Biochem. J. (1969) **115**, 441
Printed in Great Britain

Calorimetric Studies of the Haemoglobin–Haptoglobin Reaction

BY ERNEST C. ADAMS AND MARGARET R. WEISS
Ames Research Laboratory, Ames Company Division Miles Laboratories Inc., Elkhart, Ind. 46514, *U.S.A.*

(*Received* 12 *May* 1969)

Haptoglobin binds haemoglobin so firmly that there is practically no dissociation. It would be expected that the heat of the reaction would be relatively large. The development of the microcalorimeter by Benzinger offered the opportunity to measure the heat of reaction. The experiments were carried out in the Beckman 190B Microcalorimeter in two ways: (1) a constant amount of haptoglobin (Kabi; 65mg.) with different amounts of haemoglobin, and (2) a constant amount of haemoglobin (32·5mg.) with different amounts of haptoglobin. The proteins, each in 5ml. of 0·15M-phosphate buffer, pH 7·4, were placed in equal-volume calorimeter cells. The heat produced/mg. of haemoglobin was calculated from the slope of the curve for a constant amount of haptoglobin and from the maximum heat for a constant amount of haemoglobin. This heat is about 70kcal./mole at 37°. ΔH varies with temperature, being $-70{\cdot}2$ at 37°, $-29{\cdot}7$ at 20° and 7·2 at 4°. From the amount of haptoglobin required to attain maximum heat with 32·5mg. of haemoglobin and the amount of haemoglobin required to attain maximum heat with 65mg. of haptoglobin, it appears that at excess of haptoglobin there is competition between the reactions of 2moles of haptoglobin with 1mole of haemoglobin (or 2 $\alpha\beta$-chains) and 1mole of haptoglobin with 1mole of haemoglobin.

Polonovski & Jayle (1938) reported a substance in plasma that potentiated the peroxidase-like activity of haemoglobin in certain systems. Subsequently they identified this as a serum protein that reacts stoicheiometrically with haemoglobin, forming a very stable complex, and named it haptoglobin (Polonovski & Jayle, 1940). Investigations of this protein up to 1959 have been reviewed by Nyman (1959). It was realized early that there are different types of haptoglobin. Smithies & Walker (1956) identified three types of haptoglobin, Hp 1-1, Hp 2-1 and Hp 2-2, which are under genetic control. It is generally assumed that the molecular weight of haptoglobin 1-1 is 85000, that haptoglobins 2-1 and 2-2 are multiples of units of 85000, and that haemoglobin and haptoglobin combine in equimolecular proportions (Laurell & Gronvall, 1962). As shown in the present paper, this is only true when haemoglobin is in excess.

The affinity between haptoglobin and haemoglobin is strong. If the driving force for the reaction is enthalpy, the heat produced should be sufficient to be measured calorimetrically. The development of the microcalorimeter by Benzinger (Benzinger & Kitzinger, 1954) provides a tool for this measurement. This is a constant-temperature rather than the usual adiabatic calorimeter. The heat is measured during its rapid transfer through numerous thermocouples to a large heat sink. Steiner & Kitzinger (1956) used the instrument to measure heat of reaction of albumin with its homologous antiserum. Green (1966) measured the heat produced by the reaction of avidin with biotin. This latter study is particularly important since the avidin–biotin reaction appears to be remarkably similar to the haemoglobin–haptoglobin reaction.

It was the objective of these studies to measure the heat of reaction between haptoglobin and haemoglobin, to determine the enthalpy of the reaction and to interpret the mechanism from the ratios of haptoglobin to haemoglobin at which maximum heats are obtained.

MATERIALS

Haemoglobin. Blood was drawn, oxalated and the haemoglobin concentration determined by a cyanide method (Bauer, Toro & Ackerman, 1962). The cells were centrifuged off and washed several times with 0·85% NaCl. The washed cells were haemolysed by dilution with several volumes of water. The cell stroma and debris were removed by centrifugation at 32000*g* for 30min. The haemoglobin solution was diluted so that it contained 16·25mg./ml. Portions of the haemoglobin solution were stored in a deep-freeze at −60° until use.

Haptoglobin. Most of the experiments were done with a preparation of human haptoglobin obtained from Kabi AB, Stockholm, Sweden. The literature on the preparation states that it contains at least 90% α-globulins and about

50% haemoglobin-combining activity. Polyacrylamide-gel and paper electrophoresis showed that the main impurity was other α-globulins. In a personal communication, Kabi reported that the preparation contained mostly haptoglobins 2-1 and 2-2. Haptoglobin preparations were also obtained from Dr Nitschmann of Berne, Switzerland. These preparations were 70%, 46% and 20% haptoglobin (based on peroxidase-like activity converted into protein equivalent, assuming mol.wt. 85000 for haptoglobin). The main impurity in these preparations, as shown by electrophoresis, was albumin.

Buffer. The buffer was 0·15M-phosphate, pH7·4, prepared by mixing 19·2ml. of 0·15M-KH_2PO_4 with 80·0ml. of 0·15M-Na_2HPO_4.

Microcalorimeter. The Beckman 190B microcalorimeter was used.

Reaction cells. The partition cells with two equal compartments holding approx. 7ml. were used. The compartments are separated at the bottom of the cell by a glass wall, but are connected at the top so that on rotation of the cell the contents of the compartments are mixed. With the partition cells it is necessary to run blanks for both reagents, since both are being diluted 1:2. When adequate amounts of reagents are available the partition cells give more accurate results than the more commonly used drop cells because more material can be used, leading to a greater heat production.

METHODS

Measurements with constant amounts of haptoglobin or haemoglobin. (1) Constant amount of haptoglobin. A 78mg. sample of haptoglobin (Kabi) was dissolved in 6ml. of buffer. Also, 0–4·8ml. of haemoglobin solution (16·25mg./ml.) was mixed with sufficient buffer to make a total volume of 12ml. Mixtures were then made as follows: blank cell: 5ml. of buffer+5ml. of haemoglobin solution; reaction cell: 5ml. of haptoglobin solution (65mg.)+5ml. of haemoglobin solution. (2) Constant amount of haemoglobin. A 2·4ml. portion of haemoglobin solution (16·25mg./ml.) was mixed with 3·6ml. of buffer. Also, 0–360mg. of haptoglobin (Kabi) was dissolved in 12ml. of buffer. Mixtures were then made as follows: blank cell: 5ml. of buffer+5ml. of haptoglobin solution; reaction cell: 5ml. of haemoglobin solution (32·5mg.)+5ml. of haptoglobin solution.

For both constant-haptoglobin and constant-haemoglobin reactions, amounts of the other haptoglobin preparations equivalent to the above were used. (The stated values of the Kabi preparation as 50% haptoglobin, and the Nitschmann preparation as 20%, were used.)

After the cells had been greased, the haptoglobin, haemoglobin and buffer were placed in the cells (syringes with polyethylene tubing were used). The cells were stoppered and placed in the calorimeter as suggested by the manufacturer's directions. After equilibration at 37° for 1–2hr. the reactants were mixed by rotation of the cell. After the heat curve had returned to a base-line the rotation was repeated; this cycle was repeated until no more heat was obtained. The heat for the reaction was obtained from the integration of the heat-decay curve. Runs of 65mg. of haptoglobin (Kabi) with 32·5mg. of haemoglobin and with no haemoglobin at 20° and at 4° were also made.

Peroxidase-like activity. Some of the reaction mixtures were removed from the calorimeter cells and assayed for peroxidase-like activity by a method based on that of Mattenheimer & Adams (1968). The reaction mixtures were diluted 1:200 in water. A 1ml. portion of the diluted mixture was mixed with 2ml. of *o*-tolidine reagent (50mg. of *o*-tolidine dihydrochloride in 100ml. of 0·1M-tartrate buffer, pH4·2). Then 0·25ml. of a 1:200 dilution of 30% (v/v) hydrogen peroxide was added and mixed well. The E_{635} was measured in a Gilford recording spectrophotometer against a water blank until a maximum was reached.

The haptoglobin preparations were assayed for peroxidase-like activity by the method of Owen (1960) and by the method of Mattenheimer & Adams (1968).

RESULTS

Peroxidase-like activity. The Kabi preparation was found to have 42·9% active material and the Nitschmann 20% to have 23% active material. This was based on a unit molecular weight of 85000 for haptoglobin and 67000 for haemoglobin.

In the initial experiments saline solutions of haemoglobin and haptoglobin were used. It became evident that a substantial part of the heat released on rotation of the cells was a result of an acid–base reaction between the acidic haptoglobin and the relatively basic haemoglobin. For example, a value of 1·04mcal./mg. of pure haptoglobin (Nitschmann 70%) in saline was decreased to 0·8mcal./mg. in the neutral buffer. It has been suggested that the only sure way to have two proteins balanced with respect to H^+ ions is to dialyse them in a common buffer. This is not practical for haemoglobin, since the haem easily dissociates from the globin, changing its conformation and consequently the reaction with haptoglobin. The pH of the mixture of haemoglobin and haptoglobin was exactly the same as the pH of the individual solutions before mixing. Also, the heats obtained for the same amount of pure haptoglobin were the same regardless of the purity of the preparation and the type of impurity. Thus it appears reasonable to assume that the heat obtained with the reaction of the buffered solutions of haemoglobin and haptoglobin was not a result of acid–base reaction.

A typical curve and integration for the haemoglobin–haptoglobin reactions is shown in Fig. 1.

Fig. 2 shows the variation in the reaction heat with amount of added haemoglobin with a constant amount of haptoglobin (Kabi). The dilution of haptoglobin in the absence of haemoglobin is endothermic whereas the reaction with haemoglobin is exothermic, so that for more than 2·5mg. of haemoglobin with 65mg. of haptoglobin (Kabi) the net reaction is exothermic. The maximum heat obtained with 65mg. of haptoglobin (Kabi) is 22·5mcal. (heat of dilution of haptoglobin is added) and the slope is 1·02mcal./mg. of haemoglobin.

Fig. 3 shows the variation of reaction heat with

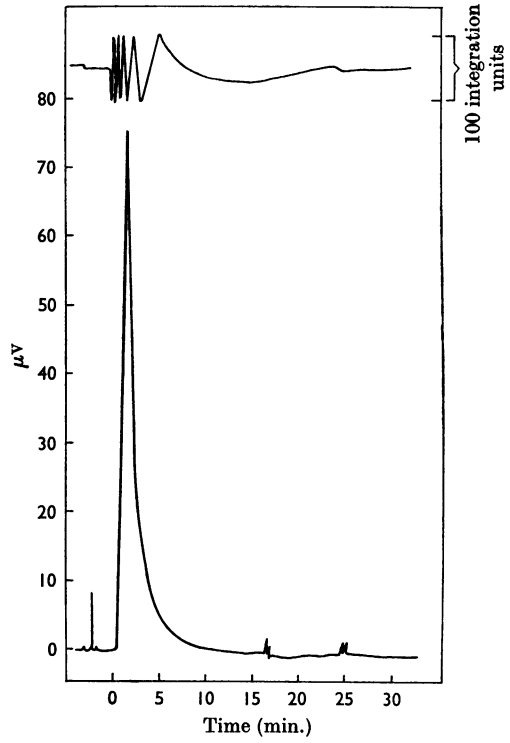

Fig. 1. Microcalorimeter measurement of the heat of reaction between 32·4 mg. of haemoglobin and 125 mg. of haptoglobin (Kabi). Each was in 5 ml. of buffer. The blank cell contained 125 mg. of haptoglobin in one side and 5 ml. of phosphate buffer, pH 7·4, in the other. The reaction temperature was 37°. The mixing sequence was 180° counterclockwise, 0° clockwise, 180° clockwise, 0° counterclockwise, 180° counterclockwise and 0° clockwise. The sensitivity setting was 100 μv. The lower curve shows the heat 'spike' and subsequent decay, and the upper one shows the integration. One integration unit = 32 mcal. (calculated from a standard heat input).

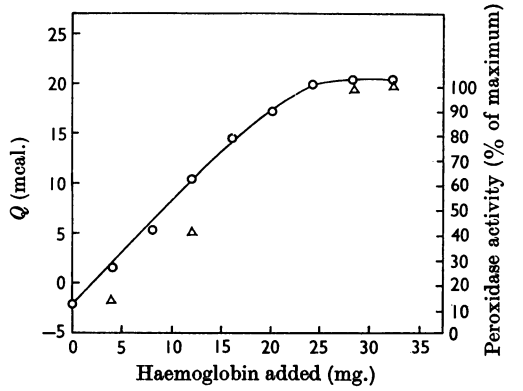

Fig. 2. Variation of reaction heat (Q) with amount of haemoglobin at a constant amount (65 mg.) of haptoglobin (Kabi). ○, Reaction heat; △, peroxidase-like activity of the mixtures after reaction.

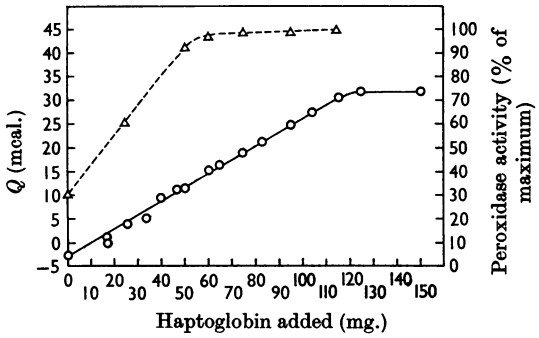

Fig. 3. Variation of reaction heat (Q) with amount of haptoglobin (Kabi) at a constant amount (32·5 mg.) of haemoglobin. ○, Reaction heat; △, peroxidase-like activity of the mixtures after reaction.

amount of added haptoglobin (Kabi) with a constant amount of haemoglobin. The dilution of haemoglobin in the absence of haptoglobin is endothermic (the dilution of haemoglobin is endothermic at this particular concentration and dilution; at other concentrations and dilutions it is exothermic); the reaction with haptoglobin is exothermic, so that for more than 15 mg. of haptoglobin (Kabi) with 32·5 mg. of haemoglobin the reaction is exothermic. The maximum heat obtained with 32·5 mg. of haemoglobin is 34·57 mcal. (heat of dilution is added), and the slope is 0·29 mcal./mg. of haptoglobin (Kabi). In both Figs. 2 and 3 the peroxidase-like activities of the mixtures after reaction are shown as percentages of the maximum.

Fig. 4 shows the variation of the reaction heat with haemoglobin with a constant amount of haptoglobin (Nitschmann 20%).

DISCUSSION

In the reactions with increasing amounts of haptoglobin, the reported mole-for-mole equivalence of haemoglobin and haptoglobin was not observed. In fact, it required at least 20 mg. more than the molecular equivalent of haptoglobin before maximum heat of reaction was attained. However, in the reverse situation, with increasing amounts of haemoglobin, approximate molecular equivalence was observed. This apparent discrepancy between molecular equivalence with haptoglobin excess and that with haemoglobin excess

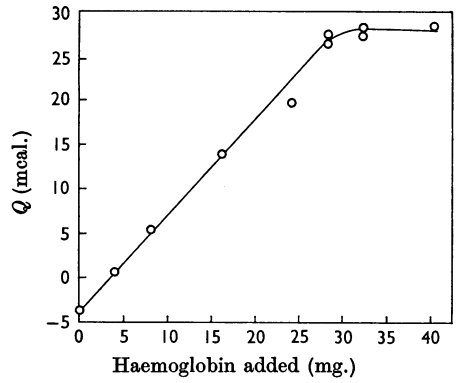

Fig. 4. Variation of reaction heat (Q) with amount of haemoglobin at a constant amount (162·5mg.) of haptoglobin (Nitschmann 20%).

can be explained by assuming that the actual reaction of haptoglobin is with the $\alpha\beta$-dimer of haemoglobin. Rossi-Fanelli, Antonini & Caputo (1961) and Guidotti (1967*a*,*b*) reported that in solution the haemoglobin (Hb) tetramer is dissociated into the dimer and at certain dilutions into the monomer:

$$Hb = \alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$$

G. Guidotti (quoted as personal communication by Bunn, 1967) reported that at concentrations of 100 mg. or less/100 ml. at least 25% of haemoglobin is present as the dimer. The exact degree of dissociation is unimportant, since reaction of the dimer with haptoglobin would cause more haemoglobin to dissociate. All of the haemoglobin can then be considered reacting as the dimer. Haptoglobin 1-1 was reported to have two sites capable of reacting with haemoglobin (Bunn, 1967). Chiancone *et al.* (1967) reported that haptoglobin 1-1 is capable of binding four α-chains and, with lesser affinity, four β-chains from haemoglobin. They found that the preferred compound is that containing two α- and two β-chains, the same as the haptoglobin–haemoglobin compound. Thus two areas of binding are required for each half molecule of haemoglobin. The reaction at haptoglobin (Hp) excess can be represented as:

$$Hp + \alpha\beta \rightarrow Hp\text{–}\alpha\beta$$

$$\text{or } Hp + \tfrac{1}{2}Hb \rightarrow Hp\text{–}Hb/2$$

and the reaction at haemoglobin excess as:

$$Hp + 2\alpha\beta \rightarrow \beta\alpha\text{–}Hp\text{–}\alpha\beta$$

$$\text{or } Hp + Hb \rightarrow Hb\text{–}Hp$$

Shim, Lee & Kang (1965) described the 'intermediate complex' between haptoglobin and haemoglobin with haptoglobin in excess as being composed of one molecule of haptoglobin and half a molecule of haemoglobin, from a study of its electrophoretic migration rate, which is intermediate between those of the slow-migrating haemoglobin and the faster-migrating fully saturated haemoglobin–haptoglobin complex, and also from its distribution on gel filtration. Chiancone *et al.* (1968), on the basis of their electrophoresis, ultracentrifugation and fluorescence-quenching studies, reported that haptoglobin combines 1:1 with haemoglobin when the molecular amounts are equal or when haemoglobin is in excess, but when haptoglobin is in excess a form that seems to be Hp–$\alpha\beta$ is formed. Nagel and co-workers, using a radioactive-tracer method (Nagel, Rothman, Bradley & Ranney, 1965) and fluorescence quenching (Nagel, 1967), reported that the reaction of haptoglobin is with the α-chain of haemoglobin, but that the β-chain, which does not itself react, increases the binding with the α-chain. Nagel (1967) also suggested that the reactive unit is the $\alpha\beta$-dimer. Bunn (1967) reported that in the reaction of the bis-(*N*-maleimidomethyl) ether derivative of haemoglobin with haptoglobin two molecules of the haemoglobin derivative were required for saturation. Other thiol reagents did not have this effect. Bis-(*N*-maleimidomethyl) ether differs from the other agents by coupling through two different groups so that the haemoglobin molecule does not readily dissociate into the dimer (Simon & Konigsberg, 1966). It was also reported that deoxyhaemoglobin does not dissociate readily (Benesch, Benesch & Williamson, 1962), and Nagel *et al.* (1965) reported that deoxyhaemoglobin did not react with haptoglobin. In our own experiments the addition of more haptoglobin to an equilibrated solution of molecular equivalents of haemoglobin and haptoglobin did not give more heat.

Even at haptoglobin excess, the preferred reaction is still of one haptoglobin molecule with two $\alpha\beta$-dimers. Otherwise the amount of haptoglobin required to give maximum heat would be close to twice that for molecular equivalence. It seems likely that a haptoglobin molecule with one $\alpha\beta$-dimer attached has a greater affinity for another $\alpha\beta$-dimer than has a haptoglobin molecule with both sites still free. As we approach an excess of haptoglobin, the two reactions:

$$\beta\alpha\text{–}Hp + \alpha\beta \rightarrow \beta\alpha\text{–}Hp\text{–}\alpha\beta$$

$$\text{and } \beta\alpha + Hp \rightarrow \beta\alpha\text{–}Hp$$

are competing, with the former favoured. Clarke (1966), using fluorescence quenching, also reported these competing reactions.

This reaction of haptoglobin with the $\alpha\beta$-dimer may explain its effect on the peroxidase-like reactions. Mattenheimer & Adams (1968) reported that at low peroxide concentrations haptoglobin makes haemoglobin a more effective catalyst of *o*-tolidine oxidation, but at the same time makes it more susceptible to destruction by peroxide. Guidotti (1967*b*) reported that in the reactions of ligands with haemoglobin the $\alpha\beta$-dimer is the unit of structure and function. Assuming that the same is true for its action as a peroxidase, we propose that the tetramer, which is normally in equilibrium with the dimer, is a less effective peroxidase and that haptoglobin functions by preventing the recombination of the dimers. The two complexes $\beta\alpha$–Hp and $\beta\alpha$–Hp–$\alpha\beta$ should have approximately the same peroxidase-like activity as long as the same amount of haemoglobin is involved, since association into the tetramer is prevented in both cases. Thus, when peroxidase activity is used as the criterion for saturation, a mole-for-mole ratio would exist with excess of either haemoglobin or haptoglobin, as shown in Figs. 2 and 3 (triangles).

The reaction heat/mg. of haemoglobin can be calculated from the slopes of the curves for a constant amount of haptoglobin or from the maximum heat for a constant amount of haemoglobin. Likewise the reaction heat/mg. of haptoglobin can be calculated from the slopes of the curve for a constant amount of haemoglobin or from the maximum heat for a constant amount of haptoglobin. Values from these calculations and the translation to ΔH/mole are shown in Table 1. The value from the slope with haptoglobin with a constant amount of haemoglobin is about 20% low, in agreement with the departure from mole-per-mole equivalence discussed above. The enthalpy measured also includes the enthalpy for the dissociation of haemoglobin into the dimers, but this was reported to be small (Kirshner & Tanford, 1964).

The value of $-70{\cdot}2$kcal./mole for ΔH is in disagreement with that of 0 ± 2kcal./mole reported by Clarke (1966). He measured fluorescence quenching when haptoglobin was titrated with haemoglobin in the temperature range 5–30°. He plotted the observed fluorescence against amount of haemoglobin added. A line drawn through the initial points determined the line where the apparent equilibrium constant is infinite, because it is assumed from the Law of Mass Action that all the haemoglobin added in the initial increments is complexed and there is no free haemoglobin. Another line was drawn through the points where fluorescence quenching had ceased. As the equivalence point was approached, the observed fluorescence deviated from the initial straight line. The apparent equilibrium constant was calculated at each point that did not lie on either of the straight lines, and the values were averaged, giving the estimated values of $K_{app.}$. Clarke found that a plot of $\boldsymbol{R}.\ln K_{app.}$ versus $1/T$ did not have a significant slope and thus ΔH must be close to 0. This large discrepancy between our calorimetric measurements and the indirect measurements of Clarke (1966) is important in understanding the mechanism of the reaction, because the hydrophobic bonding suggested for some protein–protein interactions requires a ΔH close to 0 (Nemethy & Scheraga, 1962; Nemethy, Steinberg & Scheraga, 1963). Clarke obtained his expression for the relationship between $K_{app.}$ and ΔH by integrating the Van't Hoff isobar:

$$\mathrm{d}(\ln K)/\mathrm{d}t = \Delta H/\boldsymbol{R}T^2$$

In the integration, he assumed that ΔH was practically constant over the temperature range studied. The integration then becomes:

$$\ln K = -\Delta H/\boldsymbol{R}T + C$$

If K does not change with temperature ΔH must be 0. However, if ΔH varies with temperature, the assumption is not valid and the simple integration fails. To study this assumption, the runs with 65mg. of haptoglobin and 32·5mg. of haemoglobin were repeated at 20° and 4°. As shown in Fig. 5, the ΔH value decreases from $-70{\cdot}2$kcal./mole at 37° to $-29{\cdot}7$ at 20° and to 7·2 at 4°. Thus ΔH changes by 2·3kcal./mole/deg. and is 0 at about 7°.

Table 1. *Calculation of enthalpy at* 37°

The average enthalpy obtained by the first five methods is $-70{\cdot}2$kcal./mole. (Molecular weights for haemoglobin and haptoglobin of 67000 and 85000 respectively were assumed.)

Method	Enthalpy (mcal./mg.)	Enthalpy (kcal./mole)
Maximum heat with constant amount of haemoglobin (32·5mg.)	1·064	−71·3
Slope for haemoglobin with constant amount of haptoglobin (65mg., Kabi)	1·023	−68·5
Slope for haemoglobin with constant amount of haptoglobin (162·5mg., Nitschmann 23%)	1·06	−71·0
Maximum heat with constant amount of haptoglobin (65mg., Kabi)	0·346 (0·806)*	−68·5
Maximum heat with constant amount of haptoglobin (162·5mg., Nitschmann 23%)	0·194 (0·844)*	−71·7
Slope for haptoglobin (Kabi) with constant amount of haemoglobin (32·5mg.)	0·29 (0·676)*	−57·5

* Calculated for pure haptoglobin.

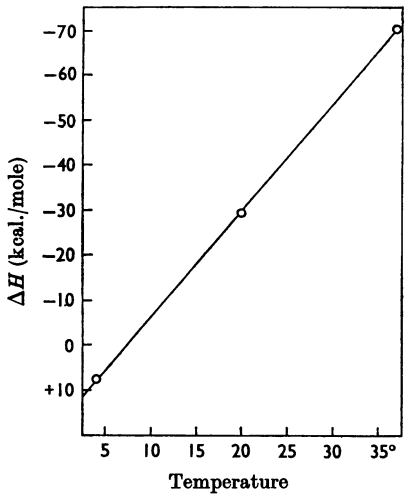

Fig. 5. Variation of ΔH of the haemoglobin–haptoglobin reaction with temperature.

This marked dependence of ΔH on temperature not only explains the discrepancy between our direct calorimetric measurements and the indirect ones of Clarke, but also is notable in itself since it resembles closely the variations of ΔH for refolding of a denatured protein (Brandts, 1964*a*,*b*; Tanford, 1968). The cited authors actually discuss the changes on denaturation, but since the denaturations are reversible, the same arguments with change of sign hold. From the relation:

$$\partial \Delta H/\partial T = \Delta C_p$$

there must be a large difference between the heat capacity of the haemoglobin–haptoglobin compound and the uncombined proteins [−2·3kcal./mole/deg. compared with 2·1kcal./mole/deg. given for denaturation of β-lactoglobulin (Tanford, 1968)]. According to the reasoning of Tanford (1968) the large value of ΔC_p explains the stability of the haemoglobin–haptoglobin compound. Changes of this magnitude would result from the removal of hydrocarbon moieties from the aqueous environment during folding of the protein (Tanford, 1968).

It is interesting to speculate whether there is a similar variation of ΔH with temperature in the case of antigen–antibody reactions. Steiner & Kitzinger (1956) reported a relatively low value of −3·5kcal./bond for albumin with its antibody measured at 22°. A similar value of 2·3kcal./mole/deg. that we found for the haemoglobin–haptoglobin reaction would give a ΔH for the albumin reaction with its antibody of −42kcal./mole at 37°. Also, it is likely that the temperature at which ΔH is 0 will be different for individual antigen–antibody reactions.

An enthalpy of −70kcal./mole is in line with that found by Green (1966), using a similar instrument and methods for the avidin–biotin reaction. He found a ΔH of −20kcal./mole of biotin and −80kcal./mole of avidin, one molecule of which combines with four of biotin:

The bonding involved in the haemoglobin–haptoglobin reaction has not been established. Van Royen (1950) (see Nyman, 1959) suggested that the bonding was between the carboxylic acid groups of haemoglobin and the amino groups of haptoglobin. Pavlicek (1966) and Kalous & Pavlicek (1965) showed there was no change in the number of carboxylic acid groups after the reaction, but the number of titratable histidine groups decreased significantly; i.e. the histidine groups were buried after the reaction. They further showed that when haemoglobin was photo-oxidized the loss of activity paralleled the loss of histidine, and when haptoglobin was photo-oxidized the loss of activity paralleled the loss of tyrosine. They suggested that hydrophobic bonding is involved in the reaction or that hydrogen bonds are formed between the tyrosine residues of haptoglobin and the histidine residues of haemoglobin, and that these bonds are reinforced by being in a hydrophobic region. Clarke (1966), on the basis of the low ΔH value he found, favoured hydrophobic bonding with entropy as the driving force. In contrast, we found the large enthalpy of −70·2kcal./mole at 37° acting as the driving force, suggesting something other than hydrophobic bonding. Hydrogen bonding reinforced by being in a hydrophobic environment is the most likely probability. If all the 26 histidine residues found by Pavlicek (1966) and Kalous & Pavlicek (1965) to be buried, and no other groups, are involved, from the ΔH value of −70kcal./mole each bond would have an energy of about −2·7kcal./mole. Other protein complexes to which ΔH makes a significant contribution to the free energy of binding are FAD by the 'old yellow enzyme' (Theorell & Nygaard, 1954), AMP by ribonuclease (Myer & Schellman, 1962), haem by apomyoglobin (Banerjee, 1962), skatole by human serum albumin (McMenamy, 1964), 1-anilinonaphthalene-8-sulphonic acid by apomyoglobin (Stryer, 1965), ε-DNP-lysine by specific antibody (Eisen & Siskind, 1964) and biotin by avidin (Green, 1966). As discussed by Kauzmann (1959) and referred to by Green (1966), 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. It appears that in the haptoglobin–haemoglobin reaction, as in the avidin–biotin

reaction, the driving force is enthalpy and not entropy, suggesting that hydrogen bonding (reinforced by the groups being in a hydrophobic region) is involved.

We attempted to determine an apparent equilibrium constant for the haemoglobin–haptoglobin reaction by using the data in Fig. 2 in the same manner that Clarke (1966) used his fluorescence-quenching data as described above. The calorimetric data were not sufficiently precise for the relatively large constant to be determined. Clarke reported that $K_{app.}$ was approximately $10^9 M^{-1}$. Chiancone *et al.* (1968) reported that the affinity of haptoglobin for oxyhaemoglobin was too high for any precise calculation of the equilibrium constant, but estimated equilibrium constants for the combination of the isolated α-chains with haptoglobin; assuming all the sites to be equivalent and independent, they found a minimum constant of approximately $5 \times 10^6 M^{-1}$. Since oxyhaemoglobin is bound much more strongly than the isolated α-chains and the sites are probably not completely independent, the actual constant is likely to be greater. If this minimum constant is substituted in the equation:

$$\Delta F = -RT.\ln K$$

a lower limit for the free energy of −9 kcal./site or −36 kcal./mole of haptoglobin can be calculated. A similar calculation from the data of Clarke (1966) gives a free energy of −12 kcal./mole.

REFERENCES

Banerjee, R. (1962). *Biochim. biophys. Acta*, **64**, 368.

Bauer, J. D., Toro, G. & Ackerman, P. G. (1962). *Bray's Clinical Laboratory Methods*, 6th ed., p. 127. St Louis: C. V. Mosby Co.

Benesch, R., Benesch, R. E. & Williamson, M. E. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2071.

Benzinger, T. H. & Kitzinger, C. (1954). *Fed. Proc.* **13**, 11.

Brandts, J. (1964*a*). *J. Amer. chem. Soc.* **86**, 4291.

Brandts, J. (1964*b*). *J. Amer. chem. Soc.* **86**, 4302.

Bunn, H. F. (1967). *J. Lab. clin. Med.* **70**, 606.

Chiancone, E., Alfsen, A., Ioppolo, C., Vecchini, P., Finazziagro, A., Wyman, J. & Antonini, E. (1968). *J. molec. Biol.* **34**, 347.

Clarke, L. G. (1966). Ph.D. Thesis: University of Vermont.

Eisen, H. N. & Siskind, G. W. (1964). *Biochemistry*, **3**, 996.

Green, N. M. (1966). *Biochem. J.* **101**, 774.

Guidotti, G. (1967*a*). *J. biol. Chem.* **242**, 3685.

Guidotti, G. (1967*b*). *J. biol. Chem.* **242**, 3704.

Kalous, V. & Pavlicek, A. (1965). *Biochim. biophys. Acta*, **111**, 337.

Kauzmann, W. (1959). *Advanc. Protein Chem.* **14**, 1.

Kirshner, A. G. & Tanford, C. (1964). *Biochemistry*, **3**, 291.

Laurell, C. B. & Gronvall, C. (1962). *Advanc. clin. Chem.* **5**, 135.

McMenamy, R. (1964). *J. biol. Chem.* **239**, 2835.

Mattenheimer, H. & Adams, E. C. (1968). *Klin. Chem. klin. Biochem.* **6**, 69.

Myer, Y. P. & Schellman, J. A. (1962). *Biochim. biophys. Acta* **55**, 361.

Nagel, R. L. (1967). *J. biol. Chem.* **242**, 3428.

Nagel, R. L., Rothman, M. C., Bradley, T. B. & Ranney, H. M. (1965). *J. biol. Chem.* **240**, PC4543.

Nemethy, G. & Scheraga, H. A. (1962). *J. phys. Chem.* **66**, 1773.

Nemethy, G., Steinberg, I. Z. & Scheraga, H. A. (1963). *Biopolymers*, **1**, 43.

Nyman, M. (1959). *Scand. J. clin. Lab. Invest.* **11**, Suppl. 39.

Owen, J. A. (1960). *J. clin. Path.* **13**, 163.

Pavlicek, Z. (1966). *Abh. dtsch. Akad. Wiss. Berl., kp. Med.* **4**, 217.

Polonovski, M. & Jayle, M. F. (1938). *C. R. Soc. Biol., Paris*, **129**, 457.

Polonovski, M. & Jayle, M. F. (1940). *C. R. Acad. Sci., Paris*, **211**, 517.

Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1961). *J. biol. Chem.* **236**, 391.

Shim, B. S., Lee, T. H. & Kang, Y. S. (1965). *Nature, Lond.*, **207**, 1264.

Simon, S. R. & Konigsberg, W. H. (1966). *Proc. nat. Acad. Sci., Wash.*, **56**, 749.

Smithies, O. & Walker, N. F. (1956). *Nature, Lond.*, **178**, 694.

Steiner, R. F. & Kitzinger, C. (1956). *J. biol. Chem.* **222**, 271.

Stryer, L. (1965). *J. molec. Biol.* **13**, 482.

Tanford, C. (1968). *Advanc. Protein Chem.* **23**, 244.

Theorell, H. & Nygaard, A. P. (1954). *Acta chem. scand.* **8**, 1649.

Van Royen, A. H. (1950). Dissertation: University of Delft.