Biochem. J. (1966) 101, 774

Thermodynamics of the Binding of Biotin and some Analogues by Avidin

By N. M. GREEN*
Laboratory of Chemistry, National Institute for Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda 14, *U.S.A.*

(*Received* 11 *May* 1966)

1. The reaction between avidin and biotin was found to be exothermic, ΔH being $-20{\cdot}3$kcal./mole of biotin bound. The corresponding value of ΔH for streptavidin was -23kcal./mole. 2. The heat evolved was independent of the pH (between 5 and 9), of the buffer (borate or ammonia) and of the fractional saturation of the avidin with biotin. 3. The entropy change for the reaction was zero, and it is suggested that the entropy increase to be expected from hydrophobic interactions was counterbalanced by a decrease in entropy accompanying the formation of buried hydrogen bonds. 4. Modification of the potential hydrogen-bonding sites of the imidazolidone ring led to a decreased heat output and a positive entropy of reaction.

The binding of biotin by avidin is accompanied by one of the largest decreases in free energy yet observed for a non-covalent interaction (20kcal./mole; Green, 1963*a*). Spectroscopic studies of the reaction led to the conclusion that hydrophobic interactions made a large contribution to the free energy of binding (Green, 1963*b*). Such a contribution would be accompanied by only a small change in heat content, but by a large increase in entropy (Kauzmann, 1959), whereas other types of non-covalent bonding give rise to decreases in both heat content and entropy. To provide further evidence on the possible role of hydrophobic interactions, the heat of binding has been measured calorimetrically, since it was not possible to measure the low dissociation constant with sufficient accuracy to obtain ΔH from the dependence of ΔG on temperature.

The binding of 2′-thiobiotin, 2′-iminobiotin and the diaminocarboxylic acid from biotin were also studied, since modifications of the imidazolidone ring should give information on the interactions of the hydrophilic part of the biotin molecule with avidin.

The protein streptavidin, which binds biotin and was isolated by Chaiet & Wolf (1964) from filtrates of cultures of *Streptomyces avidinii*, resembles avidin in several respects. Each molecule binds four molecules of biotin. The binding by streptavidin is accompanied by changes in the absorption spectrum and in the chemical reactivity of tryptophan similar to those observed with avidin (N. M. Green, unpublished work). The optical rotatory dispersion and circular dichroism spectra of the two proteins show similar Cotton effects due to aromatic amino acids (Green & Melamed, 1966). The heat of reaction with biotin was therefore measured for further comparison of its properties with those of avidin.

* Present address: National Institute for Medical Research, London, N.W.7.

METHODS AND MATERIALS

Avidin. The relatively large amounts of avidin required for these experiments (about 10mg. for each calorimetric measurement) were generously supplied by Dr R. Herz and Mr F. G. Dhyse. The partially purified material (Dhyse, 1954), which closely resembled commercial avidin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), both in specific activity (2·5units/mg.) and in its low solubility in water, was further purified by chromatography on CM-cellulose (Melamed & Green, 1963). The avidin (5g.) was dissolved in 250ml. of 0·1M-ammonium acetate, pH9·0. Insoluble material was centrifuged off and discarded and the supernatant was run on to a column (6cm.$\times$75cm.) of CM-cellulose. Most of the protein impurities were not adsorbed. The column was washed with 0·1M-ammonium acetate, pH8·6 (1·5l.), and then eluted with a linear gradient of 0·4–1·6% (w/v) ammonium carbonate (total volume 5l.). Avidin emerged with the first peak. The fractions were pooled, concentrated on CM-cellulose, dialysed and freeze-dried (183mg.; activity 12·5units/mg.). Most of the avidin emerged with the turbid effluent from the ammonium acetate wash, before the commencement of the ammonium carbonate gradient. This was diluted to 2·5l., readsorbed on the same column and eluted as before. Two active peaks emerged. They were pooled together, concentrated and dialysed. A yellow sticky precipitate was deposited on the walls of the dialysis sac and was separated from the clear supernatant. This precipitate (120mg.) became dark brown

on drying, was soluble at pH 9 and, surprisingly, possessed an activity of 10 units/mg. The supernatant was freeze-dried to give 300 mg. of white water-soluble avidin (activity 11 units/mg.). The calorimetric measurements were all made on the most active material (12·5 units/mg.).

Streptavidin. This was a gift from Dr F. J. Wolf.

Biotin and analogues. Synthetic (+)-biotin was obtained from Roche Products Ltd., Welwyn Garden City, Herts. The diaminocarboxylic acid, δ-(3,4-diaminothiophan-2-yl)-pentanoic acid, was prepared from biotin (Hofmann, Melville & du Vigneaud, 1942). The guanidino analogue of biotin, 2′-iminobiotin, was prepared from the diaminocarboxylic acid (Hofmann & Axelrod, 1950).

2′-Thiobiotin was prepared from CS_2 and the sulphate of δ-(3,4-diaminothiophan-2-yl)pentanoic acid (cf. Brown & du Vigneaud, 1946). The diamine sulphate (316 mg.) was converted into the free base with the theoretical amount of 0·5 M-$Ba(OH)_2$. The solution was evaporated to dryness, the residue was dissolved in 1 ml. of aq. 50% (v/v) ethanol, and 0·08 ml. of CS_2 was added. The mixture was refluxed at 60° for 1 hr. and then heated at 80° in a sealed tube for 3 hr. The mixture, which became solid, was acidified to pH 3 with 0·02 ml. of conc. HCl and the heating continued for a further 30 min., by which time no further H_2S was evolved. The product was purified by several recrystallizations from hot water and by solution in NaOH, decolorization with charcoal and precipitation with HCl. The yield was 80 mg., m.p. 234–235° (decomp.), $\lambda_{max.}$ 234 mμ (ϵ 15900) (Found: C, 46·5; H, 5·9; N, 10·9; S, 25·1. Calc. for $C_{10}H_{16}N_2O_2S_2$: C, 46·1; H, 6·2; N, 10·8; S, 24·5%).

Spectrophotometric titrations and measurement of dissociation constants. These were carried out at room temperature (25°) as described by Green (1963*b*), with a Cary model 11 recording spectrophotometer. Where possible, dissociation constants were calculated at four points along each curve corresponding to the addition of 0·5, 1·0, 1·5 and 2 mol. of analogue/binding site. When K was less than 10^{-7} M the extent of dissociation was too small for this procedure to be applicable and K was calculated from the extent of dissociation at the equivalent point. Since determination of K within a factor 2 suffices to define the free energy of formation of the complex within ± 0·2 kcal./mole, the estimates of K obtained by this method were sufficiently precise.

Calorimetric measurements. These were performed at room temperature (24–27°) with a prototype Beckman model 190 micro-calorimeter. This instrument is based on the design of T. H. Benzinger and its operation and construction are described by Benzinger & Kitzinger (1960) and Ross & Scruggs (1965). The instrument was on loan to Dr P. D. Ross, to whom I am greatly indebted for advice and assistance with the measurements. Matched pairs of glass cells described by Benzinger as 'drop' vessels were used. Small volumes (drops) of one reactant were separated during the preliminary equilibration from a large volume of the second reactant in two cup-shaped depressions in the inner wall of the cell. The contents were mixed by rotation of the whole calorimeter about two axes at right angles to each other. Various programmes of rotations could be selected to ensure adequate mixing of the reactants.

A solution of avidin in the appropriate buffer (15 ml.) was introduced into the main compartment of the experimental cell with a syringe fitted with polythene cannula tubing. The matched reference cell contained 15 ml. of buffer. Biotin or the appropriate analogue, equivalent to 90–95% of the binding capacity of the avidin, was dissolved in the same buffer, and was introduced into the small cup compartments with calibrated 100 μl. or 200 μl. Carlsberg pipettes. The vessels were stoppered, greased, inserted in the calorimeter and allowed to come to equilibrium (about 75 min.). When the integrator on the recorder showed no drift over a period of several minutes the contents of vessels were mixed. When the integrator again showed no difference between experimental and reference cells (10–15 min.). the mixing programme was repeated. A further heat output (usually less than 5% of the total) was observed. A third and fourth mixing usually gave a small response (less than 0·3 mcal.) because of heat produced by the mixing process. The total heat evolved was obtained by summing the heat outputs and correcting for the heat of mixing. Under the conditions of the experiment (excess of avidin, concn. 12 μM) the binding of biotin or analogue was essentially complete and ΔH was calculated/mole of biotin or analogue added to the reaction vessel. The temperature of each run was measured by a thermistor incorporated into the calorimeter.

Determination of ionization constants. The ionization constants of the diaminocarboxylic acid were obtained from the titration curve determined with a Radiometer TTT 1b pH-meter. The titration vessel was maintained at 25° and N-NaOH was added from an Agla micrometer syringe. The ionization constant of the guanidino group of iminobiotin in 0·1 M-NaCl was determined spectrophotometrically by using the increase in E_{220} accompanying the formation of the free base ($\Delta\epsilon_{220}$ 3600). The solution of iminobiotin (0·5 mM) in 0·1 M-NaCl was titrated with N-NaOH from an Agla syringe. The E_{220} value was measured after addition of the corresponding amount of NaOH to the reference cuvette. The pH was measured with a G.202B high-pH glass electrode and was corrected for the small Na^+ ion error.

RESULTS

The results of allowing avidin to react with slightly less than 4 molar equivalents of biotin are shown in Table 1. The reaction was exothermic to the extent of 20 kcal./mole of biotin or 80 kcal./mole of avidin. This change in heat content is sufficient to account for all of the free energy of binding. One run was carried out in 2 mM-ammonium acetate, pH 5·0, with avidin preparation D.31 (Melamed & Green, 1963), for direct comparison with the kinetic measurements from which the value of ΔG had been calculated. The heat evolved was not significantly different from that obtained with the 'Dhyse' avidin preparation at higher pH and ionic strength. A single run was also made with streptavidin (Chaiet & Wolf, 1964) which showed a slightly higher ΔH than avidin.

All the evidence at present available (Green, 1964*a*,*b*) suggests that the four binding sites of avidin are similar and that they combine in a random manner with biotin. It would therefore be expected that the heat of reaction would be independent of the fractional saturation of avidin with biotin. It was not possible to test this hypothesis

Table 1. *Heat of reaction of avidin and streptavidin with biotin*

Avidin sample	Buffer	pH	Ionic strength	Temp.	Biotin (μmole)	Heat evolved (mcal.)	ΔH of binding (kcal./mole of biotin)
'Dhyse' (this paper)	Ammonium acetate	9·0	0·10	24·0°	0·736	15·9	−21·5
'Dhyse' (this paper)	Ammonium acetate	9·0	0·10	24·0	0·750	14·9	−19·9
D.31	Ammonium acetate	5·0	0·002	23·4	0·613	13·2	−21·5
'Dhyse'	Borate	9·0	0·15	27·5	0·597	12·5	−20·9
Streptavidin	Borate	9·0	0·15	28·0	0·465	10·8	−23·2

Table 2. *Effect of partial saturation of avidin with biotin on the heat of reaction*

These reactions were performed in 0·1 M-ammonium acetate buffer, pH 9·0.

Fractional saturation of avidin sample	Temp.	Biotin (μmole)	Heat evolved (mcal.)	ΔH of binding (kcal./mole of biotin)
0	24·2°	0·632	12·7	−20·2
0·25	24·1	0·719	13·8	−19·3
0·5	24·1	0·650	13·4	−20·7
0·75	23·6	0·581	12·1	−20·8

reliably by allowing avidin to react with 1, 2, 3 and 4 molar equivalents of biotin, since the mixing in the calorimeter vessel was not sufficiently rapid to ensure initial random distribution of the biotin among the available binding sites. Once the biotin combined in a non-random fashion the rate of redistribution would be extremely low (Green, 1963*a*). To circumvent this problem the avidin was first partially saturated with biotin by the slow addition of 1, 2 or 3 molar equivalents of biotin to a well-stirred solution. The heat evolved when the remaining sites were saturated in the calorimeter was then determined in the usual way. Table 2 shows that ΔH was independent of the number of sites that had already reacted. The mean value of ΔH for the 'Dhyse' avidin from the six experiments in ammonium acetate was −20·3 kcal./mole (S.E.M. ±0·3 kcal./mole).

Dissociation constants of biotin analogues. The binding of the three analogues of biotin by avidin had not previously been studied in detail. Spectrophotometric titrations were therefore carried out and dissociation constants were calculated from the titration curves. Since both iminobiotin and the diaminocarboxylic acid could be protonated, it was desirable to know whether their binding was affected by their state of ionization. Moreover, it was essential to know which ionic species were bound by avidin, to interpret the calorimetric measurements.

The pK of the guanidino group of iminobiotin was found to be 11·9 by spectrophotometric titration at 220 mμ. The diaminocarboxylic acid gave a titration curve that could be fitted by three groups ionizing with pK values 4·0, 5·1 and 8·3. The two upper pK values assignable to the two amino groups are remarkably low, presumably owing to the close proximity of these *cis*-α-amino groups to each other.

At pH 9·0 spectrophotometric titration with avidin showed almost stoicheiometric binding of iminobiotin. Extrapolation of the linear portion of the curve showed that 1 mol. of avidin bound 4 mol. of iminobiotin. The dissociation constant, which was 3×10^{-8} M at this pH, increased markedly as the pH was lowered. A plot of log K against pH is shown in Fig. 1. In the region of pH 9 the slope approaches unity, the value to be expected if one proton were to be released for each molecule of iminobiotin bound. The release of protons accompanying the binding was also measured approximately by determining the amount of base required to restore the pH to its initial value after the addition of iminobiotin to avidin (15 mg.) at pH 8·5: 0·8 proton was released/mol. of iminobiotin bound. From evidence discussed below, it appears unlikely that these protons are derived from avidin, so that these results show that only the free base form of iminobiotin is firmly bound by avidin. The free energy of binding of this form was calculated from

the observed dissociation constant (K') at pH 9 and the pK of the guanidino group, as described in Table 3.

The effect of pH on binding of the diaminocarboxylic acid also suggested that only the uncharged form was bound. The dissociation constant increased from $1{\cdot}2 \times 10^{7}$-M at pH10 to about 10^{-2}M at pH4·6. Therefore, to simplify the interpretation, the calorimetric measurements and spectrophotometric titrations were performed at pH10·0, where neither of the amino groups was ionized.

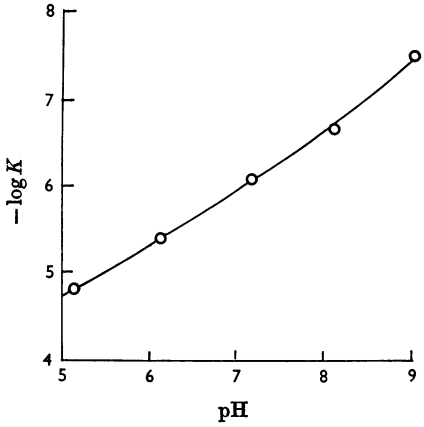

Fig. 1. Effect of pH on the dissociation constant of the complex between 2′-iminobiotin and avidin. Dissociation constants were calculated from spectrophotometric titrations carried out at different pH values in 0·05M-sodium phosphate or 0·025M-sodium borate (pH 9·0 only) buffers. The value of $\Delta E_{233}/E_{282}$ was $0{\cdot}76 \pm 0{\cdot}02$ and was independent of pH.

Spectrophotometric titration of thiobiotin with avidin in tandem cells showed the stoicheiometry to be identical with that of biotin (i.e. 4mol. was bound/mol. of avidin). There was no detectable dissociation at the concentration used in the experiment (10μM). The change in E_{233} value was 15% greater than that given by biotin. This surprisingly large change may be an effect of the binding on the absorption of the thiobiotin, which is maximal at 234mμ (ϵ15900). (The spectra of the avidin–biotin and avidin–thiobiotin complexes showed a maximum difference at 238mμ, the $\Delta E_{238}/E_{280}$ ratio being 0·27, which tends to confirm that the difference between them is not just a difference in magnitude of $\Delta\epsilon_{233}$.) Addition of excess of biotin to the titration mixture produced a slow first-order decrease in E_{238}, due to displacement of the thiobiotin. From the slope of the first-order plot the rate constant for dissociation of the complex was found to be $3{\cdot}2 \times 10^{-5}$sec.$^{-1}$ as compared with 7×10^{-8}sec.^{1} for biotin (Green, 1963*a*). It is unlikely that the rates of combination will differ very much since the forward reaction is almost diffusion-controlled (Green, 1963*a*) and the two molecules are almost identical in size and shape, so that the ratio of the dissociation rate constants will be an approximate measure of the ratio of the equilibrium constants. Therefore for thiobiotin this constant is about 5×10^{-13}M, corresponding to a free energy of binding of −16·9kcal./mole.

Heat of binding of biotin analogues. The measurement of ΔH for the binding of thiobiotin and of the diaminocarboxylic acid at pH10 was straightforward, but for the iminobiotin it was complicated

Table 3. *Thermodynamic parameters for binding of analogues of biotin by avidin*

	Buffer system	K(25°)(M)	ΔG (25°) (kcal./mole)	ΔH (27·5°) (kcal./mole)	$T\Delta S$ (kcal./mole)	ΔS (e.u./mole)
Biotin	Ammonium acetate, pH5·0	1×10^{-15}	−20·5	−20·3	0	0
Thiobiotin	Borate, pH9·0	5×10^{-13}	−16·9	−17·8	−0·9	−3
Iminobiotin	Borate, pH9·0	3×10^{-8}	−10·4	−2·8		
Iminobiotin	Ammonium acetate, pH9·0	5×10^{-8}	−10·0	−11·5		
Iminobiotin	Corrected values for free base*	$3{\cdot}5 \times 10^{-11}$	−14·3	−11·6	+2·7	+9
Diaminocarboxylic acid (basic form)	Borate, pH10·0	$1{\cdot}2 \times 10^{-7}$	− 9·5	−4·7	+4·8	+16

* ΔH and ΔG for binding of the iminobiotin were calculated for the form in which the guanidino group was uncharged. ΔG was calculated from the dissociation constant in the usual way, where K for the free base is given by, $K = K'/\{1 + ([H^+]/K_a)\}$, where K' is the constant measured at any $[H^+]$ and K_a is the acid dissociation constant of the guanidinium group. ΔH was calculated by correcting the observed heat production for the heat of ionization of the guanidinium ion (−12·4kcal./mole) and for the heat of ionization of the buffer [borate, ΔH −3·3kcal./mole (Harned & Owen, 1958); ammonia, ΔH −12·8kcal./mole (Edsall & Wyman, 1958)]. The values given are mean values from experiments in borate and ammonia buffers.

by the changes in ionization accompanying binding. The relevant reactions are:

$$\mathrm{Im} + \mathrm{H^+} \rightleftharpoons \mathrm{ImH^+} \quad (1)$$
$$pK = 11{\cdot}95;\ \Delta H = \Delta H_1$$
$$\mathrm{H^+} + \mathrm{B} \rightleftharpoons \mathrm{BH^+} \quad (2)$$
$$\Delta H = \Delta H_2$$
or $$\mathrm{H^+} + \mathrm{B^-} \rightleftharpoons \mathrm{BH}$$

where Im is iminobiotin and B is the buffer

$$\mathrm{Av} + \mathrm{Im} \rightleftharpoons \mathrm{AvIm} \quad (3)$$
$$\Delta H = \Delta H_3$$

where Av is avidin.

The observed heat change ($\Delta H_{obs.}$) for the overall reaction:

$$\mathrm{Av} + \mathrm{ImH^+} + \mathrm{B} \rightleftharpoons \mathrm{AvIm} + \mathrm{BH^+}$$

is therefore $\Delta H_3 - \Delta H_1 + \Delta H_2$ and the required quantity $\Delta H_3 = \Delta H_{obs.} + \Delta H_1 - \Delta H_2$.

ΔH_1 was calculated from experiments in which the iminobiotin hydrobromide (6 μmoles) was mixed with excess of 0·1 N-sodium hydroxide–0·1 M-sodium chloride in the calorimeter vessel. Under these conditions the carboxyl groups and 88% of the guanidino groups (determined from the final E_{220} of the reaction mixture) lost protons, to give 1·88 mol. of water/mol. of iminobiotin. ΔH_1 was found to be −12·4 kcal./mole, correcting for the heat of formation of 1·88 moles of water and taking the heat of ionization of the carboxyl group as zero, by analogy with that of ϵ-aminohexanoic acid (Edsall & Wyman, 1958). The measurements of the heat of reaction with avidin were carried out both in borate and in ammonia buffers, in which the values of ΔH_1 differ by 9·1 kcal./mole. The observed heat changes differed by 8·7 kcal./mole, confirming that, within the limits of error, one proton was liberated for each molecule of iminobiotin bound.

The observed heats of formation of the complexes of avidin with analogues of biotin are shown in Table 3, together with the free energy of binding obtained from spectrophotometric titration curves. It appears that as the free energy of binding decreased the relative contribution of the heat content also decreased, while the entropy change became more positive, contributing more both in relative and in absolute terms to the stability of the complex.

DISCUSSION

Although the dissociation constants of complexes of proteins with small molecules have often been measured, until recently relatively very little was known of their temperature-dependence. The relative magnitudes of the contributions of heat and entropy to the stability of these complexes was unknown except for those of serum albumins and γ-globulins, which were extensively studied. Most of the earlier results with these two classes of protein showed the binding of a wide variety of small molecules to be accompanied by a small change in heat content and a large increase in entropy (Kauzmann, 1959). In comparison with this behaviour the binding of avidin by biotin appears exceptional. However, more recent observations on a variety of systems have shown many examples where ΔH makes a major contribution to the free energy of binding. For example, the binding of FAD by the 'old yellow enzyme' (Theorell & Nygaard, 1954), AMP by ribonuclease (Myer & Schellman, 1962), haem by apomyoglobin (Banerjee, 1962), 1-anilinonaphthalene-8-sulphonic acid by apomyoglobin (Stryer, 1965), skatole by human serum albumin (McMenamy, 1964) and ϵ-DNP-lysine by specific antibody (Eisen & Siskind, 1964) are all exothermic. In the last example ΔH (−20 kcal./mole) was as great as that observed here for avidin and biotin. However, the free energy of binding of the ϵ-DNP-lysine was only about −10 kcal./mole, on account of an unfavourable entropy term. Since even a small molecule may interact with its binding site in different ways at different points and since it may also affect the conformation of the protein, the observed contributions of heat and entropy to the free energy of binding represent the sum of many individual terms. The relatively simple saturated structure of biotin limits direct interactions to hydrogen bonds and hydrophobic interactions (including in this term the effect of dispersion forces). Following the discussion of Kauzmann (1959) it is probable that hydrogen-bond formation is accompanied by decreases in both entropy and heat content, whereas hydrophobic interactions lead to a large entropy increase and little change in heat content. There may also be heat and entropy changes accompanying any change in conformation of avidin induced by the biotin. Although it is likely that such conformational changes do occur, they are not large enough to produce significant changes in the sedimentation and the diffusion coefficients (Green, 1964*b*), in the rotational relaxation time (Green, 1963*c*) or in the optical rotatory dispersion (Green & Melamed, 1966) of avidin. Large thermodynamic effects from this source are therefore unlikely. Nevertheless, there remains a possibility that some of the heat of binding arises from a conformational change and this should be kept in mind during the following discussion.

Apart from conformational changes it is essential to consider whether there are any changes in the ionization of amino acid residues in avidin when biotin is bound, since such changes can be accompanied by large heat changes. (The binding of iminobiotin provides a clear example of the large effect of ionization on the observed heat of reaction.)

Ideally this requires data on the pH-dependence of the dissociation constant, but the strength of the binding of biotin makes this difficult to obtain. However, related evidence indicates that changes in ionization have little influence on ΔG or ΔH. Thus there was no obvious effect of pH on the binding of biotin in the range pH3–13 and the ultraviolet difference spectrum showed only minor changes throughout this range (Green, 1963*b*). The dissociation constant of the uncharged analogue of biotin, 3-*n*-butylimidazolidone, decreased only slightly from 3×10^{-6}M to $1{\cdot}5 \times 10^{-6}$M on raising the pH from 4 to 10 (N. M. Green, unpublished work). The results in Table 1 show no significant effect of buffer (borate or ammonia at pH 9·0) or of pH (ammonium acetate at pH 5 or 9) on the observed ΔH. It can therefore be concluded that ΔH is not significantly affected by secondary ionizations and is mainly the result of hydrogen-bond formation and hydrophobic interactions. By the same arguments it is unlikely that any ionizing groups are involved in the hydrogen bonds, unless they remain in the same state of ionization over the whole pH range in both avidin and the avidin–biotin complex. The observation that the positive ions of iminobiotin and of the diaminocarboxylic acid were not bound by avidin suggested that the binding site could not accommodate a positively charged group. This could result from either the presence of a positively charged group in the binding site of the imidazolidone ring or the difficulty of burying an isolated positive charge in a closely-fitting uncharged binding site. The second explanation is favoured, since it is consistent with the suggestion (Green, 1963*b*) that the imidazolidone ring of the biotin is buried in the interior of the protein. This contrasts with the behaviour of the carboxyl group of the biotin, whose state of ionization of substitution has little effect on the binding and which presumably lies near the surface of the avidin–biotin complex.

The main conclusion of this paper, that the combination of avidin with biotin is accompanied by no net change of entropy and a large decrease in heat content, conflicts with the hypothesis that hydrophobic interactions play a large part in stabilizing the avidin–biotin complex. The main evidence for this hypothesis was the nature of the biotin molecule itself, the spectral changes that accompanied its binding, and the binding of certain dyes and other non-polar molecules (Green, 1963*b*, 1965). This evidence still stands, but it appears that the increase in entropy expected from hydrophobic interactions is not observable, no doubt because of a compensating decrease accompanying the formation of other bonds, probably involving the imidazolidone ring.

The effects of replacing the 2′-carbonyl group of biotin by >C=S or >C=NH confirm earlier results that suggested that hydrogen bonds to the imidazolidone ring were of some importance (Wright, Skeggs & Cresson, 1951; Fraenkel-Conrat, Snell & Ducay, 1952; Green, 1963*b*). Since these bonds are probably buried in the interior of avidin they can make a much larger negative contribution to ΔH than the corresponding bonds in aqueous solution (Nemethy, Steinberg & Scheraga, 1963), provided that the groups on the protein which participate in this hydrogen-bond formation do not form buried hydrogen bonds, among themselves or with water, in the absence of biotin. The entropy terms of hydrophobic and hydrogen-bond formation will be of opposite sign and could lead to a zero net entropy change. This model can account qualitatively for the results obtained although it is still difficult to explain the magnitude of ΔH, when current estimates for the strength of buried hydrogen bonds are used [Nemethy *et al.* (1963) suggest ΔH −3 to −5 kcal./mole and ΔS −3 to −5 e.u./mole]. Eisen & Siskind (1964) have put forward a similar hypothesis to explain their results on the binding of ϵ-DNP-lysine by specific antibody.

Although the binding of thiobiotin by avidin is considerably weaker than that of biotin the relative magnitudes of ΔG and ΔH are similar. This would be consistent with the ability of both >C=O and >C=S to act as similar acceptor groups in hydrogen-bond formation. Omission of the >C=O group or its replacement by >C=NH have considerably greater effect on the binding, which is now accompanied by a much smaller heat change and an entropy increase. This would be consistent with formation of fewer or weaker buried hydrogen bonds.

I am grateful to Dr B. Witkop for his support of this work in his Laboratory, and to Dr P. D. Ross and Dr R. L. Scruggs for the use of the micro-calorimeter and for their advice and assistance. I also thank the Wellcome Foundation for a Research Travel Grant. I was a Visiting Scientist of the U.S. Public Health Service, 1963–64, while this work was being carried out.

REFERENCES

Banerjee, R. (1962). *Biochim. biophys. Acta*, **64**, 368.
Benzinger, T. H. & Kitzinger, C. (1960). *Meth. biochem. Anal.* **8**, 309.
Brown, G. B. & du Vigneaud, V. (1946). *J. biol. Chem.* **163**, 761.
Chaiet, L. & Wolf, F. J. (1964). *Arch. Biochem. Biophys.* **106**, 1.
Dhyse, F. J. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 515.
Edsall, J. T. & Wyman, J. (1958). *Biophysical Chemistry*, vol. 1, p. 452. New York: Academic Press Inc.
Eisen, H. N. & Siskind, G. W. (1964). *Biochemistry*, **3**, 996.
Fraenkel-Conrat, H., Snell, N. S. & Ducay, E. D. (1952). *Arch. Biochem. Biophys.* **39**, 97.

Green, N. M. (1963*a*). *Biochem. J.* **89**, 585.
Green, N. M. (1963*b*). *Biochem. J.* **89**, 599.
Green, N. M. (1963*c*). *Biochem. J.* **89**, 609.
Green, N. M. (1964*a*). *Biochem. J.* **90**, 564.
Green, N. M. (1964*b*). *Biochem. J.* **92**, 16 c.
Green, N. M. (1965). *Biochem. J.* **94**, 23 c.
Green, N. M. & Melamed, M. D. (1966). *Biochem. J.* **100**, 614.
Harned, H. S. & Owen, B. B. (1958). *Amer. chem. Soc. Monogr: Physical Chemistry of Electrolytic Solutions*, p. 667. New York: Reinhold Publishing Corp.
Hofmann, K. & Axelrod, A. E. (1950). *J. biol. Chem.* **187**, 29.
Hofmann, K. & Melville, D. B. & du Vigneaud, V. (1942). *J. biol. Chem.* **144**, 513.
Kauzmann, W. (1959). *Advanc. Protein Chem.* **14**, 1.
McMenamy, R. (1964). *J. biol. Chem.* **239**, 2835.
Melamed, M. D. & Green, N. M. (1963). *Biochem. J.* **89**, 591.
Myer, Y. P. & Schellman, J. A. (1962). *Biochem. biophys. Acta*, **55**, 361.
Nemethy, G., Steinberg, I. Z. & Scheraga, H. A. (1963). *Biopolymers*, **1**, 43.
Ross, P. D. & Scruggs, R. L. (1965). *Biopolymers*, **3**, 491.
Stryer, L. (1965). *J. molec. Biol.* **13**, 482.
Theorell, H. & Nygaard, A. P. (1954). *Acta chem. scand.* **8**, 1649.
Wright, L. D., Skeggs, H. R. & Cresson, E. L. (1951). *J. Amer. chem. Soc.* **73**, 4144.