cell, placed in the glass-electrode vessel for a pH determination, then returned to the cell. Later, a polarographic cell was constructed which would accommodate the pH-meter electrodes. These were connected to the pH meter by 30 in. shielded leads. The following procedures were used.

(*a*) The pH of an ammonia buffer depends upon the equilibrium $NH_4^+ \rightleftarrows NH_3 + H^+$ so that $[H^+]$ can be maintained at a relatively constant value and various concentrations of ammonium ion and ammonia used if $[NH_4^+]$ and $[NH_3]$ are maintained in constant proportion to one another by dilution of the buffer. The results reported in section A were obtained with this method. 0·001 M-$CoCl_2$ and 1·52 M-