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# THE EFFECTS OF NATIVE AND MODIFIED BOVINE SERUM ALBUMIN ON THE PERMEABILITY OF FROG MESENTERIC CAPILLARIES

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SUMMARY

1. Single capillaries in the mesenteries of pithed frogs were perfused sequentially with two frog Ringer solutions. The first solution contained no protein; the second solution contained either native or chemically modified bovine serum albumin (BSA) at a concentration of 3–5 mg $ml^{-1}$. During each perfusion capillary permeability was assessed from the hydraulic conductivity of the capillary wall ($L_p$) which was determined from measurements of fluid filtration rate at two or more different capillary pressures (Michel, Mason, Curry, Tooke & Hunter, 1974).

2. $L_p$ measured during perfusion with protein-free Ringer solution was on average three times greater than its value for the same vessel perfused with Ringer solution containing native BSA. This confirms the findings of Mason, Curry & Michel (1977).

3. BSA, which had been succinylated to modify the free amino groups of its lysine residues, appeared to be as effective as native BSA in reducing $L_p$.

4. After modification of its arginine side chains by exposure to 1,2-cyclohexanedione (CHD) in the presence of 0·2 M-NaOH, BSA lost its property of reducing $L_p$ in capillaries perfused with Ringer solution.

5. Exposure of BSA to 0·2 M-NaOH followed by dialysis against normal Ringer solution did not affect its property of reducing $L_p$.

6. CHD-treated BSA at a concentration of 2·5 mg $ml^{-1}$ had no effect upon the effective osmotic pressure exerted across capillary walls by Ringer perfusates containing the neutral polymer Ficoll 70 at a concentration of 40 mg $ml^{-1}$. Native BSA raised the effective osmotic pressure from $7{\cdot}07 \pm 1{\cdot}93$ $cmH_2O$ to $20{\cdot}50 \pm 2{\cdot}37$ $cmH_2O$ ($n = 7$; $P < 0{\cdot}001$).

7. It is concluded that the effects of BSA on permeability depend upon specific sites in the BSA molecule. It is suggested that these sites involve positively charged arginine side chains of the albumin molecule. The results are discussed in terms of the fibre-matrix hypothesis of capillary permeability and in terms of Brown's (1976) theory for the structure of albumin.

## INTRODUCTION

Normal capillary permeability appears to depend upon the interaction of circulating plasma proteins with the capillary wall. This view is based on the observation which has been made both in single perfused capillaries (Levick & Michel, 1973*b*; Mason,

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Curry & Michel, 1977; Michel & Phillips, 1979, 1985; Clough & Michel, 1981; Huxley & Curry, 1982) and in perfused capillary beds (Drinker, 1927; Danielli, 1940; Kinter & Pappenheimer, 1963; Rippe & Folkow, 1977; Myhre & Steen, 1977; Diana, Keith, & Fleming, 1980; Schneeberger, 1982) that removal of plasma proteins from the perfusates reversibly increases vascular permeability. In single frog mesenteric capillaries, Ringer solutions containing bovine serum albumin (BSA) appear to be as effective as frog plasma in preventing this increase in permeability.

The changes in permeability which result from perfusing with protein-free solutions are very large. Thus in single frog mesenteric capillaries, the hydraulic conductivity of the capillary wall ($L_p$) is increased 3–5-fold when the vessels are perfused with protein-free Ringer solutions (Mason, Curry & Michel, 1977). This increase can be prevented by addition to the perfusate of BSA at a concentration of 1 mg $ml^{-1}$. Complete reversal of the increase in $L_p$ appears to require a higher BSA concentration (Huxley & Curry, 1982).

While the phenomena are well described, the underlying mechanism is not understood. Danielli (1940) proposed that plasma proteins narrowed the pores or channels conducting fluid through the capillary wall by binding to their walls and this theory has been examined quantitatively (Landis & Pappenheimer, 1963; Levick & Michel, 1973*b*; Mason *et al.* 1977). More recently it has been suggested that the molecular filter at the capillary wall is the cell coat or glycocalyx which covers and fills the channels conducting fluid and hydrophilic molecules through the endothelium (Michel, 1978; Curry & Michel, 1980; Curry, 1980; Michel, 1980). According to this model, the plasma proteins lower permeability by binding to the matrix, reducing the space within it, and possibly by rearranging the fibrous molecules into a more regular lattice and thus into a more efficient filter (Curry, 1981; Michel, 1981, 1983).

Evidence that proteins which bind to the endothelial surface coat may reduce capillary permeability has been provided by the observation that a highly cationized form of ferritin which binds evenly to the surface coat of frog mesenteric capillaries reduces $L_p$ after perfusion with Ringer solutions (Turner, Clough & Michel, 1983). It also raises the effective osmotic pressure which perfusates containing the neutral macromolecule Ficoll 70 can exert across the capillary walls (Michel & Phillips, 1979, 1985). These properties of cationized ferritin resemble those of BSA but whereas cationized ferritin is believed to bind to the capillary wall through its positively charged groups, BSA carries a net negative charge at physiological pH.

In spite of its net charge, BSA does bind anions and in some cases the binding has been shown to depend upon the positively charged lysine and arginine side chains of the albumin molecule (see for example Kragh-Hansen, 1981). The experiments described in this paper were designed to see whether chemical modification of these positively charged side chains of BSA affects its permeability-reducing properties. The lysine side chains were modified by succinylation of the free amino groups and the arginine residues were modified by exposing the BSA to 1,2-cyclohexanedione (CHD) in the presence of 0·2 M-NaOH. The effects of BSA upon permeability were assessed by measuring the $L_p$ value of single frog mesenteric capillaries first when they were perfused with protein-free Ringer solutions and then when they were perfused with Ringer solutions containing BSA (3–5 mg $ml^{-1}$). Five series of experiments were carried out to test the different forms of BSA which were: native

BSA; succinylated BSA; CHD-treated BSA; succinylated and CHD-treated BSA; and BSA which had been exposed to 0·2 M-NaOH and then dialysed against normal Ringer solution. A further series of experiments were performed to investigate the effects of CHD-treated BSA upon the effective osmotic pressures exerted by perfusates containing the neutral macromolecule Ficoll 70 across the walls of single capillaries. Viscometry of solutions of native, succinylated and CHD-treated BSA was carried out to check the extent to which these chemical modifications had affected the conformation of the BSA molecules.

Preliminary results of these experiments have been communicated to the Physiological Society (Michel, Phillips & Turner, 1982).

## METHODS

*Ringer solutions.* The Ringer solutions used as superfusates had the following composition (mmol $l^{-1}$): NaCl, 111·1; KCl, 2·4; $MgSO_4 . 7H_2O$, 1·0; $CaCl_2$, 1·1; glucose, 5·5; pH adjusted to 7·30–7·50 by addition of $NaHCO_3$ (10 mmol $l^{-1}$) and bubbled with 3 % $CO_2$ in oxygen. Similar Ringer solutions were used as perfusates. When they contained albumin, the $NaHCO_3$ was omitted and the pH was adjusted by titration to 7·4 using 0·23 M-NaOH after addition of the protein. All perfusates were filtered through Millipore filters (type GS, pore size 0·22 $\mu$m) before being used.

*Native and chemically modified albumin solution*

Native bovine serum albumin (crystallized, B.D.H. Chemicals Ltd.) was dissolved in the Ringer solution in known concentrations as the control perfusate. This same sample of BSA was used for the preparation of chemically modified forms of albumin.

The positively charged free amino groups on the side chains of the lysine residues of the albumin molecules were modified by the method of Klapper & Klotz (1972) which replaces the positively charged free amino groups with negatively charged succinyl residues. BSA was dissolved in 25 ml 0·2 M-phosphate buffer (pH 8·0) to a concentration of 60 mg $ml^{-1}$. The pH was maintained at 8–9 by addition of NaOH and the solution continuously stirred as 0·4 g succinic anhydride was added in small portions over a period of 30 min. The solution was stirred for a further 30 min and then dialysed for 24–48 h at 4 °C against several changes of frog Ringer solution. The concentration of succinylated albumin in the dialysate was estimated from the optical density of the solution at 220 nm using solutions of native albumin as standards. The dialysate was then diluted with frog Ringer solution to give the concentration of succinylated albumin required for an experiment (5 mg $ml^{-1}$), filtered through Millipore filter paper and titrated with 0·23 M-NaOH to a pH of 7·4–7·6. The extent to which the albumin had been modified was assessed by estimating the free amino group content of the succinylated specimen using Habeeb's (1966*a*) modification of the TNBS (2,4,6-trinitrobenzene 1-sulphonic acid) method.

The positively charged guanidino groups of the arginine residues of albumin were modified to give an uncharged product by treatment of the protein with CHD using the procedure of Toi, Bynum, Norris & Itano (1967). A solution of 0·125 g native BSA in 25 ml 0·2 M-NaOH (in deionized water) was poured into a beaker containing 0·05 g CHD (Sigma) and stirred gently for several minutes with a glass rod. The resulting clear pale yellow solution was left to stand for 4 h at room temperature. It was then stirred rapidly and carefully titrated with HCl to neutral pH. The solution was dialysed at 4 °C for 48 h against several changes of frog Ringer solution and on some occasions it was concentrated by ultrafiltration across an Amicon PM-10 membrane. The dialysed solution of modified albumin was finally filtered through a Millipore filter and titrated to pH 7·4–7·6. The concentration of albumin was estimated from the optical density at 220 nm using native BSA as standards and in all perfusion experiments adjusted to 3–5 mg $ml^{-1}$. The extent to which the arginine groups had been modified by this procedure was assessed by Messineo's (1966) method.

In some experiments, both the lysine and arginine side chains of the albumin were modified. For this preparation, 4 ml succinylated albumin solution (50 mg $ml^{-1}$) which had been dialysed against frog Ringer solution, was mixed with 36 ml 0·22 M-NaOH (in deionized water). 25 ml of the resulting solution (5 mg succinylated albumin $ml^{-1}$ 0·20 M-NaOH) was then added to 0·05 g CHD and the procedure described above for modifying the arginine groups was followed.

Because the modification of the arginine groups involved exposure to 0·2 M-NaOH which may have irreversibly denatured the albumin, control experiments were carried out using albumin which had been exposed to 0·2 M-NaOH but not to CHD. These solutions were prepared by following the procedure for modifying the arginine groups with the single difference that the beaker into which the albumin solution was poured did not contain CHD. After dialysis, and in some cases ultrafiltration, the albumin concentration was estimated from its optical density at 220 nm using solutions of native BSA as standards.

*Viscometry.* An Ostwald viscometer, with a delivery time of approximately 145 s for distilled water at room temperature, was used to estimate the relative viscosities of the albumin solutions. The viscometer was mounted in a water bath which was stirred continuously by a stream of bubbles from an oxygen cylinder. Relative viscosity was calculated from the ratio of the delivery time for a given volume of BSA solution to the delivery time for the same volume of protein-free Ringer solution. Measurements were made at a series of BSA concentrations from 3·5 to 63·0 mg $ml^{-1}$.

*Animal preparation.* Capillary permeability was estimated in single capillaries in the mesenteries of frogs (*Rana temporaria* and *Rana pipiens*) whose brains and upper spinal cords had been destroyed by pithing. The mesenteries were carefully laid over the polished surface of a short Perspex pillar so that they could be transilluminated (Michel, Baldwin & Levick, 1969; Levick & Michel, 1973*a*) and the upper surface of the tissue was cooled and kept moist by a continuous flow of superfusate Ringer solution. Tissue temperature was monitored by a thermistor glued to the upper surface of the pillar and was kept within the range 18–24 °C.

The micropipettes and the technique of cannulating and perfusing single mesenteric capillaries with suspensions of red cells have been described previously (Michel *et al.* 1969; Michel, Mason, Curry, Tooke & Hunter, 1974).

*Measurement of capillary permeability in single vessels.* Capillary permeability was assessed by measuring the hydraulic conductivity ($L_p$) of the capillary walls. In a few experiments the molecular sieving properties of the capillary walls were assessed by measuring the effective osmotic pressures exerted by perfusates containing the neutral polymer Ficoll 70 at a concentration of 40 mg $ml^{-1}$ in the presence and absence of CHD-modified albumin.

The methods for measuring $L_p$ and the effective osmotic pressures across capillary walls were those described by Michel and his colleagues (Michel *et al.* 1974; Curry, Mason & Michel, 1976; Michel, 1980) and based upon the Landis micro-occlusion technique. The transilluminated mesentery was viewed through a Wild M5 dissecting microscope with a trinocular fitting for simultaneous observation and video recording. Long (1000 $\mu$m) unbranched straight capillaries of diameter 15–30 $\mu$m were cannulated with sharpened glass micropipettes (tip diameter 10–15 $\mu$m). The pipettes were filled with Ringer solutions which were either protein-free or contained native or treated albumin at a concentration of 3–5 mg $ml^{-1}$. Small numbers of washed human red cells were added to all perfusates to act as flow markers. To estimate fluid filtration, a vessel was perfused with the test solution at a high flow rate and then occluded with a glass microrod at a site which was initially 1000 $\mu$m or more from the cannulation site. Subsequent occlusions were made progressively closer to the cannulation site but never approached it more closely than 400 $\mu$m. The period of occlusion lasted approximately 5–20 s and during this time the movements of the red cells within the occluded region of the capillary were recorded on video tape for subsequent analysis. The rate of filtration of fluid across the capillary wall ($J_v$) was estimated from the movements of the red cells measured from the video recording. Knowing the diameter of the capillary and the distance between the marker red cell and occlusion site, the filtration rate per unit area of capillary wall ($J_v/A$) may be calculated. The hydrostatic pressure within the capillary, $P_c$, during occlusion was assumed to equal that applied to the micropipette. By estimating $J_v/A$ at a series of values of $P_c$, $L_p$ (which is the increment of $J_v/A$ per unit of $P_c$) can be calculated. The linearity of the relationship between $J_v/A$ and $P_c$ has been established in previous studies on single frog mesenteric capillaries (see e.g. Michel, 1981) so that in many of the experiments reported in the present paper a series of determinations of $J_v/A$ were made at two values of $P_c$ only.

The effective osmotic pressure exerted across the capillary walls was estimated from the value of $P_c$ when $J_v/A = 0$, which was measured either directly or by extrapolation or interpolation of the relation between $J_v/A$ and $P_c$.

*Protocol of microperfusion experiments.* In each experiment a chosen capillary was perfused first with a Ringer solution, when $L_p$ was determined from several estimates of $J_v/A$ at each of at least two values of $P_c$. The same vessel was then recannulated and perfused with a Ringer solution

containing either native BSA or chemically modified BSA at a concentration of 3–5 mg $ml^{-1}$ and $L_p$ redetermined. In five experiments the effective osmotic pressures across the capillary walls were determined when the initial perfusate contained Ficoll 70 at a concentration of 40 mg $ml^{-1}$ and the second perfusate contained Ficoll 70 at the same concentration together with CHD-modified BSA at a concentration of 2·5 mg $ml^{-1}$.

*Statistics.* The effects of albumin on the hydraulic conductivity of the capillary wall was expressed as the ratio of $L_p/L_{p_0}$, where $L_p$ was the hydraulic conductivity during perfusion with an albumin-containing solution and $L_{p_0}$ was the hydraulic conductivity when the vessel was perfused with protein-free Ringer solution. For a series of experiments on different capillaries with a particular form of albumin, the mean value of $L_p/L_{p_0}$ was calculated as the mean $L_p(\bar{L}_p)$ divided by the mean $L_{p_0}(L_{p_0})$. The deviations from the ratio found in individual experiments were calculated as the fractional deviations of the measured $L_p$ from that calculated from $\bar{L}_p/\bar{L}_{p_0}$ at the appropriate value of $\bar{L}_{p_0}$, i.e.

$$\left(\frac{\bar{L}_{p_0} L_p}{\bar{L}_p L_{p_0}} - 1\right).$$

The standard error of the deviation was calculated in the usual manner.

## RESULTS

### *Estimation of lysine and arginine groups in native and chemically modified BSA*

To test the effectiveness of the succinylation procedure in modifying the lysine residues of the albumin, the presence of free amino groups in native and succinylated albumin was assessed from the optical densities of solutions of the trinitrophenylated proteins (Habeeb, 1966*a*). Fig. 1 shows the optical densities at 335 nm of solutions of native and succinylated albumin after treatment with TNBS, plotted against albumin concentration. The results indicate that succinylation modified virtually all the free amino residues, and since these are on the lysine side chains and the terminal amino acid we may conclude that all the lysine residues were modified.

Messineo's (1966) modification of the Sakaguchi reaction was used to assess the effectiveness of CHD treatment in modifying the arginine residues of albumin. Fig. 2 shows a calibration curve of optical density at 520 nm for a range of arginine concentrations. The optical densities of solutions containing native albumin (1 mg $ml^{-1}$), succinylated albumin (0·98 mg $ml^{-1}$) and CHD-modified albumin (0·94 mg $ml^{-1}$) were respectively 0·35, 0·32 and 0. Thus, treatment with CHD appeared to modify all the arginine groups of the albumin. Succinylation of the albumin had no effect on its arginine groups.

### *Viscometry of solutions of native and chemically modified BSA*

Estimates of the relative viscosity, $\eta_{rel}$, of solutions of CHD-treated BSA did not differ significantly from those of solutions containing the same concentration of native BSA. The concentration intrinsic viscosity, i.e. $(\eta_{rel}-1)$ divided by the protein concentration in g $ml^{-1}$, had a value of 5·6 ml $g^{-1}$ for both native and CHD-treated BSA.

Since $\eta_{rel}$ is related to the fraction of the volume of a solution occupied by the solute, it may be used to calculate the equivalent molecular radius (Einstein, 1906). Taking the molecular weight of BSA as 67,000, the concentration intrinsic viscosity of 5·6 gives a value of 3·9 nm for the molecular radius. This is a little larger than the usual figure of 3·5–3·6 nm.

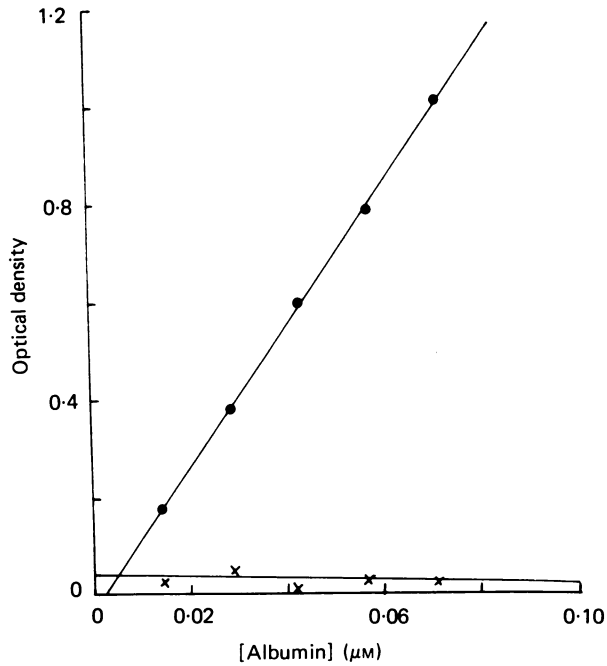


Fig. 1. The relationship between optical density of albumin solutions and albumin concentration (μM). The optical density is that of an albumin solution minus that of protein-free Ringer solution after treatment with TNBS, measured at 335 nm against deionized water. ●, native albumin; ×, succinylated albumin.

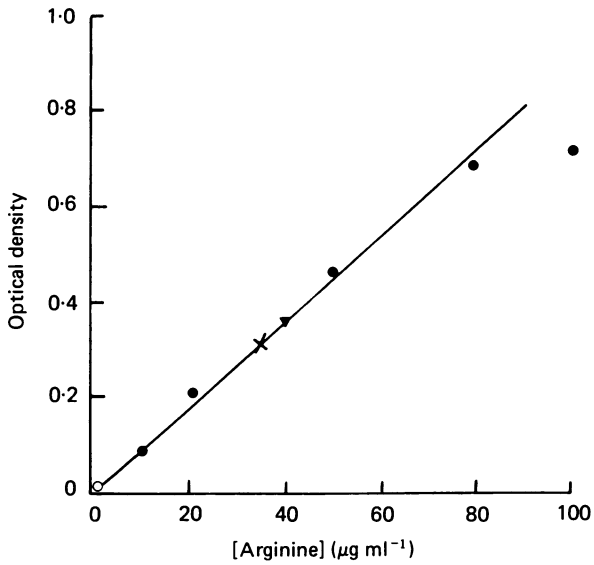


Fig. 2. The relationship is shown (●) between optical density of arginine solutions and arginine concentration. The optical density was measured at 520 nm against deionized water after exposure of the arginine to an alkaline solution of 2,4-dichloronaphthol (Messineo, 1966). The other symbols indicate the optical densities of albumin also at 520 nm after exposure to 2,4-dichloronaphthol: ▼, native albumin (1 mg ml$^{-1}$); ×, succinylated albumin (0·98 mg ml$^{-1}$); ○, CHD-treated albumin (0·94 mg ml$^{-1}$). Superimposition of the latter points on the line allows the number of free arginine residues in the albumin to be calculated.

By contrast, concentration intrinsic viscosity had a value of 19·24 for solutions of succinylated BSA when the protein concentration was less than 10 mg $ml^{-1}$. This yields a value of 5·9 nm for the equivalent molecular radius of BSA, and indicates that succinylation has led either to a major conformational change in the albumin molecule or to an association of BSA molecules which might behave as larger particles in solution (e.g. dimers, tetramers, etc.).

*Effects of native and modified albumin on $L_p$ of single vessels*

In these experiments, $L_p$ was determined in a chosen capillary after it had been perfused first with protein-free Ringer solution for 3–5 min. The vessel was then recannulated and perfused with a Ringer solution containing either native or chemically modified BSA at a concentration of 3–5 mg $ml^{-1}$ for further 3–5 min to allow the protein to have its full effect on permeability before $L_p$ was redetermined. Fig. 3 shows examples of experiments which illustrate the effects of native and treated BSA upon the relation between $J_v/A$ and $P_c$. The slope of this relation is $L_p$, and it is seen that whereas the addition to the perfusates of native, succinylated and NaOH-treated BSA reduced $L_p$, addition of CHD-treated BSA had no effect.

The results of all the experiments in this series are summarized in Table 1. The effect of BSA on permeability is expressed as the ratio of $L_p$ determined in a vessel perfused with a solution containing BSA to its value for the same vessel perfused with a protein-free solution. Thus a value of 1·0 indicates that BSA had no effect on $L_p$ and this result was obtained only when the arginine groups of BSA had been modified with CHD.

In addition to the effects on $L_p$ of succinylated BSA and CHD-treated BSA, Table 2 includes data from experiments where the BSA was modified by both succinylation and CHD treatment. It is worth explaining why these experiments were carried out. In this series, the first experiments to be performed were those with succinylated BSA. When it was found that BSA could still reduce $L_p$ after succinylation, the succinylated BSA was treated with CHD to see whether modification of all the positively charged groups on BSA might affect its permeability-reducing properties. When this was found to do so, we examined the effects on $L_p$ of BSA in which the arginine but not the lysine had been modified. The findings showed that BSA lost its property of reducing $L_p$ after modification of the arginine groups alone by CHD treatment.

*The effects of BSA modified by CHD on the effective osmotic pressures of perfusates containing Ficoll 70*

In previous studies, we (Michel & Phillips, 1979, 1985) found that concentrations of native BSA as low as 1 mg $ml^{-1}$ greatly increased the effective osmotic pressures exerted across frog capillary walls by Ringer perfusates containing Ficoll 70 at a concentration of 40 mg $ml^{-1}$. To see whether this property of BSA was retained after treatment with CHD, we performed five experiments where a single vessel was perfused first with a Ringer solution containing Ficoll 70 (40 mg $ml^{-1}$) and no BSA and then with a Ringer solution containing Ficoll 70 (40 mg $ml^{-1}$) and CHD-treated BSA (2·5 mg $ml^{-1}$). Both $L_p$ and the effective osmotic pressure of the perfusate were measured during each perfusion. The data showing the relationship between filtration rate and capillary pressure are shown for one experiment in Fig. 4. It is seen that

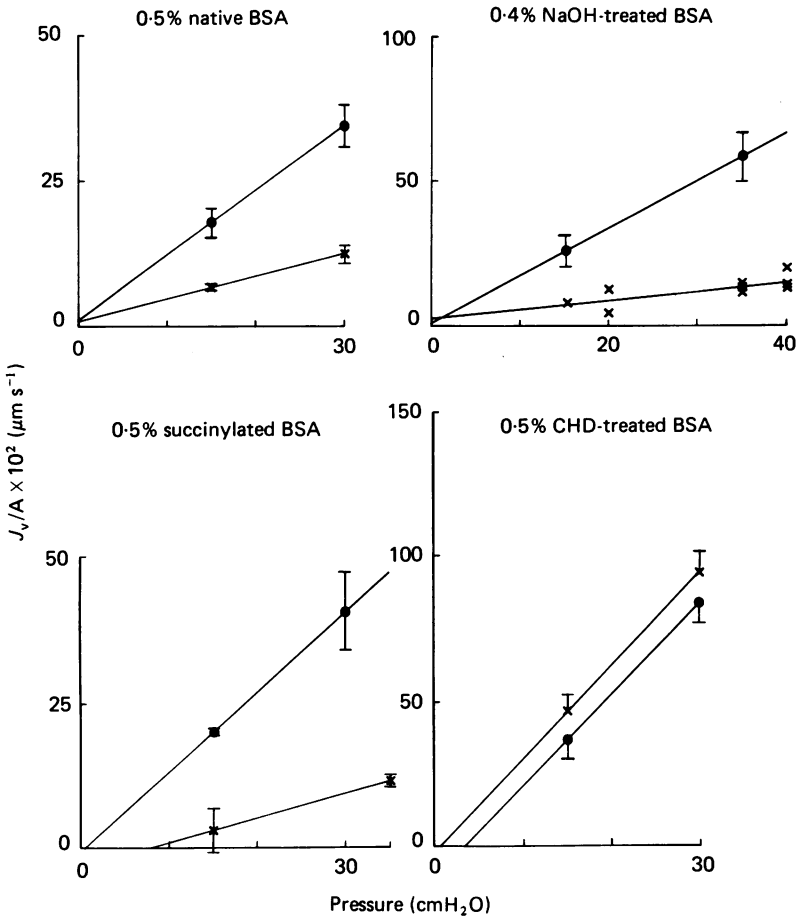


Fig. 3. The relationship between filtration rate per unit area of capillary wall ($J_v/A \times 10^2$ μm s$^{-1}$) and capillary pressure (cm$H_2O$), in single capillaries perfused with Ringer solution (●) and albumin (×) solutions. The bars represent S.E. of mean; each point associated with the bars is the mean of at least four measurements.

TABLE 1. Effects of native and chemically modified bovine serum albumin (BSA) on the hydraulic conductivity of the walls of single capillaries perfused with Ringer solutions

| Perfusate | Native BSA | NaOH-treated BSA | Succinylated BSA | CHD-treated BSA | Succinylated and CHD-treated BSA |
|---|---|---|---|---|---|
| $L_p/L_{p_o}$ | 0·300 | 0·353 | 0·300 | 1·171 | 0·879 |
| No. of experiments | 8 | 13 | 6 | 7 | 4 |
| S.E. of mean | ±0·104 | ±0·170 | ±0·02 | ±0·158 | ±0·158 |

$L_p/L_{p_o}$ is the ratio of hydraulic conductivity in vessel perfused with BSA ($L_p$) to its value for the same vessel perfused with protein-free solution ($L_{p_o}$).

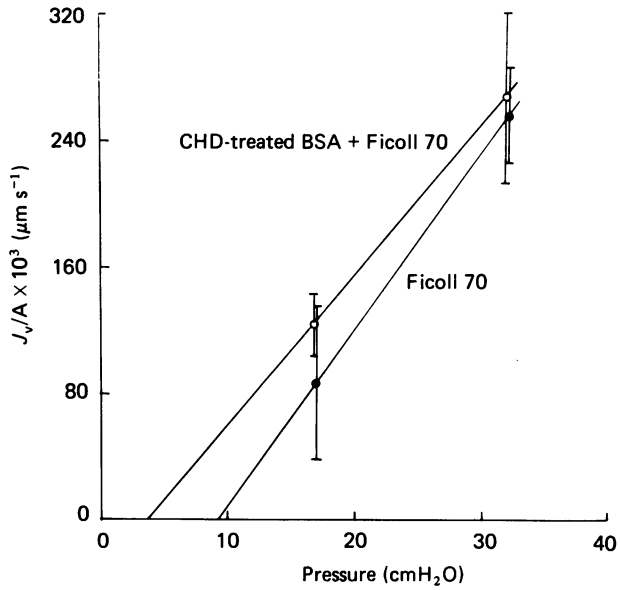


Fig. 4. The relationship between filtration rate per unit area of capillary wall ($J_v/A \times 10^3$ $\mu$m s$^{-1}$) and capillary pressure (cm$H_2O$) in a single capillary perfused first with Ficoll 70 (40 mg ml$^{-1}$) (●) and then with CHD-treated BSA (2·5 mg ml$^{-1}$) and Ficoll 70 (40 mg ml$^{-1}$) (○). The bars represent S.E. of mean; each point is the mean of at least four measurements.

TABLE 2. Effects of native and CHD-treated bovine serum albumin (BSA) on the effective osmotic pressures of perfusates containing Ficoll 70

| | Effective osmotic pressure (cm$H_2O$) | |
|---|---|---|
| Perfusate macromolecules | *In vitro* | *In vivo* (±S.E. of mean) |
| Ficoll 70 (40 mg ml$^{-1}$) | 22·5 | 7·07±1·93 |
| Ficoll 70 (40 mg ml$^{-1}$) and native BSA (1–10 mg ml$^{-1}$) | 23–25·5 | 20·50±2·37 |
| No. of experiments | | 7 |
| *P* | | < 0·001 |
| Ficoll 70 (40 mg ml$^{-1}$) | 22·5 | 3·50±1·50 |
| Ficoll 70 (40 mg ml$^{-1}$) and CHD-treated BSA (2·5 mg ml$^{-1}$) | 23·5 | 4·50±1·20 |
| No. of experiments | | 5 |
| *P* | | Not significantly different |

the presence of CHD-treated BSA in the perfusate changed neither the slope ($L_p$) nor the intercept (the effective osmotic pressure) of the perfusate to a significant extent. This was also found in the other experiments and the results of this series are summarized in Table 2. By contrast, the addition of low concentrations of native BSA to perfusates containing Ficoll 70, reduced $L_p$ and raised the effective osmotic pressure exerted across the capillary walls approximately 3-fold.

## DISCUSSION

The experiments described in this paper show that BSA loses its property of reducing the permeability of capillaries perfused with Ringer solution after its arginine side chains have been chemically modified by exposure to CHD in the presence of 0·2 M-NaOH. Chemical modification of BSA by succinylation of the free amino groups (which does not affect the arginine residues) or by exposure to 0·2 M-NaOH alone, did not affect its permeability-reducing properties. Treatment

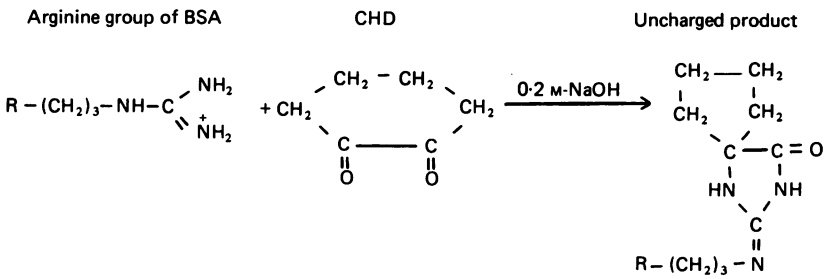


Fig. 5. The modification of the guanidino group of one arginine with 1,2-cyclohexanedione (CHD) in 0·2 M-NaOH, to give an uncharged product. There are many such arginine groups in albumin and R represents the remainder of the BSA molecule.

of proteins with CHD in the presence of 0·2 M-NaOH modifies the arginine side chains by covering the guanidino groups with a non-polar ringed structure (see Fig. 5).

This reaction appeared to be complete in our preparations of CHD-modified albumin since we were unable to detect guanidino groups of the BSA after CHD treatment.

Modification of the arginine side chains could give rise to major changes in the conformation of the BSA molecule, but our failure to detect a significant difference between the concentration intrinsic viscosity of solutions of native BSA and CHD-treated BSA suggest that such conformational changes were relatively small.

In view of these findings it is reasonable to suggest that BSA's property of reducing the permeability of capillaries perfused with Ringer solution depends upon sites in the BSA molecule which involve, or are closely associated with, some or all of the arginine residues. Evidence that specific sites on the albumin molecule rather than its general conformation are responsible for its permeability-reducing properties is provided by our finding that BSA can still reduce permeability after succinylation of its free amino groups. Our own measurements of the viscosity of solutions of succinylated albumin and the work of others (Habeeb, Cassidy & Singer, 1958; Habeeb, 1966*b*, 1967) indicate that succinylation of albumin results in considerable changes in its molecular configuration. Thus the over-all shape of the albumin molecule does not appear to be crucial for its permeability-reducing effects.

Our results are consistent with the hypothesis that BSA reduces capillary permeability by binding to sites on the capillary wall (Danielli, 1940; Landis & Pappenheimer, 1963; Levick & Michel, 1973; Mason *et al.* 1977). Recently, more direct evidence for this hypothesis has been provided by Schneeberger (1982) and

Schneeberger & Hamelin (1984). Using an immunoperoxidase technique to detect native serum albumin, they have demonstrated a slowly exchanging layer of albumin at the luminal surface of rat lung capillaries. Perfusion with protein-free solutions greatly reduces the amount of albumin in the layer and this is associated with increased penetration of ferritin molecules through the endothelium and into the endothelial cell vesicles (cf. Clough & Michel, 1981).

It has been suggested that albumin binds to the cell coat of the endothelium, and that the cell coat or glycocalyx forms a lattice or fibre matrix which covers and possibly fills the channels through the endothelium, acting as the molecular filter (Michel, 1978, 1980; Curry & Michel, 1980; Curry, 1980). Turner *et al.* (1983) found that when cationized ferritin reduced the $L_p$ value of frog mesenteric capillaries perfused with Ringer solutions, the cationized ferritin formed a uniform layer at the luminal surface of the endothelium. From electron micrographs of these vessels it was possible to estimate the concentration of cationized ferritin molecules in the surface coat and this was found to be constant and independent of the luminal concentration of cationized ferritin once the latter exceeded 1 mg $ml^{-1}$. Similarly the effects of cationized ferritin upon $L_p$ were independent of the luminal concentration of ferritin at concentrations greater than 1 mg $ml^{-1}$. Michel & Phillips (1985) demonstrated that, as well as reducing $L_p$, the cationized ferritin increased by 3-fold the effective osmotic pressure exerted across the capillary walls by perfusates containing Ficoll 70, presumably by raising the reflexion coefficient to Ficoll 70. Using the data of Turner *et al.* (1983) for the concentration of cationized ferritin molecules in the surface coat, Michel & Phillips (1985) attempted to predict the change of reflexion coefficient which would result from the presence of cationized ferritin molecules acting as additional obstacles within the fibre matrix. They concluded that the observed increase in perfusate osmotic pressure was too large to be accounted for by the presence of the cationized ferritin molecules alone. It could be accounted for, however, if the cationized ferritin cross-linked the fibrous molecules of the cell coat to convert their arrangement from a random network into a more regularly spaced lattice (see also Michel, 1983).

It is possible that BSA similarly acts as a 'spacer' between adjacent chains of the glycoproteins of the cell coat, converting it into a regular lattice and thus a more efficient filter to fill the wide regions of the intercellular clefts or other transendothelial channels (Michel, 1983). To act in this way it would be likely for there to be at least two binding sites on the BSA molecule separated from one another by the 'spacing distance'. In pursuing this speculation we are limited by the lack of detailed knowledge of the structure of serum albumin. BSA is a single polypeptide chain of 582 amino acids of known sequence (Brown, 1976, 1977; Reed, Putnam & Peters, 1980). On the basis of the distribution of the disulphide bridges and of the amino acid sequence itself, Brown (1976) has suggested that BSA is composed of three homologous domains linked together by peptide chains. When comparing the three domains of BSA, Brown (1976) observed that in several homologous positions the amino acids are identical. Domains 1 and 2 resemble each other more closely than either resembles domain 3.

When Brown's model of serum albumin is examined for the distribution of arginine residues, one striking feature is the sequence arginine–arginine–histidine at positions

143, 144 and 145 in domain 1 and at 334, 335 and 336 which are the equivalent positions in domain 2. Although histidine has a pK of 6·9 in serum albumin (Cantor & Schimmel, 1980) and is weakly anionic at neutral pH, the pH in the micro-environment at the cell surface may be considerably lower than the pH of the bulk phase of extracellular fluid, as a result of the fixed negative charges on the membrane. Thus at the cell surface, histidine could be neutral or even weakly cationic and the grouping arginine–arginine–histidine should be very strongly cationic. A similar concentration of basic residues is seen at the equivalent positions in domain 3 (viz. 532, 533, 534), though here the amino acid sequence is lysine–histidine–lysine. All these three cationic regions are found at the ends of the Cx helices of each of the three domains and are presumably at the surface of the molecule. It seems reasonable therefore to consider them as possible sites which might link BSA to negatively charged groups at the endothelial cell surface. Since succinylation should affect the site on domain 3, it would seem that binding between the capillary wall and the sites on domains 1 and 2 is all that is necessary for BSA to retain its permeability-reducing property. The actual distances between these sites on the BSA molecule are unknown, so that even if they are the regions of linkage between BSA and the fibrous molecules of the endothelial surface coat, it is not possible to say whether they would enable BSA molecules to act as 'spacers' and so order the surface coat into a more regular lattice. Nor is it known whether the distances between these sites are affected by succinylation of the BSA, which alters the conformation of the molecule but not its effects on permeability.

It is nevertheless of interest that the experiments described in this paper indicating the importance of the arginine residues of BSA in relation to its property of reducing capillary permeability, and present theories of the structure of BSA, are both consistent with a hypothesis which arose from the interaction of a different protein, cationized ferritin, with the capillary wall. It is hoped that by casting the hypothesis in more specific terms, both the speculations presented in this paper and the hypothesis itself may be more accessible to experimental test.

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