is not an adsorption phenomenon but probably involves diffusion into the blood cells.

The relationship between D_p/D_c and x_p is possibly non-linear (see Fig. 1); this could be caused by an adsorption of diaminodiphenyl sulphone on to the cell surface which was inhibited by the presence of blood plasma (cf. Kono, 1953). Hypothesis (1) above may therefore be valid only to a first approximation; the deviation is not, however, necessarily significant.

In the plasma the solubility data alone show that in this part of the system compound formation between the sulphone and a component does take place. A numerical calculation of the equilibrium constants has been attempted by Linderstrøm-Lang (1962).

SUMMARY

1. The solubility of 4,4'-diaminodiphenyl sulphone in water is 38 mg./100 g. at 37° and decreases by a factor of two with a 12° decrease in temperature.

2. Equilibrium between human blood cells and plasma with respect to 4,4'-diaminodiphenyl sulphone is established within 5 min.

3. The amount of the sulphone contained in, or in other ways attached to, the blood cells is four times an estimated free concentration inside the cells.

4. 4,4'-Diaminodiphenyl sulphone forms complexes with a component in human blood plasma.

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4,4'-Diaminodiphenyl sulphone: Binding to Serum Albumin

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It was shown in the preceding paper (Linderstrøm-Lang & Naylor, 1962) that 4,4'-diaminodiphenyl sulphone, dissolved in blood plasma, forms one or more complexes with a component of the plasma. The present paper describes an attempt to verify that the component is plasma albumin.

EXPERIMENTAL

Materials. 4,4'-Diaminodiphenylsulphone was a recrystallized (from ethanol) laboratory-grade product (m.p. 176–

179°). The serum albumin used was crystallized bovine plasma albumin from Armour Pharmaceutical Co.

Solubility determinations. Solid diaminodiphenyl sulphone and the solvent were shaken in a thermostat overnight. The supernatant liquid was isolated by suction through a sintered-glass disk (G4) and concentration of the sulphone was determined.

Dialysis experiments. The cellophan bags employed were, at the upper end, provided with a short piece of conical glass tubing fitted with a small rubber bung. Thus each bag could be used several times and an effective check for impurities in the cellophan wall was made possible.

Usually, 0.5 ml. of albumin solution in iso-osmotic (0.9%) sodium chloride was added to the weighed bags as the inner phase. These were then immersed in 2 ml. of

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standard solutions of the sulphone (iso-osmotic with sodium chloride) of differing concentration and placed in a thermostat. After equilibration the tubes were withdrawn and quickly weighed (the operation taking about $\frac{1}{2}$ min.) and a sample was taken. A knowledge of the final volume of the inner phase (weighings were corrected for the probable amount of water in the cellophan wall) was necessary for the determination of percentage recovery of the sulphone and for the calculation of the final concentration of albumin.

The equilibration period was from 1 to 2 days, except in experiments on the rate of penetration.

Determinations of diaminodiphenyl sulphone. The extinction at 305 m μ of the sulphone was measured directly. The sensitivity of this method was slightly less than the one employed in the preceding paper and it was necessary to correct for albumin, which has a small absorption at this wavelength (the edge of the very strong absorption band at 280 m μ).

When standard solutions (10 mg./100 g.; 0.4 mm) of the sulphone were prepared, the rate of dissolution was so small that it was almost impossible to bring all the solid particles into solution without heating. This may, of course, be due to impurities; but the solubility, as determined spectrophotometrically, was independent of the amount of solid material present.

RESULTS

Solubility determinations. The solubility determinations were reproducible with s.d. 2–3 %. The approach to equilibrium from a higher temperature, and therefore a higher concentration, was, however, very slow; this may have been caused by the adsorption of albumin on the surface of the crystals of diaminodiphenyl sulphone.

The present determinations of the solubility in water agree with those of the preceding paper; this may serve as the analytical basis for the comparison to be made.

Dialysis experiments. These experiments show a somewhat higher accuracy than the blood experiments. Results, with s.d. of the recovery, are shown in Table 1.

The results indicate that the sulphone is adsorbed on the cellophan, a phenomenon often encountered in dialysis experiments (Rosenberg & Klotz, 1960). The possibility of a systematic error in the spectrophotometric procedure was eliminated by control experiments.

Table 1. *Percentage recovery of diaminodiphenyl sulphone in dialysis experiments*

Results of these experiments are shown in Figs. 1 and 2.

Strength of albumin soln. (%)	Average recovery (%)	s.d. (%)	No. of expts.
0 (blanks)	93.9	1.4	15
2.5	98.9	2.4	19
8–10	101.5	2.7	37

The results of the dialysis experiments with diaminodiphenyl sulphone and serum albumin are shown in Figs. 1 and 2, together with a combination of solubility values for water and albumin solutions of differing strength.

The equations used for the plasma-cell relationship (see preceding paper) are also applicable here, where $K' = 1$. The somewhat higher accuracy of these experiments makes a more detailed calculation permissible, equation (3) (Linderström-Lang & Naylor, 1962) being modified:

$$r \equiv \frac{D_0 - D''}{D} = A_p K_1 \frac{1 + 2DK_2 + 3D^2K_2K_3 \dots}{1 + DK_1 + D^2K_1K_2 + D^3K_1K_2K_3 + \dots} \quad (1)$$

where D_0 is the total concentration of the sulphone and D'' is free sulphone in the albumin solution (which may be calculated from D on the basis of available water in the albumin phase); D is the concentration outside (i.e. D/D'' is a measure of the

Fig. 1. Relative amount of diaminodiphenyl sulphone bound to serum albumin as a function of albumin concentration, at 25°. $\circ$, $0.025 < D$ [concentration (mm) of the sulphone in albumin-free solution] < 0.075 ; $\bullet$, $0.100 < D < 0.200$; $\square$, $0.200 < D < 0.300$; $\blacksquare$, $0.300 < D < 0.40$; $\triangle$, $0.40 < D < 0.50$; $\blacktriangle$, $0.50 < D < 0.60$; ∇ , $0.60 < D < 0.70$; $\diamond$, $D = 0.75$. Ordinate: ratio of concentration of diaminodiphenyl sulphone bound to serum albumin to that of free diaminodiphenyl sulphone (D) in the albumin-free solution. (1) Distribution of the sulphone between NaCl solution and a serum albumin solution, separated by cellophan, was determined after equilibration for 1 or 2 days; (2) the solubility of the sulphone in each of these solutions was determined. For further explanation see text.

Fig. 2. Relative amount of diaminodiphenyl sulphone bound to serum albumin as a function of albumin concentration, at 37°. ♦, $D = 1.3$ mm. See Fig. 1 for further identification of symbols and explanation.

activity coefficient of the sulphone in the albumin solution). It is assumed that the addition of one, two etc. molecules of diaminodiphenyl sulphone to one albumin molecule does not change its properties appreciably; so that, for example, for

$$K_1 = \frac{AD \cdot f_{AD}}{D'' \cdot f_D \cdot A \cdot f_A}$$

f_{AD} and f_A are equal and therefore cancel, i.e.

$$K_1 = \frac{AD}{D \cdot A}$$

similarly for K_2 etc. A combination of these equations yields equation (1).

According to equation (1), r is proportional to A_p at constant D ; the straight lines in Figs. 1 and 2 represent this relationship.

The influence of pH and the presence of sodium chloride on the compound-formation has also been investigated. The solubility of the sulphone is about 5% higher in pure water than in 0.9% sodium chloride, and the corresponding solubilities in the presence of albumin differ less (a few per cent). The effect of sodium chloride on the ratio of concentrations (r) in Figs. 1 and 2 is therefore less than the experimental error.

The same is true for pH. Normally the pH of the albumin solution is between 5.7 and 5.9, but changing the pH to 5.4 or 6.1 with hydrochloric acid and sodium hydroxide respectively had no effect on the solubility (in 2.5% albumin) within $\pm 2\%$.

There was an osmotic-pressure difference across the membrane in all experiments with albumin and no pressure adjustments were made. The resulting

steady flow of water through the membrane was highly dependent on protein concentration: the amount of water passing through was only 0.01 ml./24 hr. when 2.5% albumin was used, and for 10% albumin the figure was 0.12 ml./24 hr.

This agrees with the molal osmotic coefficient, Φ , of serum albumin, when the rate of water transport is assumed to be proportional to the osmotic pressure difference (p). p may be written

$$p = \frac{RT \cdot \gamma_A}{v' \cdot M_A} \left(1 + \frac{\Phi - 1}{\gamma_A} \cdot \gamma_A \right)$$

where γ_A is g. of protein/1000 g. of water, M_A is the molecular weight of the protein.

$$(\Phi - 1)/\gamma_A = \frac{1}{2} (\partial \ln f_A / \partial \gamma_A)$$

(Güntelberg & Linderstrøm-Lang, 1949), where f_A is the activity coefficient of the protein; the derivative may be considered independent of γ_A .

Φ is 2.75 for $\gamma_A \approx 110$ (Scatchard, Batchelder & Brown, 1946), so the ratio of the pressures is

$$\frac{p_{10}}{p_{2.5}} = 4 \frac{1 + (2.75 - 1)}{1 + \frac{1}{4}(2.75 - 1)} = 7.6$$

The association of chloride with serum albumin (about 10 ions/molecule of protein) will also increase the ratio. A calculation carried out in accordance with the formula developed by Güntelberg & Linderstrøm-Lang (1949) leads to a 25% increase in $p_{10}/p_{2.5}$. Thus $p_{10}/p_{2.5}$ becomes approx. 10, in agreement with the experiments.

Determination of equilibrium constants. As a working hypothesis it is assumed that albumin is the component in blood plasma that reacts with diaminodiphenyl sulphone. On this basis the corresponding equilibrium constants may be calculated.

One possible approach is to assume that a limited number of sites on the albumin molecule are available to the sulphone. Equation (1) with the blood-plasma figures then yields a K_1 and K_2 when K_3 , K_4 etc. are assumed to be zero: The plasma albumin concentration, A_p , for $x_p = 1$, is assumed to be normal, i.e. 5.5 g./100 ml. (0.8 mm). The result of the calculation is: $K_1 = 2.0 \pm 0.5$ l./m-mole and $K_2 = 1.2 \pm 0.5$ l./m-mole. A value for K_1/K_2 of less than 4 implies that the addition of the second molecule of the sulphone is facilitated by the presence of the first. It is more probable, however, that there is a K_3 and perhaps even a K_4 etc.

When the same method is applied to the serum albumin data the agreement is poor and one is forced to assume the existence of at least a K_3 .

Another approach, often employed, is to assume that the more or less numerous binding sites on one albumin molecule are independent and identical. Equations (1) or (3) (preceding paper) then become

$$r = A_p K_1 \frac{1}{1 + (1/n) DK_1} \quad (2)$$

where n is the number of binding sites, and: $r = (S_p - S)/S$, blood plasma-solubility data (preceding paper); $r = (D_p K' - D_0)/D_0$, blood data (preceding paper, Fig. 1); $r = (S_0 - S'')/S$, serum albumin-solubility data; $r = (D_0 - D'')/D$, serum albumin dialysis data. Here S_0 is total solubility of diaminodiphenyl sulphone in an albumin solution and $S_0 - S''$ is the amount bound to the albumin.

For $n = 3$, $K_1:K_2:K_3 = 9:3:1$.

According to equation (2) A_p/r is a linear function of D . This is shown by the lines in Fig. 3, where the points represent the reciprocal gradients of the lines in the three Figures [Fig. 1 (preceding paper), Figs. 1 and 2 (this paper)] and the data on solubility given in the preceding paper. Hence (for blood) $K_1 = 2.5$ l./m-mole and $n = 2.7$; $n = 3$ is the most likely interpretation.

The serum albumin results at 25° yield

$$K_1 = 6.0 \pm 0.2 \text{ l./m-mole}$$

and $n = 2.5$, and at 37° $K_1 = 4.5 \pm 0.2$ l./m-mole and $n = 2.9$. A possible interpretation within experimental error is again $n = 3$.

From the dependence of K_1 on the temperature, the heat of reaction for diaminodiphenyl sulphone-serum albumin may be calculated:

$$-RT^2 \text{ d} \ln K_1 / \text{d}T = +4.5 \text{ kcal.}$$

Fig. 3. Comparison of blood results with serum albumin results (A_p/r) as a function of the concentration of diaminodiphenyl sulphone in albumin-free solution (D). □, Blood results at 37°; ○, serum albumin results at 37°; ●, serum albumin results at 25°. Ordinate: A_p/r (see equation 2) (mm). Abscissa: D (mm). Blood results are taken from the preceding paper (Linderstrøm-Lang & Naylor, 1962: Results section and Fig. 1); A_p for $x_p = 1$, i.e. no dilution of the blood plasma, is assumed to be 5.5 g./100 ml.; S and D_0/K' respectively are taken as D . The serum albumin results are taken from Figs. 1 and 2, where the reciprocal gradients of the lines are A_p/r .

DISCUSSION

The deviation of the experimental points in Figs. 1 and 2 from linearity is significant. A systematic error in the analytical procedure is not impossible but is unlikely, and other explanations should be sought.

The lack of any appreciable effect of pH and sodium chloride on the solubility eliminates any possibility that Donnan effects across the cellophan membrane (i.e. unequal distribution of H^+ , Na^+ and Cl^- ions) may have caused this disagreement between the results for experiments with 2.5% and 10% albumin.

Another explanation would be the departure from true equilibrium which is caused by the osmotic pressure difference across the membrane. The resulting water transport is much smaller at $A_p = 2.5\%$ than at $A_p = 10\%$; thus the higher A_p values of r are the more affected.

Experiments showed that the final state reached was independent of which phase the diaminodiphenyl sulphone was added to at the start. The system thus reaches a quasi-stationary stage, and any concentration gradient across the membrane will be mainly a function of the rate of water transport. The kinetics of the penetration of the sulphone was studied experimentally and a probable concentration difference calculated; but it was too small (about 5%) to account for the discrepancies found in Figs. 1 and 2. However, the permeability of the sulphone was determined when it was far from being in equilibrium, so the possibility remains that the permeability is altered when equilibrium is approached, thereby making an extrapolation impossible.

If the system for all practical purposes was in equilibrium, the bending of the curves in Figs. 1 and 2 may be the result either of compound-formation of the type A_2D or of changes in the properties of the albumin molecule when the sulphone is added to it (and thereby changes of the activity coefficients). In neither case is equation 1 applicable, and, indeed, the difference between the two explanations may be one of description rather than of physical properties.

The limited accuracy of the data makes further calculations unprofitable. The lines drawn are based mainly on the high albumin values as they are the more accurate.

Fig. 3 shows that the hypothesis that the sulphone reacts with albumin in blood plasma leads to identical n values for the two systems, and the equilibrium constants become of the same order of magnitude. The fairly small discrepancy may be caused by minor structural differences between the two albumins, human and bovine, and to some extent by a deviation from normal plasma albumin concentration in the blood used.

On the basis of this similarity in the two systems it would seem to be a justifiable inference that plasma albumin in human blood is the active component.

n should, according to theory, be independent of temperature, as also, the results indicate, may be the case.

SUMMARY

1. 4,4'-Diaminodiphenyl sulphone forms complexes with bovine serum albumin.

2. From a comparative study of these and of the complexes formed in human blood plasma (see preceding paper), it is inferred that, in human blood plasma, plasma albumin is the active component.

3. The data are compatible with the existence of three identical and independent binding sites ($K_1:K_2:K_3 = 9:3:1$), available to 4,4'-diaminodiphenyl sulphone on each albumin molecule.

4. The first complexity constant for the sulphone and human plasma albumin at 37° is 2.4×10^3 l./mole; for bovine serum albumin at 25°, it is 6.0×10^3 l./mole and at 37°, 4.5×10^3 l./mole.

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Studies on the 'Incorporation Factor' with *Bacillus megaterium*

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Gale & Folkes (1958) showed that ultrasonically disrupted cells of *Staphylococcus aureus* (strain Duncan) lost much of their ability to incorporate amino acids into trichloroacetic acid-precipitable material after incubation with deoxyribonuclease. The addition of nucleic acid isolated from the same organism or from *Bacillus megaterium* restored this ability. They further demonstrated that a trace component, which was purified 1000-fold from nucleic acid digests, was capable of replacing nucleic acid. This material, called 'incorporation factor', has also been reported to stimulate the rate of mitosis of chicken fibroblasts (Gale, 1959).

Our initial attempts to investigate the structure of this potent material were hampered by the difficulties of the assay with disrupted *S. aureus* cells [see Gale & Folkes (1958)]. We discovered that the incorporation of [¹⁴C]glycine into the trichloroacetic acid-precipitable material of washed and starved intact *B. megaterium* cells was stimulated by an incorporation-factor preparation. We have thus developed a simple and more reproducible assay which has properties similar to the disrupted staphylococcal system of Gale & Folkes (1958).

With this assay, a 5-month-old sample of incorporation factor was shown to consist mainly of glycerol by Kuehl, Demain & Rickes (1960). Since the properties of free glycerol do not correlate with some of the characteristics of incorporation factor as described by Gale (1959), Kuehl *et al.* (1960) mentioned the possibility that, in nucleic acid, the incorporation factor might exist as a bound form of glycerol with high specific activity. This possibility is now being studied by E. F. Gale (personal communication). This paper presents the details of our work with the *B. megaterium* system.

METHODS

Organism. *B. megaterium* was kindly supplied by Dr E. F. Gale of Cambridge University. The culture was maintained by serial transfer on nutrient agar (Difco) slants which were stored at 4°.

Growth and preparation of suspensions. The medium used for growth was similar to the 'deficient medium B' of Gale (1951) and contained 1% of glucose, 0.1% of yeast extract (Difco) and 0.12% of L-arginine hydrochloride in buffered salts mixture. The salts mixture contained 0.1% of KH_2PO_4 , 0.33% of Na_2HPO_4 , 0.07% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1% of