Br. J. Pharmac. (1973), **47**, 133–137.

Further studies of binding of bromosulphthalein sodium by human serum albumin: effects of albumin concentration and buffer composition

J. M. THOMPSON

Department of Anaesthetics, University of Birmingham, Queen Elizabeth Hospital, Birmingham, B15 2TH

Summary

1. Binding isotherms of equilibrium solution concentration of bromosulphthalein (BSP) determined on the number of moles of BSP bound per mole of human serum albumin (HSA) in 310 ideal milliosmolar pH 7·4, Krebs–Henseleit and Krebs improved mammalian Ringer number 1 buffers at 37° C were determined using continuous diafiltration. The albumin concentration range was from about 10 to 30 g/litre.

2. The results indicate a competition between HSA polymerization and HSA binding BSP, confirming in more physiological conditions, the findings of Crawford, Jones, Thompson & Wells (1972) with pH 7·4 phosphate buffer.

3. The results in Krebs–Henseleit buffer were markedly different from those in Krebs mammalian Ringer buffer and it is suggested that the differences in ionic composition influence the HSA conformation and so affect the competition between HSA polymerization and HSA binding BSP.

Introduction

Crawford & Hooi (1968a) and Crawford, Davies & Davies (1971) have shown that the binding of bromosulphthalein sodium (BSP) by serum and also by serum albumin varies with albumin concentration. Crawford, Jones, Thompson & Wells (1972), with the continuous diafiltration technique (Blatt, Robinson & Bixler, 1968), obtained binding isotherms for three different albumin concentrations at 22° C in pH 7·4 isotonic phosphate buffer and proposed that the binding of BSP by human serum albumin was competitive with the polymerization of the albumin.

This hypothesis has now been tested under more physiological conditions of temperature (37° ±0·05° C) and buffer composition. The buffers used were Krebs improved mammalian Ringer number 1 (Krebs, 1950), which contains organic acids and glucose, and Krebs–Henseleit buffer (Krebs & Henseleit, 1932). The range of albumin concentrations investigated was from about 10 to 30 g/litre.

Methods

Materials

The albumin used was dried purified human serum albumin (100% pure standard for electrophoretic analysis) supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks. Bromosulphthalein sodium was supplied by Koch-Light Laboratories Ltd. and was analytical reagent grade.

Inorganic Krebs-Henseleit buffer is an isotonic buffer containing the major inorganic ions of human serum in the proportions recommended by Krebs & Henseleit (1932): NaCl 115 mM, KCl 4·6 mM, $CaCl_2$ 2·54 mM, KH_2PO_4 1·15 mM, $MgSO_4$ 1·15 mM, $NaHCO_3$ 24·2 mM (the bicarbonate solution was equilibrated with 5% CO_2 for one hour before mixing with the other components). Krebs improved mammalian Ringer buffer number 1 (hereafter referred to as Krebs-Ringer buffer (Krebs, 1950)) contains glucose and, in addition, the sodium salts of three organic acids (the latter replacing a proportion of the chloride ion); its composition was as follows: NaCl 92 mM, KCl 4·6 mM, $CaCl_2$ 2·54 mM, KH_2PO_4 1·15 mM, $MgSO_4$ 1·15 mM, $NaHCO_3$ 24·2 mM, sodium L-lactate: 4·92 mM, sodium fumarate: 5·39 mM, sodium L-glutamate 4·92 mM, D-glucose: 11·5 mM (the $NaHCO_3$ was treated with CO_2 to a pH of 7·4).

Glass-distilled water was used in the preparation of all the buffer solutions. Calcium chloride, sodium bicarbonate sodium L-lactate and sodium fumarate were general purpose reagent grade, the other reagents were analytical grade.

The method of continuous diafiltration and the equation for calculation of the binding isotherm is described by Crawford *et al.* (1972). The apparatus used for these experiments was washed in 30% aqueous ethanol (this prevented fermentation of the Krebs mammalian Ringer buffer) and rinsed with sterile distilled water. All buffers were sterilized by ultrafiltration.

Albumin concentrations of 9·8, 19·6 and 29·3 g/l. were used for Krebs-Henseleit buffer and 9·9, 19·7 and 28·3 g/l. for Krebs-Ringer buffer. The eluate fractions from the ultrafiltration cell were analysed for BSP content by the method of Crawford *et al.* (1972). The temperature of the ultrafiltration cell was 37° ±0·05° C.

Results

The binding isotherms expressed as moles of BSP bound per mole of albumin (molar binding ratio) versus the equilibrium solution concentration of unbound BSP are shown in figures 1A (for Krebs–Henseleit buffer) and 1B (for Krebs–Ringer

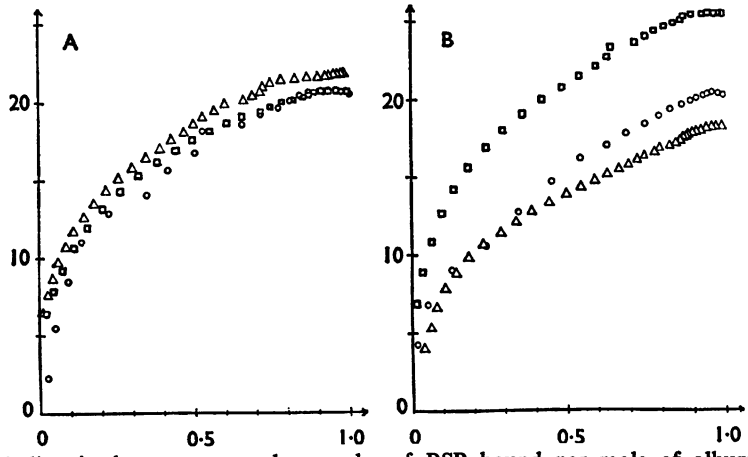

FIG. 1. Binding isotherms expressed as moles of BSP bound per mole of albumin plotted against the equilibrium solution concentration of unbound BSP. Ordinates BSP bound in moles per mole of albumin; abscissae: equilibrium BSP concentration in (mM). A, for Krebs–Henseleit buffer: ○, 9·8 g/l. albumin; □, 19·6 g/l. albumin; △, 29·3 g/l. albumin. B, for Krebs–Ringer buffer: ○, 9·9 g/l. albumin; □, 19·7 g/l. albumin; △, 28·3 g/l. albumin.

buffer). The isotherms of total BSP concentration (free plus bound) versus free BSP concentration are shown in figures 2A (Krebs–Henseleit buffer) and 2B (Krebs–Ringer buffer). The inverse isotherms showing the reciprocal of the number of moles of BSP bound by each mole of albumin plotted against the reciprocal of the free solution concentration of BSP are illustrated in Fig. 3A (for Krebs–Henseleit buffer) and 3B (for Krebs–Ringer buffer).

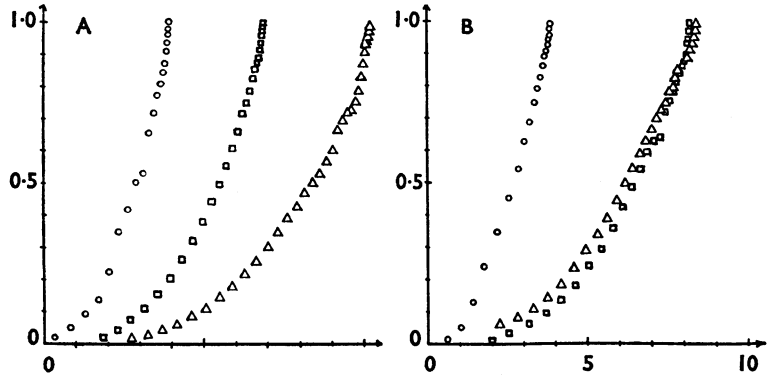

FIG. 2. Isotherms of total (bound+unbound) BSP versus free (unbound) BSP concentrations. Ordinates: Free BSP concentration (mM); abscissae: Total BSP concentration (mM). A, for Krebs–Henseleit buffer: ○, 9·8 g/l. albumin; □, 19·6 g/l. albumin; △, 29·3 g/l. albumin. B, for Krebs–Ringer buffer: ○, 9·9 g/l. albumin; □, 19·7 g/l. albumin; △, 28·3 g/l. albumin.

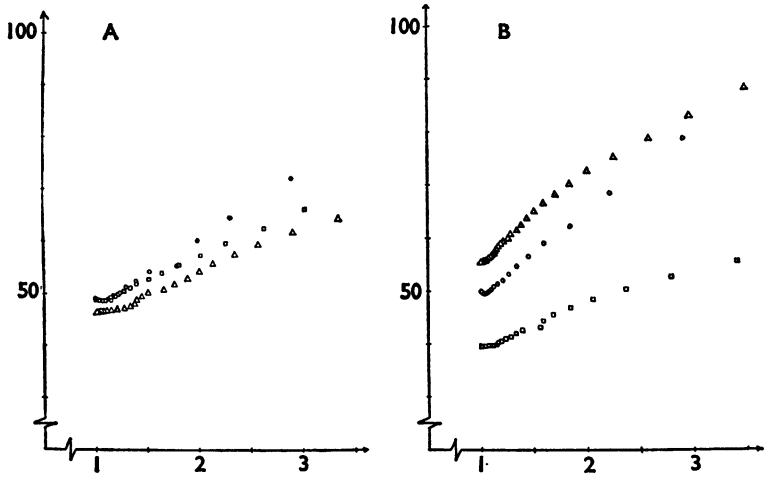

FIG. 3. Inverse isotherms showing the reciprocal of the moles of BSP bound per mole of albumin versus the reciprocal of the free solution concentration of BSP. Ordinates: mmol/mol; abscissae: ml/mol. A, for Krebs–Henseleit buffer: ○, 9·8 g/l. albumin; □, 19·7 g/l. albumin; △, 29·3 g/l. albumin. B, for Krebs–Ringer buffer: ○, 9·9 g/l. albumin; □, 19·7 g/l. albumin; △, 29·3 g/l. albumin.

Discussion

These results agree with those observed in isotonic phosphate buffer at pH 7·4 and room temperature (Crawford *et al.*, 1972). The influence of buffer composition on the binding behaviour of various concentrations of albumin is illustrated in

Figs. 1A and B. In the Krebs–Henseleit buffer system (Fig. 1A), the molar binding capacity of the albumin increases gradually with increasing albumin concentration, whereas in Krebs–Ringer buffer (Fig. 1B) the molar binding capacity of the albumin at first increases with increasing albumin concentration and then decreases sharply. This phenomenon is also strikingly illustrated in Fig. 2B which shows the isotherms of the free drug concentration versus the total (free plus bound) drug concentration. In concentrations of 19·7 and 28·3 g/l. the albumin is acting to hold the isotherm of free drug concentration versus total drug concentration in a particular position on the free drug/total drug co-ordinate system. Thus the number of available binding sites at these two protein concentrations is the same, and a ' negative feedback ' system is operating to hold the affinity of the protein for BSP at a constant value irrespective of the protein concentrations in this particular range. This is in contrast to the situation in the Krebs–Henseleit buffer (Fig. 2A) where no such behaviour occurs.

In the Krebs–Ringer buffer, a small proportion of the chloride ions of the Krebs–Henseleit buffer are replaced by three organic acid ions: L-lactate, fumarate, L-glutamate (Krebs, 1950). That buffer composition affects protein binding of small molecules is known (Klotz & Urquhart, 1949 and Ganguly & Westphal, 1968) but the present results are new in two respects. First, in previous studies the changes in buffer composition have been much larger as also have those used in studies of the influence of various neutral salts on macromolecule conformations (see the recent review of von Hippel & Schleich, 1969). Second, although variations of the molar binding ratio with protein concentration have been recorded for a number of systems (Klotz & Urquhart, 1949 ; Brunkhorst & Hess, 1965 ; Kerkay & Westphal, 1969 ; Crawford & Hooi, 1968a, and b ; Crawford *et al.* 1971, 1972), influence of buffer composition on this phenomenon has not been recorded previously.

The inverse isotherms, showing variation of the reciprocal of the molar binding capacity of the albumin with the reciprocal of the equilibrium concentration of unbound BSP at various albumin concentrations (Figs. 3A and 3B), when examined using the criteria of Nichol, Jackson & Winzor (1967) confirm previous observations (Crawford *et al.*, 1972) that there is competition between albumin polymerization and binding of BSP. Nichol *et al.* (1967) in their theoretical study of binding equilibria showed that the shape of the inverse isotherms, as they approach the axis of the reciprocal of binding capacity (at high equilibrium solution concentrations of the substance being bound) is indicative of the presence or absence of competitive or non-competitive isomerization or polymerization of the protein along with binding of the ligand. Their criterion for competition between protein polymerization and ligand binding is that the isotherms for different protein concentrations should curve towards the reciprocal binding capacity axis and should not be coincident. This was the case in the present experiment with both buffers.

Thus the hypothesis of Crawford *et al.* (1972) that the binding of BSP to human serum albumin in isotonic phosphate buffer (pH 7·4) at room temperature is competitive with albumin polymerization is also valid under the more physiological conditions of temperature and buffer composition of the present work. In addition, it was further observed that the binding process is influenced by small changes in the buffer composition. Thus, protein conformation and hence the availability of binding sites, is influenced by the buffer composition.

The author wishes to thank Professor J. S. Robinson, Drs. J. S. Crawford and P. J. Tomlin of the Birmingham University Anaesthetics Department for their interest and encouragement. Mr. Richard Jones is thanked for his technical assistance and the Birmingham Regional Hospital Board for financial support.

REFERENCES

BLATT, W. F., ROBINSON, S. M. & BIXLER, H. J. (1968). Membrane ultrafiltration: the diafiltration technique and its application to microsolute exchange and binding phenomena. *Anal. Biochem.*, **26**, 151.

BRUNKHORST, W. K. & HESS, E. L. (1965). Interaction of cortisol with serum albumins. *Arch. Biochem.*, **111**, 54.

CRAWFORD, J. S. & HOOI, H. W. Y. (1968a). Binding of bromosulphthalein by serum albumin from pregnant women, neonates and subjects on oral contraceptives. *Br. J. Anaesth.*, **40**, 723.

CRAWFORD, J. S. & HOOI, H. W. Y. (1968b). Binding of salicylic acid and sulphonamide in serum from pregnant patients, cord blood and subjects taking oral contraceptives. *Br. J. Anaesth.*, **40**, 825.

CRAWFORD, J. S., DAVIES, P. & DAVIES, P. (1971). Further studies of the binding of bromosulphthalein by serum albumin. *Br. J. Anaesth.*, **43**, 344.

CRAWFORD, J. S., JONES, R. L., THOMPSON, J. M. & WELLS, W. D. E. (1972). The binding of bromosulphthalein sodium by human serum albumin using a continuous diafiltration technique. *Br. J. Pharmac.*, **44**, 80.

KERKAY, J. & WESTPHAL, U. (1969). Steroid protein interactions XXI. Metal ion inhibition of association between progesterone and α_1-acid glycoprotein. *Arch. Biochem.*, **129**, 480.

KREBS, H. A. (1950). Body size and tissue respiration. *Biochim. biophys. Acta*, **4**, 249.

KREBS, H. A. & HENSELEIT, K. (1932). Untersuchungen über die Harnstoffbildung im Tierköper. *Hoppe-Seyler's Zeitschrift für physiologische Chemie*, **210**, 33.

NICHOL, L. W., JACKSON, W. J. H. & WINZOR, D. J. (1967). A theoretical study of the binding of small molecules to a polymerising protein system. A model for allosteric effects. *Biochemistry*, **6**, 2449.

VON HIPPEL, P. H. & SCHLEICH, T. (1969). The effects of neutral salts on the structure and conformational stability of macromolecules in solution. In: *Structure and Stability of Biological Macromolecules*. Ch. 6. ed. Timasheff, S. M. & Fasman, G. D. New York: Dekker.

(*Received June* 16, 1972)