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Thermodynamic Quantities for the Dissociation of Three Ferrihaemoproteins from Soya-Bean Nodules

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A spectrophotometric study of the two primary ferrihaemoproteins isolated from soya-bean root nodules provided evidence for multiple haem-linked ionizations (Thorogood, 1963). Though there is considerable overlap in these ionizations, the main dissociation extending from pH 6 to 10 was delineated in both pigments, and it was argued that the pK for the latter ionization could be obtained under stipulated conditions. If this were so, it would permit comparison with similar ionizations occurring in vertebrate ferrihaemoglobins.

The nodule pigments are acidic proteins with one haem unit per molecule. By analogy with the vertebrate haemoglobins, it is assumed that in aqueous solution a water molecule is co-ordinated to the haematin $Fe(III)$ atom; in alkaline solution this hydrate is presumed to dissociate (eqn. 1) so

that the formal charge on the $Fe(III)$ atom changes from +1 to 0. The apparent ionization constant is given by eqn. (2), where the ratio of the concentration of base to that of acid is measured spectrophotometrically, and the H^{+} (H_3O^{+}) ion activity,

$$K = \alpha_{H^{+}} \cdot [Fe \cdot OH] / [Fe^{+} \cdot OH_2] \quad (2)$$

$\alpha_{H^{+}}$, is obtained from pH measurements. Though this value of K is expected to vary with I , it will approach the thermodynamic constant, K^0 , as I tends to 0.

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Thermodynamic parameters have now been evaluated for this ionization in the two major ferrihaemoproteins of soya-bean root nodules, and a partial study has been made of a third ferrihaemoprotein component not previously examined. The technique used is that applied to horse ferri-myoglobin (George & Hanania, 1952) and horse ferrihaemoglobin (George & Hanania, 1953). The thermodynamic and charge properties deduced from this study, together with the oxygenation property (Appleby, 1961), appear to justify the use of the term 'haemoglobin' to describe one attribute of the nodule pigments. The term does not take into account the observed tendency of the pigments to discharge an electron ($Fe^{2+} \rightarrow Fe^{3+} + e$).

EXPERIMENTAL

Preparation of pigments. Over a 10-day period, 2.5 kg. of soya-bean nodules, *Glycine max* var. Hawkeye, were collected and extracted with precautions to minimize deterioration (Thorogood, 1963). The nodules, grown in Champaign County, Ill., U.S.A., were picked directly into ice-water in the field. Four-hourly collections were brought to the laboratory and homogenized, in a Waring Blendor, in cold 0.05 M-sodium veronal containing ice chips. Block-tin-distilled water was used for the extraction and purification procedures and, unless otherwise stated, all manipulations were carried out at 5°. The solution was clarified by centrifuging (at 16 000g for 1 hr.) and the water-soluble pigment (kept at 0° to -5°) was precipitated with ammonium sulphate at a concentration of from 26 to 31% (w/w). The red precipitate was collected by centrifuging (at 16 000g for 15 min.) and frozen. When all the nodules were extracted, the accumulated frozen precipitates were dissolved

in water, combined, dialysed free of salt and concentrated in the dialysis sac by leaving the sac at 2° in a refrigerator and repeatedly shelling the ice that formed on it as water leaked through the pores. The concentrate was fractionated by paper electrophoresis (sodium veronal buffer, pH 8.5, I 0.05) at -2° to 5°.

Five ferrihaemoproteins were so resolved. If these are numbered in order of decreasing mobility, 1, 2 and 3 were components of a fast-moving sequence and 4 and 5 formed a slower one. The major components were 3 and 5. The separated components were eluted with water, dialysed free of salt, concentrated and stored at 5°. The three components that were analysed are designated slow (5), fast (3) and minor fast (2). Since only one pigment was analysed spectrophotometrically at a time, work was planned so that each component would be reprocessed by paper electrophoresis and dialysis (a 2-day procedure) immediately before its analysis, to ensure the removal of any newly formed green pigment and, thus, the utmost protein homogeneity. Each haemoprotein analysis was begun on what could be called a 2-day-old (electrophoretic) pigment preparation. The experimental study was completed within 3 months of the nodule harvest.

Buffer solutions. De-ionized water redistilled from KMnO_4 was used in preparing the buffers. No attempt was made to free the water of dissolved CO_2 , since accurate measurements were not made above pH 9.5. To cover the pH range 6–10, concentrated stock buffer solutions of phosphate (NaH_2PO_4 – Na_2HPO_4), veronal (sodium diethylbarbiturate– HCl), borate (boric acid– NaOH) and carbonate (NaHCO_3 – Na_2CO_3) were used. Because of the limiting solubilities of the buffer salts, the solutions were adjusted to I 0.4 with NaCl . These stock buffers were appropriately diluted to give the desired I for each series of measurements except those below I 0.01. Here a second series of buffers was prepared without NaCl , so that the maximum buffer capacity would be available. The determination of total I in the very dilute solutions required an estimate of the contribution from those charged groups on the haemoprotein molecule which would influence the immediate environment of the Fe atom. In mammalian ferrihaemoglobin solutions (10 μM), the upper limit to this contribution was estimated as I 0.001 by George & Hanania (1953). In the present paper the same value has been added to the I of the buffer in every case.

Spectrophotometric measurements. A Beckman model DU quartz spectrophotometer was used, with a pair of matched silica cuvettes of 1 cm. light-path. Constant temperatures, within 0.2°, were maintained in a water-jacketed cell compartment. The ratio of the concentration of acidic ferrihaemoprotein to that of alkaline ferrihaemoprotein was obtained by comparing the extinction at 404 $m\mu$ with the extinctions of the hydroxide complex (pH approx. 10) and the hydrated complex (pH approx. 6) at the same pigment concentration, according to the procedure of George & Hanania (1952).

Measurements of pH. These were carried out with a Beckman model G-2 pH-meter. Constant temperature, within 0.2°, was maintained by circulating water through a jacketed glass cup used for the measurement of pH. The instrument was standardized by the method of Bates, Pinching & Smith (1950). The pH of every solution was measured immediately after the extinction was measured, a procedure especially important at very low I . Occasion-

ally a test solution would give an abnormal pH reading, and the measurement was then discarded.

Determination of ionization constants. The ferrihaemoproteins were studied at a number of values of I and at several temperatures. At the higher values of I , pigment–buffer mixtures were prepared in advance over the desired pH range (6–10) at constant I , and were placed in a temperature-controlled water bath. Then, as rapidly as possible, extinction and pH values were read. However, at I values below 0.01 the buffer and pigment stock solutions were stored separately in the water bath, were diluted and mixed individually, and measurements were made without delay.

Because of the overlapping ionizations at below pH 8, the extinction of the pure acid ferrihaemoglobin species could not be measured directly (Thorogood, 1963). Likewise, because of the sensitivity of the protein to alkaline denaturation above pH 10, no direct measurement of the extinction of the pure conjugate base could be made. Accordingly, an indirect method was followed. The extinction of a water solution of pigment (pH approx. 6) was taken to represent the approximate 100% acid species, and the extinction of a buffered alkaline solution (pH slightly above 10) was taken to represent the approximate 100% conjugate base. Within these limits, pK values were calculated for each pigment–buffer mixture. However, though these values agreed within the mid-portion of the pH region, they usually drifted as they approached the acid or the alkaline end points. To counteract the drift, E values for the acid or alkaline end points or both were shifted slightly, and a new series of pK values was calculated by using the revised base:acid ratios obtained from the intermediate E values. After the testing of several such numerical approximations to the true end points, the drift in pK values was eliminated and a pK series showing a satisfactory agreement at all the measured pH values was obtained. In most cases, the mean deviation (the arithmetic mean of all deviations from the average value) of six or more pK determinations in a series was ± 0.02 . Thus, though true end points for the ionization could not be measured spectrophotometrically, the assumption is made that the mathematical operation described gives reasonably approximate end points and hence gives reasonably accurate pK values.

RESULTS

Determinations of pK were made for each of the nodule haemoproteins over a range of pH (7.5–9.5), I (0.450–0.001) and temperature (16.0°, 25.0° and 37.0°). The results are summarized in Table 1. To obtain the thermodynamic ionization constants for the three pigments, the pK values in Table 1 were plotted against a Debye–Hückel type of function (eqn. 3), where 0.001 is the estimated contribution to I from the protein itself. The resulting

$$f(I) = (I + 0.001)^{\frac{1}{2}}/[1 + (I + 0.001)^{\frac{1}{2}}] \quad (3)$$

curves (Figs. 1 and 2) were extrapolated linearly to I equals 0, giving pK^0 values which presumably approximate to the true thermodynamic constants.

Fig. 1 presents the curves obtained at three temperatures for the slow component. The curves

Table 1. *Soya-bean-nodule haemoproteins: variation of pK with ionic strength and temperature*

Experimental details are given in the text. The function $f(I)$ was obtained by eqn. (3), and pK values were calculated according to eqn. (2). Each pK is the arithmetic mean of six or more determinations, and is followed by the mean deviation. The temperature of the pigment solutions was controlled to 0.2° . The pigment concentration for observation of the γ peak at $404\text{ m}\mu$ was approx. $5\text{ }\mu\text{M}$.

Ionic strength (I)	$f(I)$	Slow component			Fast component		Minor fast component 25.0°
		16.0°	25.0°	37.0°	16.0°	25.0°	
0.001	0.032	8.50 ± 0.01	8.44 ± 0.02	8.38 ± 0.01	8.58 ± 0.02	8.51 ± 0.01	8.55 ± 0.02
0.002	0.044	8.48 ± 0.02	8.42 ± 0.02	8.35 ± 0.02	8.55 ± 0.02	8.49 ± 0.02	8.52 ± 0.01
0.003	0.053	8.47 ± 0.02	—	—	—	8.47 ± 0.01	—
0.005	0.067	8.44 ± 0.02	8.38 ± 0.02	8.30 ± 0.02	8.50 ± 0.01	8.44 ± 0.02	8.48 ± 0.01
0.007	0.078	—	—	—	—	—	8.48 ± 0.02
0.010	0.092	8.43 ± 0.02	8.35 ± 0.02	8.28 ± 0.02	8.47 ± 0.02	8.40 ± 0.01	8.47 ± 0.02
0.050	0.184	8.39 ± 0.02	8.32 ± 0.02	—	8.39 ± 0.02	8.37 ± 0.01	—
0.100	0.241	8.39 ± 0.02	8.32 ± 0.02	—	8.37 ± 0.02	8.36 ± 0.01	8.44 ± 0.02
0.200	0.309	—	8.32 ± 0.02	—	8.38 ± 0.02	—	—
0.450	0.402	—	8.36 ± 0.02	—	—	—	—

Fig. 1. Variation of pK with $f(I)$ and temperature in the slow component isolated from soya-bean nodules. The temperatures used were: A, 16° ; B, 25° ; C, 37° . The data are from Table 1. The curves are extrapolated linearly to I equals 0 to give values for pK^0 .

are linear in the region well below I equals 0.010. At 16.0° , the limiting linear slope is about -1.6 and the pK^0 is 8.55 ± 0.02 . At 25.0° , the corresponding slope is again about -1.6 and the pK^0 is 8.49 ± 0.02 . At 37.0° , the slope is about -1.8 , and the pK^0 is 8.43 ± 0.02 . The average enthalpy for the ionization of the slow component (Table 2) was calculated from the three pK^0 values, assuming constant heat capacity over the range of temperature covered (eqn. 4). The average entropy of

$$\Delta H^0 = 2.303Rd(pK^0)/d(1/T) \quad (4)$$

ionization (Table 2) was calculated (George & Hanania, 1952) from ΔH^0 and pK^0 at 25.0° (eqn. 5).

$$\Delta S^0 = (\Delta H^0 - \Delta G^0)/T \quad (5)$$

Table 1 also lists the pK values calculated for the fast component at two temperatures. By plotting

pK against $f(I)$, a pK^0 of 8.63 ± 0.02 was obtained at 16.0° , and a pK^0 of 8.57 ± 0.02 at 25.0° . Both limiting linear slopes are about -1.8 . The thermodynamic parameters were calculated as for the slow component.

The minor fast component was studied at 25.0° . A pK^0 of 8.62 ± 0.02 was obtained at this temperature, and a limiting linear slope of approximately -2.2 .

In Fig. 2, results for the three nodule pigments at 25.0° are collected to show the similarity in the effects of I . There is, however, a gradation in the values for the limiting slopes which may be significant (-1.6 for the slow, -1.8 for the fast, and -2.2 for the minor fast component). One possible interpretation of this fact is given below.

DISCUSSION

With the detailed results obtained, it is possible to compare the ionizations of root-nodule ferrihaemoproteins with those of mammalian ferrimyoglobin (George & Hanania, 1952, 1957) and ferrihaemoglobin (George & Hanania, 1953). Table 2 summarizes the thermodynamic constants of these five pigments. It also includes the available data on the haemoglobin of *Chironomus plumosus* blood (Scheler & Fischbach, 1958). The ionization processes in the plant and mammalian pigments are similar; ionization constants are close, and there is small positive enthalpy with large negative entropy. There is an approximately linear relationship between ΔH^0 and pK^0 : enthalpy increases as Table 2 is descended. Thus the slow nodule component is the strongest acid with respect to this particular ionization, and myoglobin the weakest. Yet, of the three nodule pigments, the slow component is the least acidic electrophoretic component, as its name implies. The most acidic

Table 2. Standard thermodynamic data relating to the ionization of the three pigments isolated from soya-bean nodules, of ferrimyoglobin and ferrihaemoglobin obtained from horse heart and blood respectively and of *Chironomus ferrihaemoglobin*

Experimental details are given in the text. Values cited refer to 25.0°, either directly or by interpolation between two temperatures. Limits of error, where shown, are $\pm$ mean deviations. The effective unit charge on the acidic ferrihaemoprotein, z , and the critical ionic strength are defined in the text.

Ferrihaemoprotein	pK^0	ΔH^0 (kcal./mole)	ΔS^0 (e.u.)	z	Isoelectric point	Critical ionic strength
Soya-bean-nodule pigments						
Slow component	8.49 ± 0.02	2.20 ± 0.75	-31.5 ± 2.5	-1	4.7	0.05
Fast component	8.57 ± 0.02	2.60 ± 0.90	-30.5 ± 3.5	-1	4.4	0.05
Minor fast component	8.62 ± 0.02	—	—	-2	<4.4	—
Horse-blood ferrihaemoglobin	8.81 ± 0.02	3.91 ± 0.49	-27.2 ± 1.8	-2	7.7	0.2-0.3
Horse-blood ferrimyoglobin	8.97 ± 0.02	5.75 ± 0.67	-21.6 ± 2.4	-2	7.0	0.01
<i>Chironomus</i> -blood ferrihaemoglobin	8.19	3.8	-25	-4	—	—

Fig. 2. Comparison of the variation of pK with $f(I)$ at 25.0° in three ferrihaemoproteins isolated from soya-bean nodules: S (slow component), F (fast component) and MF (minor fast component). The data are from Table 1. Linear extrapolation to I equals 0 gives values for pK^0 .

electrophoretic component, the minor fast, has the highest pK^0 of the nodule pigments but it cannot be compared with the mammalian pigments, which are considerably more alkaline proteins having still higher pK^0 values. The net electrophoretic surface charge, therefore, does not appear to influence the dissociation significantly.

The root-nodule haemoproteins are further characterized by the effect of neutral salts (i.e. I) on their ionization. Fig. 3 shows the relationship between pK and $f(I)$ for the slow nodule pigment, the mammalian pigments and the *Chironomus*-blood pigment. Though the curves differ in shape, at sufficiently low I the pK increases with decreasing I in all four cases. In terms of a simple (limiting) Debye-Hückel model this implies that, so far as this ionization is concerned, all these haemoproteins have an effective negative charge even though the formal charge on the Fe(III) atom is +1 changing to 0. The magnitude of this so-called

Fig. 3. Variation of pK with $f(I)$ for four haemoproteins. Experimental details are given in the text. M: Ferrimyoglobin (horse-heart muscle) at 20.0°; pK^0 , 9.03 ± 0.02 ; limiting linear slope, -2.3. H: Ferrihaemoglobin (horse blood) at 20.0°; pK^0 , 8.86 ± 0.02 ; limiting linear slope, -2.8. S: Slow component from soya-bean nodules at 25.0°; pK^0 , 8.49 ± 0.02 ; limiting linear slope, -1.6. C: Ferrihaemoglobin (*Chironomus plumosus* blood) at 20.0°; approximate pK^0 , 8.32; limiting linear slope, -4.7.

'ionic strength charge' (Barnard, 1944) is estimated from the limiting linear slope of each curve, assuming that a limiting Debye-Hückel equation applies to this specific ionization. On this basis, the unit charge on the acidic slow nodule component is -1, and consequently -2 on its conjugate base. The corresponding charge on both acidic mammalian pigments is -2, or -3 on the conjugate

bases. As the data on *Chironomus* haemoglobin include no limits of error, the only guide to extrapolation is the curve itself, passing through the four points of Fig. 3, and this leads to a unit charge of -4 on the acid, or -5 on the conjugate base. That the effective charge on the iron atom of the nodule pigments is no more than -1 or -2 , even 5 pH units on the alkaline (or negative) side of the isoelectric point, indicates that there is apparently as little transmission of negative (molecular surface) charge to the iron atom in the nodule pigments at the pK^0 point as there is in the mammalian haemoproteins. If the application of the Debye-Hückel model to this particular ionization is valid, this effect of I confirms the conclusion drawn tentatively from the enthalpy data. In the particular case of ferrimyoglobin, the effect of I can be examined in greater detail. George & Hanania (1957) have shown that the variation of pK with I at extremely low I depends on the range of pH within which ionization measurements are made. Thus the effective charge on ferrimyoglobin varies from $+1$, at pH below 8, to -4 , at pH approx. 10. Unfortunately, overlapping ionizations below pH 8, and the instability of the proteins at about pH 10, make it practically impossible to explore this phenomenon in the haemoproteins of soya-bean nodules or of horse blood. Full comparison of the haemoproteins cannot thus be gained from studies of the salt effect.

The source of the effective charge in haemoproteins, and the mechanism of its transmission to the iron atom, are not known. Possible sources of the negative charge have been discussed by George & Hanania (1954, 1957). The small effective negative charge, detected even in the acidic nodule pigments, does emphasize the likelihood that most haemoprotein anionic groups are well screened from the prosthetic group by distance or by counter-polar cationic groups. It also suggests that the dissociation of one or more cationic functional groups ($\equiv N:H^+ \rightarrow \equiv N:$) liberates whatever negative charge is measured at the pK^0 point, but it is not possible to specify the chemical nature of any groups actually involved. Recent work on complex ions has shown that the co-ordination of a ligand to a metal affects the ionization of acidic groups on the ligand far more than has hitherto been realized, shifts of 3 and 4 pH units having been noted in pK^0 value (Hanania & Irvine, 1962; G. I. H. Hanania, unpublished work). These changes are accompanied by large changes in the enthalpy of ionization. There is also the strengthening of the acidity of water co-ordinated to the haemoproteins, with changes in enthalpy and entropy of ionization. From this evidence it is clear that any inference about the chemical nature of haem-linked groups based solely on thermodynamic data may be misleading.

An independent measure of the interaction between the haematin iron atom and its neighbouring functional groups exists in the values for 'critical ionic strength', which is defined as the minimum I of the trough in each of the curves of Fig. 3. These values are cited in Table 2. They represent the upper limit of I of the buffer at which negative charge on the apoprotein may still influence the iron atom (at the pK point), or above which the inorganic ions in solution screen the iron atom from the influence of its nearby protein structure. In this respect the nodule haemoproteins are intermediate between haemoglobin and myoglobin, but are closer to the latter. Thus, like myoglobin, the nodule pigments show only weak interaction between the haematin iron atom and its neighbouring groups at the pK point. This inference from the critical ionic strength agrees with that from the effective negative charge on the iron atom.

Another structural feature of the nodule pigments is the trend in value of the limiting linear slopes (Fig. 2). The slopes are approximately -2.2 (minor fast), -1.8 (fast) and -1.6 (slow). One cannot be certain that the trend is genuine because of the rather wide limits of error involved, but it is suggested that the highest slope corresponds to a change of charge from -2 to -3 , whereas the lower slopes correspond to a change from -1 to -2 . This would imply that one component bears the equivalent of nearly 1 extra negative charge, somehow linked to the haematin Fe(III) atom, or that the structural environment of the haem is not identical in the three soya-bean-nodule pigments. The trend in slopes does reflect the electrophoretic mobility of the pigments, or the isoelectric point, as well as the trend in pK^0 values. However, the differences are too small to provide conclusive evidence.

The six haemoproteins (from four different sources) cited in Table 2 have in common the remarkable property of binding molecular oxygen. Yet the environment of their haematin nuclei, or their catalytic centres, is clearly not the same.

SUMMARY

1. A thermodynamic study has been made of the ionization of two ferrihaemoproteins isolated from soya-bean nodules; a partial study of a third component is presented. The equilibrium is assumed to involve the dissociation of a water molecule co-ordinated to the haematin Fe(III) atom.

2. pK was measured over a range of pH, temperature and I . The standard enthalpies and entropies of ionization were obtained for two of the plant pigments, and compared with corresponding parameters for other haemoproteins. The three

nodule ferrihaemoproteins have very similar thermodynamic properties.

3. The three pigments show marked effects of *I* that are also characteristic of mammalian ferri-myoglobin and ferrihaemoglobin. Although the formal charge on the Fe(III) atom of ferrihaemoglobins is +1, the effective charge deduced from the variation of p*K* with *I* at infinite ionic dilution is about -1 on the two major soya-bean pigments and -2 on the third component.

4. The charge properties connected with this ionization are discussed in relation to the structural environment of the haematin nucleus in the nodule pigments.

5. A gradation in the enthalpies and entropies of ionization in the several haemoproteins is discussed.

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Biosynthesis of Lecithin in Brain

PARTICIPATION OF CYTIDINE DIPHOSPHATE CHOLINE AND PHOSPHATIDIC ACID

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Previously it was shown that the phosphorus of [³²P]phosphorylcholine, but not that of α-glycero-[³²P]phosphate, was incorporated into the lecithin of mitochondria and dispersions prepared from rat brain (McMurray, Strickland, Berry & Rossiter, 1957*b*). The incorporation of radioactivity from [³²P]phosphorylcholine was dependent upon the presence of CTP in the reaction mixture. Subsequently, similar rat-brain preparations were shown to incorporate the radioactivity of CDP-choline labelled with ³²P into lecithin (Rossiter, McLeod & Strickland, 1957*a*). The incorporation was increased by the addition of D-αβ-diglyceride prepared enzymically from each of several naturally occurring lecithins. These findings strongly suggested that brain lecithin is formed *in situ* from smaller molecules by reactions similar to those proposed by Kennedy & Weiss (1956) and by Kennedy (1957) for the biosynthesis of phosphatide in fowl liver.

The experiments described in the present paper show that, as previously reported for [³²P]phosphorylcholine, the radioactivity of [¹⁴C]phosphorylcholine is incorporated into lecithin. On the other hand, in contrast with the findings for DL-α-glycero-

[³²P]phosphate, the radioactivity of L-α-[¹⁴C]-glycerophosphate could be recovered from lecithin. Other experiments with ¹⁴C-labelled CDP-choline or [¹⁴C]phosphatidic acid showed that under the appropriate conditions each of these substances may be the precursor of brain lecithin. Experiments of this type indicate that rat-brain preparations are capable of catalysing a sequence of reactions leading to the formation of lecithin.

Preliminary reports on some of these findings have already appeared (Rossiter, McMurray & Strickland, 1957*b*; Rossiter & Strickland, 1959).

METHODS

Tissue preparation and incubation. Rats of the Sprague-Dawley strain (150–250 g.) were decapitated. The cerebral hemispheres were rapidly removed and homogenized in 4 vol. of water at 0° in a Potter-Elvehjem-type homogenizer. Portions of this dispersion were incubated under conditions similar to those described by McMurray, Strickland, Berry & Rossiter (1957*a*). Normally the complete system contained: glucose, 28 mM; ATP (potassium salt), 0.7 mM; NAD, 1 mM; nicotinamide, 40 mM; MgCl₂, 8 mM; tris buffer (pH 7.4), 50 mM; potassium phosphate (pH 7.4), 10 mM. In most experiments an amount of dispersion equivalent to