tractors in combination with a fourfold difference in the time intervals used. The abscissa scales are then in the proportion of 2·5:1. These protractors are illustrated in Figs. 2 and 3. The data used in making them are given in Tables 2 and 3, which also include an indication of a convenient size for each protractor, though this may be varied to suit any particular requirement.

The actual protractor scales illustrated cover the range of s values most commonly encountered, and they may be extended by a number of devices. For example, the 4 min. scale, if used with 2 min. exposure intervals in the ultracentrifuge, will give half the correct S value, and so S values as high as 500s may be determined. Furthermore, the low gear-speeds from 3397 to 9341 rev./min. (numbered in red) may be used in conjunction with the corresponding normal speeds (numbered in white), when the S value determined from the protractor needs to be multiplied by 36 (the square of the gear-box ratio). This extends the range of values which may be measured to 9000s.

It is important in preparing the scales that a stable material should be used. Ideally they should be drawn on card at least twice the size required, and reduced photographically onto a process-type cut film (Kodaline KS3, Kodak Ltd., London). Both protractors (Figs. 2, 3) can be incorporated onto the same sheet of film. The most important consideration in preparing the protractors is that the ordinate and abscissa scales should be exactly at right angles to each other.

HOW TO USE THE METHOD

The experimental values of x are read off from the ultracentrifuge plates in the usual way, and plotted on the logarithmic graph paper (Fig. 1). The appropriate protractor (Fig. 2 or 3) is then placed on the graph with its abscissa scale along a horizontal graph line (in Fig. 1, left, this is on line $x = 6{\cdot}3$ cm.), with the line corresponding to the rotor speed at the intersection of the experimental curve with the latter. The experimental curve will then intersect the ordinate scale of the protractor at the required value of S (Fig. 1, right).

The objective of the system described above was first and foremost convenience. It is quite obvious that the problem may be solved by using simpler equipment, e.g. by making the protractor scales integral with the graph paper, and using parallel scales to transfer the slopes, but it was thought that this method was not quite so convenient in use.

As far as accuracy is concerned, it has been found in practice that results are reliable within about ± 1s in 100s if boundary positions are estimated to within 0·01 cm. The step involving the plotting of log x against t is particularly valuable because it serves as a check that sedimentation is progressing uniformly, besides ensuring that the value for S is based on a weighted assessment of all the experimental observations. If two or more components are present during a run, it is possible to check that the two curves intersect at the level of the meniscus; and, if a peak is just resolved at the end of a run, it is possible to draw a line from its position to the meniscus, and so estimate the slope accurately.

SUMMARY

1. A graphical method for the evaluation of sedimentation coefficients is described. Its principle involves the employment of a special logarithmic graph paper and protractors calibrated directly in Svedberg units.

I should like to thank Mr S. Frey for the trouble which he has taken with the photographic problems involved.

REFERENCE

Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, p. 17. Oxford University Press.

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The Reactions of some Aromatic *C*-Nitroso Compounds with Haemoglobin

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Although the reactions of many ligands with methaemoglobin have been described, work with haemoglobin has, until recently, been confined to the low molecular weight molecules of oxygen, carbon monoxide and nitric oxide. St George & Pauling (1951) have, however, examined the equilibrium between haemoglobin and a series of *iso*-cyanides, finding a sharp decrease in affinity on passing from *iso*propyl *iso*cyanide to *tert.*-butyl *iso*-cyanide. With free haem, on the other hand, they found much smaller differences in affinity. To explain their results, they put forward the idea that the haem groups of haemoglobin are located in a 'crevice' in the protein which hinders the approach

of the bulky *tert.*-butyl *iso*cyanide molecule, but allows easier access to the smaller *iso*propyl *iso*cyanide molecule. Recently, Ainsworth, Gibson & Roughton (1960) have given an account of kinetic measurements with the series of alkyl *iso*cyanides, showing that the difference in affinity of the several members is wholly accounted for by differences in their rates of combination with reduced haemoglobin; this result is exactly contrary to the position for oxygen, carbon monoxide and nitric oxide where the million-fold difference in affinity depends chiefly on differences in the rate of dissociation.

It has been known for nearly 40 years that nitrosobenzene will combine with haemoglobin, and Jung (1940) has shown that in nitrosobenzene–haemoglobin the ratio nitrosobenzene to iron is 1:1 and that nitrosobenzene and carbon monoxide compete with one another for combination. The nitrosobenzene molecule, with its bulky aromatic nucleus and the facility of introducing substituents with known relations to the active centre, offers an opportunity to extend the observations already made with the *iso*cyanides to a further group of compounds, and this paper describes the results obtained with nitrosobenzene and the nitrosotoluenes.

EXPERIMENTAL

Blood solutions. Stock solutions of sheep reduced haemoglobin, of undetermined electrophoretic type and corresponding to blood diluted 1:4, were prepared exactly as described by Gibson & Roughton (1957*a*), and were stored in a refrigerator for up to a week. Working solutions were prepared by diluting the stock solution with a 2% (w/v) soln. of $Na_2B_4O_7,10H_2O$, and all determinations were made with this borate buffer solution. Crystalline whale myoglobin was a gift from Professor S. R. Elsden.

Nitroso compounds. Nitrosobenzene was prepared according to Vogel (1948), *o*-nitrosotoluene by the method of Bamberger & Rising (1901*a*), and *m*- and *p*-nitrosotoluenes as described by Bamberger & Rising (1901*b*). In each case the corresponding hydroxylamine was separated from the reaction mixture and crystallized from light petroleum before oxidation to the nitroso compound. All the melting points were within 1·5° of those given in the references quoted. The nitroso compounds were stored at −12°, and appeared to be stable for several months at that temperature.

Solutions were prepared by shaking an excess of nitroso compound with the 2% soln. of borate warmed to about 50°. The mixture was then cooled, kept for 10 min. and excess of nitroso compound was filtered off. The concentration of solutions prepared in this way was determined by dilution with water (1:50 was usually convenient) and measurement of the extinction at 320 mμ in 1 cm. cells. Conversion of the readings into concentrations of nitroso compounds was done by comparison with a standard curve obtained by dissolving weighed amounts (about 10–15 mg.) in 50 ml. of ethanol and then diluting with water. Dilution to 1:50 with water gave a reading at 320 mμ of about 0·4. The solutions of nitroso compounds do not seem to be very stable and were always prepared immediately before use.

Stopped-flow determinations. These were made with the apparatus and procedure of Gibson (1954). The combination reactions with haemoglobin were followed at 435 mμ and the replacement of nitroso compounds by CO was followed at 600 mμ.

Concentrations of haemoglobin solutions. These were determined spectrophotometrically with a Unicam SP. 600 at 576 mμ, taking the millimolar extinction coefficient of O_2Hb as 15·6. All concentrations are stated in terms of haemin, not in molarity of haemoglobin of molecular weight 68 000.

Notation. The combination of nitroso compounds with haemoglobin, like that of other ligands, is believed to take place in four stages as suggested by Adair (1925). Following Antonini & Gibson (1960) the individual velocity constants are written as lower-case letters with two subscript numbers the first of which is the number of ligand molecules bound before reaction, and the second the number after. Thus the first step in the combination reaction is written h_{01}, and the first dissociation reaction is h_{43}. The letter h is used to distinguish the nature of the ligand, k and l being used for O_2 and CO respectively. The four equilibrium constants are written as capital italic letters, the subscript giving the number of ligand molecules combined in the more fully saturated of the two compounds concerned. Thus the equilibrium constant of the first reaction between reduced haemoglobin and a nitroso compound is written H_1.

RESULTS

Combination of reduced haemoglobin with nitroso compounds. The rate constant for this reaction was determined by mixing together, in the stopped-flow apparatus, reduced haemoglobin and an oxygen-free solution of nitrosobenzene dissolved in the buffer used for the haemoglobin. When the reaction was followed with several different concentrations of nitrosobenzene the initial value for the rate constant was greater the more dilute the nitrosobenzene solution (Fig. 1). It is well known [see, for example, the recent review of Gowenlock & Lüttke (1958) for references] that aromatic nitroso compounds readily form unreactive dimers both in solution and in the solid state. If the dimers do not react with haemoglobin, dependence of the apparent rate constant on the concentration of nitroso compound would be expected. The curve in Fig. 1 has been calculated assuming a monomer–dimer equilibrium, and agrees well with the experimental points. The value of the rate constant for the combination of haemoglobin with each of the compounds studied was obtained by carrying out an experiment similar to that shown in Fig. 1 and extrapolating to infinite dilution of nitroso compound.

The values are collected in Table 1. This shows that the rates are all similar except for *o*-nitrosotoluene which combines only about one-fifth as rapidly as the other compounds.

Course of the reaction between nitrosobenzene and haemoglobin. Plotting the logarithm of the ratio of

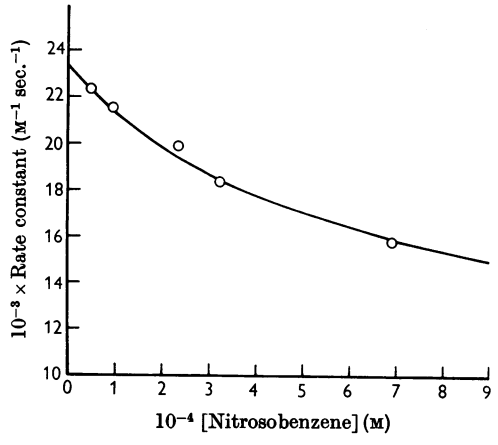

Fig. 1. Combination of reduced haemoglobin with nitrosobenzene. The figure shows the effect on the second-order rate constant of varying the concentration of nitrosobenzene. The concentration of haemoglobin was 30 μM in borate buffer, pH 9·1, The observations were made at 20° with light of mean wavelength 435 mμ with a depth of solution of 2·34 mm. The curve was calculated on the assumption that the variation in rate is due to an equilibrium in the solution between dimeric and monomeric forms of nitrosobenzene with dissociation constant $2{\cdot}2 \times 10^{-3}$ M. The ordinate is the overall rate constant, equal to 0·25 h_{01}.

Table 1. *Combination of nitroso compounds with haemoglobin and myoglobin*

The figures give the rate of combination extrapolated to zero concentration of ligand. The figure for haemoglobin is h_{01}, giving the rate of combination of the first molecule of ligand. Borate buffer, pH 9·1; temp. 20°. Myoglobin: observations at 437 mμ, 2·34 mm. light path; concn. 19 μM after mixing. Nitroso compounds: concn. 0·1–0·5 mM.

Compound	Rate of combination: Myoglobin ($10^{-4} \times M^{-1}$ sec.$^{-1}$)	Haemoglobin ($10^{-4} \times M^{-1}$ sec.$^{-1}$)
Nitrosobenzene	9·2	9·2
o-Nitrosotoluene	8·0	1·7
m-Nitrosotoluene	10·8	10·5
p-Nitrosotoluene	10·0	10·5

the concentration of reactants in a second-order reaction against time should give a straight line. When this procedure was applied to the combination of haemoglobin with nitrosobenzene a gently rising curve was obtained (Fig. 2, line *A*), a result reminiscent of Gibson & Roughton's (1955) finding for the combination of haemoglobin with carbon monoxide. They attributed the deviation from the second-order equation to differences in the rate with which successive molecules of carbon monoxide combined with a single molecule of haemoglobin, and later gave an account of methods by which the four velocity constants for the successive reactions

$$Hb_4 + CO \rightarrow Hb_4CO,$$
$$Hb_4CO + CO \rightarrow Hb_4(CO)_2,$$
$$Hb_4(CO)_2 + CO \rightarrow Hb_4(CO)_3,$$
$$Hb_4(CO)_3 + CO \rightarrow Hb_4(CO)_4$$

could be determined (Gibson & Roughton, 1957*a*). Applying their methods, values of the first constant h_{01} were obtained from the initial slope of plots similar to that in Fig. 2, and h_{34} was taken to be $1{\cdot}3 \times 10^5 M^{-1}$ sec.$^{-1}$, as determined independently by methods to be described later. Values of the two remaining constants h_{12} and h_{23} were selected by

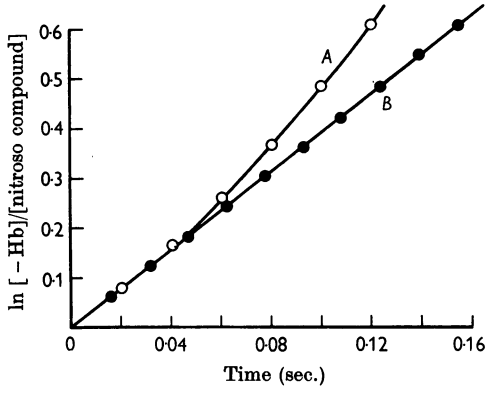

Fig. 2. Plot showing that *o*-nitrosotoluene reacts with reduced haemoglobin in an accurately second-order way (*B*), whereas the reaction with nitrosobenzene speeds up as it progresses (*A*). The units of the ordinate are arbitrary and have been adjusted to make the initial slopes coincide for the two compounds. The reactions were followed with light of mean wavelength 435 mμ, with 30 μM-haemoglobin dissolved in borate buffer pH 9·1, at a temperature of 20°.

Table 2. *Comparison of the course of the reaction between nitrosobenzene and reduced haemoglobin as observed and as calculated*

The calculated values were obtained by Desk Method II of Gibson & Roughton (1957*a*), taking the ratios of the velocity constants for the combination of successive molecules of nitrosobenzene to be 4:3:2·5:5·5. The experimental values were obtained with the stopped-flow apparatus with 1·03 mM-nitrosobenzene and 50 μM-haemoglobin at 20·3° and pH 9·1. Concentrations given are those before mixing.

Time (msec.) ...	20	40	60	80	100	120
Nitrosobenzene–haemoglobin observed (%)	16·0	31·5	45·1	57·9	67·5	75·8
Nitrosobenzene–haemoglobin calculated (%)	16·7	31·6	45·1	57·0	67·4	75·5

trial and error, and the calculated and observed courses of the reaction compared with Desk Method II of Gibson & Roughton (1957*a*). An example of the results is given in Table 2, which shows that a reasonable fit was obtained with $h_{01}:h_{12}:h_{23}$ as 4:3:2·5. These ratios do not differ significantly from those (4:6:2) found by Gibson & Roughton (1957*a*) for sheep haemoglobin combining with carbon monoxide, though they are closer to the ratios (4:3:2) which would be obtained if there were no haem–haem interaction until three molecules of nitrosobenzene had combined.

Course of the reaction between haemoglobin and the nitrosotoluenes. When the results with *m*- and *p*-nitrosotoluenes are compared with those for nitrosobenzene, it is found that the curves for all three are superimposable, and the ratios of the combination velocity constants must therefore be similar. This is not true of *o*-nitrosotoluene, however, where the reaction appears to be accurately second-order throughout its course (Fig. 2, line *B*). This difference between *o*-nitrosotoluene and the other compounds examined has been confirmed in experiments with different samples of blood and in each case the result of Fig. 2 has been reproduced. There are two main ways of explaining this result: either there is no haem–haem interaction with *o*-nitrosotoluene, when values for successive velocity constants would be in the ratio 4:3:2:1, or haem–haem interaction takes place between pairs of haems and gives rise to ratios of, say, 4:10:2:10, where the exact numerical value of the second and fourth constants is not important, provided only that they are both substantially larger than the first and third velocity constants. The choice between these alternatives is considered in the Discussion.

Effect of temperature on the combination of haemoglobin with nitrosobenzene. Determinations of the rate of combination, at three temperatures and two concentrations of nitrosobenzene, were made. The natural logarithms of the rate constants are shown plotted in the usual way against the reciprocal of absolute temperature in Fig. 3. It is clear that the 6·4-fold difference in concentration of nitrosobenzene did not influence the temperature dependence of the reaction so that the slope of the lines in Fig. 3 is a measure of the activation energy of the nitrosobenzene–haemoglobin reaction, and not of the effect of temperature on, for example, the monomer–dimer equilibrium of nitrosobenzene. The separate lines for the two concentrations of nitrosobenzene are due to the dependence of the observed rate constant on concentration shown in Fig. 1. The value of the activation energy derived from the slope of the lines in Fig. 3 is 13·8 kcal.

Combination of myoglobin with nitroso compounds. The rates of combination with reduced myoglobin

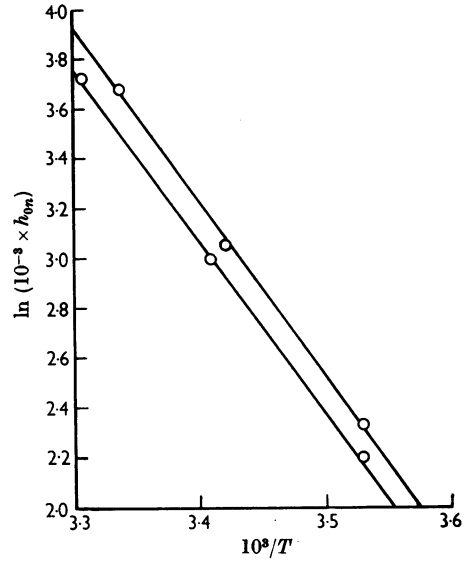

Fig. 3. Effect of temperature on the combination of nitrosobenzene with reduced haemoglobin. The natural logarithm of the overall rate constant (= 0·25 h_{01}) is plotted against 1000/T, where T is the absolute temperature of the experiments. The lower line was obtained with 0·59 mM-nitrosobenzene, the upper line with 92 μM. The concentration of haemoglobin was 31 μM in borate buffer, pH 9·1. The reaction was followed with light of mean wavelength 435 mμ.

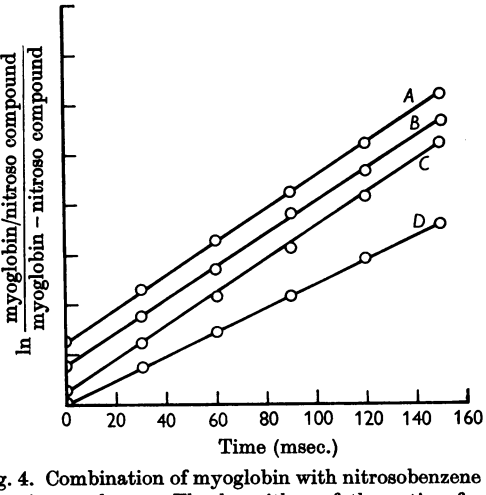

Fig. 4. Combination of myoglobin with nitrosobenzene and the nitrosotoluenes. The logarithm of the ratio of myoglobin remaining to nitroso compound remaining, divided by the difference in concentration between the myoglobin and the nitroso compound, is plotted against time. The slope of the lines is proportional to the second-order velocity constant. The ordinate is arbitrary. The concentration of myoglobin, determined as metmyoglobin cyanide, was 19 μM, the temperature was 20°, and the reaction was followed with light of mean wavelength 437 mμ. *A*, Nitrosobenzene, 66 μM; *B*, *p*-nitrosotoluene, 131 μM; *C*, *m*-nitrosotoluene, 112 μM; *D*, *o*-nitrosotoluene, 86 μM.

are given in Table 1 and are similar to those for haemoglobin (with the exception that the rate of combination of *o*-nitrosotoluene is much nearer to that for the other compounds than with haemoglobin). The course of the reaction is accurately second-order in every case, and examples for the four compounds are given in Fig. 4. This result shows that changes in the concentration of the monomer, which might be expected to follow on mixture of the solution of nitroso compound with an equal volume of myoglobin solution, are either much faster or much slower than the combination reactions with myoglobin. This point is important in justifying the kinetic analysis of the reactions with haemoglobin where the complex nature of the reaction might obscure changes due to dissociation of the nitroso compounds.

Dissociation velocity constants. The rate of dissociation of the first molecule of nitroso compound from fully saturated haemoglobin has been determined by displacement with carbon monoxide. The principles of the method have been fully discussed by Gibson & Roughton (1957*b*); the ratio l_{34}/h_{34} is sufficiently high to allow direct determinations of h_{43} in just the same way as nitric oxide can be used to determine l_{43} (Gibson & Roughton, 1957*b*) without the extrapolation procedure of Gibson & Roughton (1955) [l_{34} is the velocity constant for the reaction $Hb_4(CO)_3 + CO \rightarrow Hb_4(CO)_4$, and l_{43} that for the reverse reaction]. The results, given in Table 3 show that nitrosobenzene and *m*-nitrosotoluene dissociate at about the same low rate, which is about half that for carbon monoxide (Gibson & Roughton, 1957*b*). *o*-Nitrosotoluene dissociates about twice as fast as nitrosobenzene, whereas *p*-nitrosotoluene dissociates about 12 times as fast. The temperature coefficient for the dissociation of nitrosobenzene is 4·1/10°, which is similar to that for carbon monoxide.

Relative affinity of haemoglobin for oxygen and nitroso compounds. Where the numbers of free ligand-binding groups are negligible, Roughton (1934) has shown that the partition of haemoglobin between two ligands is given by [HbY][X] = [HbX][Y] × partition constant, where X and Y are the two ligands. The partition constant is itself equal to the ratio of the equilibrium constants for the combination of the fourth molecules of the two ligands. In the case of carbon monoxide and oxygen this relation is $L_4/K_4 = M$, where L_4 is the equilibrium constant for the reaction $Hb_4(CO)_3 + CO \rightleftharpoons Hb_4(CO)_4$ and K_4 that for the corresponding reaction with oxygen. Exactly analogous relations apply to any pair of ligands which do not react with one another, and, as the value of K_4 is known, the equilibrium constant for the combination of the fourth molecule of a nitroso compound may be obtained by examining its competition with oxygen.

In practice, a solution of oxyhaemoglobin in buffer had a measured amount of nitroso compound added, and was then equilibrated with a known partial pressure of oxygen. The proportions of oxyhaemoglobin and of nitrosohaemoglobin were then determined spectrophotometrically, and this allowed H_4, the equilibrium constant for the combination of the fourth molecule of nitroso compound to be calculated.

The equation [HbY][X] = [HbX][Y] × constant, may, if the total concentration of haemoglobin is taken as 1, be re-written log [HbY/(1−HbY)] = log constant + log Y − log X; if the concentration of X is kept constant, a plot of log [HbY/(1−HbY)] against log Y should give a straight line of unit slope. Fig. 4 shows such plots for the four nitroso compounds examined; the values of H_4 derived from them are given in Table 3.

Velocity of combination of the fourth molecule of the nitroso compounds with haemoglobin. By combining the data for H_4 and h_{43} given in Table 3, values of h_{34}, the combination velocity constant for the fourth molecule of nitroso compound to combine with haemoglobin, may be obtained. These values are given in Table 3, together with the ratio $4h_{34}/h_{01}$, which ranges only between 5 and 9 and has an average value of 7. This average value is to be compared with the statistical value of 1 which would be expected in the case that no haem–haem interaction existed.

Table 3. *Rates of combination and dissociation of the fourth molecule of nitroso compounds reacting with haemoglobin*

h_{43} was determined by CO displacement: blood diluted/100 in borate buffer, pH 9·1, in the form of nitrosohaemoglobin was mixed with mM-CO in borate buffer, 20°; observations were made at 600 mμ. H_4 was determined by competition between nitroso compounds and O_2: blood diluted/100 in borate buffer, in equilibrium with air (1 atm. pressure), was mixed with varied quantities of nitroso compounds, shaken with air and the extinction was read at 600 mμ; final concentration of haemoglobin, 38 μM.

Compound	h_{43} (sec.$^{-1}$)	$10^{-5} \times H_4$ (M^{-1})	$10^{-4} \times h_{34}$ (M^{-1} sec.$^{-1}$)	$4h_{34}/h_{01}$
Nitrosobenzene	0·016	80	13	5·5
o-Nitrosotoluene	0·032	9·5	3·0	7·1
m-Nitrosotoluene	0·021	108	23	8·8
p-Nitrosotoluene	0·20	7·0	14	5·3

DISCUSSION

The experiments have been made more laborious and less certain by the tendency of the nitroso compounds to dimerize in aqueous solution. No changes in extinction were, however, observed when making spectrophotometric measurements of the concentration of solutions, nor was a wait necessary when making dilutions of the stock nitroso compound solutions in carrying out experiments of the kind shown in Fig. 1. If there were a rapidly changing monomer–dimer equilibrium during the combination reactions, the calculation of second-order rate constants would be disturbed in experiments similar to those shown in Figure 1, because, as the combination reaction proceeded, the dimer initially present would decrease, owing to the taking up of part of the monomer by the haemoglobin. This effect may be rendered unimportant either by measuring initial rates, or by working with a considerable excess of nitroso compound, and one or other of these practices has been followed throughout.

One other source of difficulty requires discussion. Jackson & Thompson (1954) have shown that iodonitrosobenzene and iodophenylhydroxylamine can combine with haemoglobin at sites other than those occupied by ligands such as oxygen and carbon monoxide. They find, for example, a stoicheiometric uptake of the radioactive iodo compounds by carboxyhaemoglobin, unaccompanied by spectral changes. No evidence of interference, due to combination reactions of this kind, has been found in the experiments described here. It is not possible to say with certainty if this is because the conditions used here were unsuitable for the combination or because the tests available were not adequate to detect it. The experiments on the competition of oxygen with nitrosobenzene and *m*-nitrosotoluene offer the best chance of detecting combination with groupings other than the haem group. In these experiments a small measured quantity of nitroso compound was mixed with a known amount of haemoglobin. The quantity of free nitroso compound in equilibrium with the oxyhaemoglobin was calculated from the amount of nitrosohaemoglobin formed and the amount of nitroso compound added. If, besides combining with the haem group, some of the nitroso compound had combined with another group in the haemoglobin molecule, the calculation of the amount of free nitroso compound would be inaccurate, and this inaccuracy would be reflected in plots of the kind given in Fig. 5. The effect to be expected depends on the relative affinity of the haem group and the second site. In the limiting case where the affinity of the two groups is widely different, if the haem group has the lower affinity, no combination will be observed until one equivalent of nitroso compound has been added; in less extreme cases the plots would be concave upwards; Fig. 5 shows no tendency of this kind, suggesting that the affinity of the second site may be lower than that of the haem group. Such a suggestion is entirely compatible with Jackson & Thompson's (1954) findings, since in their example they used 25 mg. of iodophenylhydroxylamine in 10·5 ml., giving an initial concentration of about 10 mM, compared with the concentration of about 20 μM required in the experiments of Fig. 5 for half-displacement of oxygen.

It would also be possible for effects, due to the combination of further molecules of nitroso compounds, to show up in the course of the combination reaction with reduced haemoglobin and myoglobin. As the reaction with haemoglobin is kinetically complex, no conclusions can be drawn. With myoglobin, no side-effects were observed, the course of the combination being accurately second-order throughout. There does not seem to be any reason for thinking that reactions of the kind described by Jackson & Thompson (1954) have interfered measurably with the results given here.

The results for the compounds examined show surprising variations in the rates of the reactions with haemoglobin, whereas with myoglobin the rate is about the same with all four compounds (Table 1). With haemoglobin, the rate of combination of both the first and fourth molecules of *o*-nitrosotoluene is about one-fifth of that for the other three compounds, and both rates differ widely from that for myoglobin. With the other three nitroso compounds the numerical values of h_{01}, h_{34} and h_{0n} (for myoglobin) all agree within a factor of

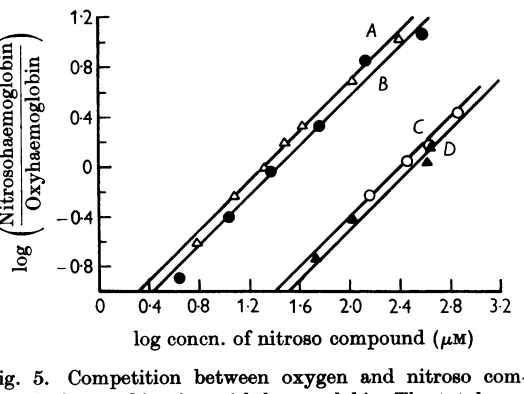

Fig. 5. Competition between oxygen and nitroso compounds for combination with haemoglobin. The total concentration of haemoglobin was 0·8 mM, in borate buffer, pH 9·1 at 20°. Determinations of the concentration of nitrosohaemoglobin were made spectrophotometrically at 600 mμ. *A*, *m*-Nitrosotoluene; *B*, nitrosobenzene; *C*, *o*-nitrosotoluene; *D*, *p*-nitrosotoluene.

two, and the agreement between h_{01} and h_{0n} is almost exact. This result is very different from that reported by Ainsworth *et al.* (1960) for a series of alkyl *iso*cyanides (ethyl, *iso*propyl, *tert.*-butyl) where the rate of combination with myoglobin correlated well with the rate of combination of the fourth molecule with haemoglobin, but showed poor correlation with the rate of combination of the first molecule of *iso*cyanide. In this respect the nitroso compounds resemble the gaseous ligands more closely than the *iso*cyanides.

It is surprising that *p*-nitrosotoluene should show a rate of dissociation about ten times as high as that for the other three compounds: just as *o*-nitrosotoluene forms an exception so far as the rates of combination are concerned, so *p*-nitrosotoluene is exceptional in its rate of dissociation.

The behaviour of *o*-nitrosotoluene offers the further puzzle that the course of the combination reaction with haemoglobin is accurately second order. As the $4h_{34}/h_{01}$ ratio is 7 (Table 3) the possibility that there is no haem–haem interaction is excluded, since this would require that $4h_{34}/h_{01} = 1$. The alternatives which remain are that h_{12} and h_{23} are both considerably greater than h_{01} [in such a case the extrapolation procedure of Gibson & Roughton (1957*a*), would yield high values for h_{01}] or that interaction occurs between pairs of haems, i.e. $h_{12} \gg h_{01}$ and $h_{34} \gg h_{23}$. The data do not permit a choice to be made between these possibilities, but interaction between pairs of haems is the more likely.

The haem–haem interaction, as measured by the ratio of the first and last velocity constants and given in Table 3, is about the same for all four compounds and gives the average value 7. In the *iso*cyanide series already mentioned, the comparable ratio varies from 2·3 for ethyl *iso*cyanide to 78 for *tert.*-butyl *iso*cyanide (Ainsworth *et al.* 1960), whereas for the gaseous ligands the values are CO = 60, O_2 = 30, NO = 4 (Gibson, 1959). The value for the nitroso compounds is thus similar to that for nitric oxide. It is tempting to take the series of values for haem–haem interaction as a whole and try to account for them by a single hypothesis. For the gaseous ligands, whose molecules occupy much the same volume, but which show wide fluctuations in interaction, the size of the interaction varies inversely with the absolute rate of combination of the first ligand molecule. Such an effect would be expected if, as a result of haem–haem interaction, the rate of combination of the fourth ligand molecule reached a maximum largely independent of the chemical nature of the ligand molecule. This idea can readily be extended to the *iso*cyanides where the absolute value of the velocity constant for the combination of the fourth molecule of *iso*cyanide decreases only tenfold on passing from ethyl to *tert.*-butyl *iso*cyanide, whereas the rate of combination of the first molecule decreases 500-fold in the same series. The nitroso compounds examined do not show large variations in haem–haem interaction, but the average of 7 is within the range for the *iso*cyanide series. Consideration of the absolute rates for the combination of the fourth ligand molecule is, however, less satisfactory, for the rate for the nitroso compounds is considerably larger than that for the *iso*cyanides, which must presumably be put down to the chemical differences between them.

The recent striking success of Perutz *et al.* (1960) and of Kendrew *et al.* (1960) in constructing models showing the relation of the haem groups to the polypeptide chains for haemoglobin and myoglobin lends additional interest to the discussion of haem–haem interaction, for the models show that the structures of oxyhaemoglobin and oxymyoglobin are closely similar. The question then arises whether reduced myoglobin more nearly parallels, in its behaviour, fully reduced haemoglobin or haemoglobin combined with three molecules of ligand. The evidence on this point is not yet complete and appears contradictory. If attention is given only to the rates of reaction, the gaseous ligands and the nitroso compounds react with myoglobin as though it were one-quarter of fully reduced haemoglobin, whereas the *iso*cyanides behave as though myoglobin corresponded to haemoglobin in which three places were already occupied by ligand molecules. Further work, including particularly the determination of the activation energies of the reactions, is required to clear up this point.

The rate of combination of the first molecule of nitrosobenzene and the activation energy lead to a value of the frequency factor similar to that found for other ligands (with the notable exception of oxygen combining with haemoglobin). A summary of the results and a figure has been given by Gibson (1959). The *iso*cyanides have been found by Ainsworth *et al.* (1960) to give a similar value for the frequency factor as does the rate of dissociation of the first molecule of nitrosobenzene from fully saturated haemoglobin. Such data are difficult to interpret unambiguously, but it seems that there is no special difficulty about the approach of the nitrosobenzene molecules to within reaction radius of the haem group. Certainly the compounds examined here are well below the limiting size for approach to the haem group, for Kiese & Plattig (1958) have shown that haemoglobin is able to react both with benzylphenylhydroxylamine and the corresponding nitroso compound. Detailed kinetic studies with haemoglobin would be difficult because of the insolubility of these compounds in water.

SUMMARY

1. Reduced haemoglobin combines successively with four molecules of nitrosobenzene and the nitrosotoluenes.

2. The rate constants for the combination of the first molecule of ligand at pH 9·1 and 20° are (M^{-1} sec.$^{-1}$): nitrosobenzene, $9·2 \times 10^4$; *o*-nitrosotoluene, $1·7 \times 10^4$; *m*-nitrosotoluene, $10·5 \times 10^4$; *p*-nitrosotoluene, $10·5 \times 10^4$.

3. The fourth molecule to combine does so more rapidly than the first, the average increase in rate is sevenfold. The increase compared with the first molecule is about the same for all four compounds.

4. The rate of dissociation of the first molecule of the same compounds from fully saturated haemoglobin has been measured. For nitrosobenzene, and *o*- and *m*-nitrosotoluene, the rates lie within the range 0·016–0·032 sec.$^{-1}$ at 20°. The value for *p*-nitrosotoluene is 0·2 sec.$^{-1}$.

5. The activation energies for the combination of the first molecule of nitrosobenzene and the dissociation of the first molecule of nitrosobenzene give a normal value to the frequency factor in both cases.

6. The rates of combination of the four nitroso compounds with myoglobin are numerically equal to those for the combination of the first molecule with reduced haemoglobin, with the exception of *o*-nitrosotoluene which combines with myoglobin almost as fast as do the other compounds.

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The Estimation of Phospholipase A Activity in Aqueous Systems

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The action of snake-venom phospholipase A on phospholipid fractions was first investigated in aqueous reaction systems. Levene & Rolf (1923) and Levene, Rolf & Simms (1924) carried out the reaction in phosphate buffer, which has been used extensively since that time (Chargaff & Cohen, 1939; Fairbairn, 1945; Rimon & Shapiro, 1959). In phosphate buffer, however, the conversion of lecithin into lysolecithin by venom preparations is slow and incomplete. For example, Chargaff & Cohen (1939) reported a lysolecithin yield of from 15 to 50% of the original lecithin employed in a reaction system maintained at 38° for a period of 24 hr.

The finding that phospholipase A can split lecithin in ether solution (Hanahan, 1952; Hanahan, Rodbell & Turner, 1954) has been of great practical importance. The ethereal reaction system was further studied and improved by Long & Penny (1957), and is used generally at the present time for the preparation of lysolecithin. The system is somewhat inflexible, however, as some diacyl phospholipids are relatively insoluble in ether, and pH adjustment is difficult.

The rate at which phospholipase A attacks lecithin or other phospholipids has been followed by a

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