J. Pharm. Pharmacol. 1989, 41: 222–226
Received July 14, 1988

© 1989 J. Pharm. Pharmacol.

Physicochemical Characterization of Methotrexate–Bovine Serum Albumin Conjugates

GAVIN W. HALBERT AND ALEXANDER T. FLORENCE*

Department of Pharmacy, University of Strathclyde, Glasgow, G1 1XW, UK

Abstract—Physicochemical parameters have been determined for a series of methotrexate-bovine serum albumin conjugates produced using a water-soluble carbodiimide. Drug coupling produces a heterogeneous product consisting of mixtures of albumin and albumin polymers with varying quantities of covalently bound drug. Due to this heterogeneity, the measurement of absolute physicochemical values was not possible but two factors producing change were evident. The water soluble carbodiimide is responsible for major changes by producing polymers that dominate the properties of the conjugates. The changes induced by the covalently attached drug are less dramatic but still appreciable. The results emphasize that both the attached drug and the method of coupling are significant factors in altering the physicochemical properties of proteinaceous drug carriers.

Currently much attention is being directed to the use of drug targeting in cancer chemotherapy as a means of reducing the systemic toxicity and increasing the therapeutic benefit of antineoplastic agents (Goldberg 1983). This treatment modality includes approaches using drug carriers which have ranged from protein microspheres to small molecular weight polymers such as poly-L-lysine. The incorporation of cytotoxic drugs into the larger colloidal carriers (i.e. microspheres) is unlikely to result in any alteration of the physicochemical properties of the carrier involved. However, the attachment of appreciable quantities of cytotoxic drugs to macromolecules is likely to result in a change in the physicochemical properties of the carrier. Since macromolecular carriers are chosen often for their ability to discriminate between normal and tumorous cells, this side effect of drug attachment is obviously undesirable. Kulkarni et al (1981) for example, have demonstrated that the antigen binding capacity of antibodies is reduced after drug coupling. The use of macromolecular conjugates has nevertheless been extensively investigated (Poznansky & Cleland 1980), especially in relation to such factors as the degree of drug coupling and biological activity both in-vitro and in-vivo. Very little attention, however, has been paid to the physicochemical consequences of covalent drug attachment to macromolecular carriers.

To assess relevant changes to the carriers we have studied covalent conjugates of methotrexate (MTX) and bovine serum albumin (BSA) which have aroused interest as drug targeting agents (Garnett & Baldwin 1986; Marriott & Pouton 1986; Halbert et al 1987). BSA provides a carrier with well characterized physicochemical properties which can act as a baseline to gauge the effects of covalent drug coupling. In this study the effect of coupling MTX to BSA using a water-soluble carbodiimide has been measured. Studies were performed on a range of albumin conjugates containing varying amounts of bound methotrexate which have previously been produced and partially characterized (Halbert et al 1987).

Correspondence to: G. W. Halbert, Department of Pharmacy, University of Strathclyde, Glasgow G1 1XW, UK.
* Present address: School of Pharmacy, University of London, Brunswick Square, London WC1N 1AX, UK.

Materials and Methods

Materials

All buffer salts and other reagents were of Analar grade and purchased from BDH; the acrylamide and sodium dodecylsulphate were biochemical grade. Bovine serum albumin (BSA; Fraction V 96–99% albumin) was obtained from Sigma Chemical Co., Dorset, and used without further purification. Methotrexate (MTX) was the kind gift of Lederle Laboratories. Buffer, pH 7·4, consisted of NaCl 75·3 mM, Na_2HPO_4 53·4 mM, NaH_2PO_4 15·3 mM.

Methods

Preparation of conjugates. High Strength Conjugates. To BSA (100 mg) dissolved in $NaHCO_3$ buffer (2 mL 0·05 M pH 7·6) was added MTX (40 mg, 0·09 mM) in 4 mL of buffer and 1-ethyl-3-(3′dimethylaminopropyl)carbodiimide HCl (25 mg, 0·13 mM) in 1 mL of buffer, and the mixture made to a final volume of 12 mL, then incubated at room temperature (20°C) in the dark for 4 h. The mixture was then applied to a BioGel P100 gel exclusion chromatography column (2·2 × 35 cm) and eluted with 0·1 M potassium tetraborate (pH 8·2) containing 1 M urea. Collection of 4 mL fractions afforded the conjugate in fractions 6–10 and unreacted MTX in fractions 16–24. The first conjugate fraction (containing approximately 5 percent of the total conjugate) was discarded, the remainder pooled and dialysed (Visking Tubing 18/32) against distilled water; in the dark at 4°C; the solution filtered (0·22 μm) then lyophilized (Halbert et al 1987). The recovery of conjugate after freeze drying was on average 80 percent of the theoretical yield. Low strength conjugates were produced by the above method but utilizing half the quantities of MTX and carbodiimide. BSA C was produced in a method analogous to the high strength conjugates but in the absence of MTX. The MTX content of the conjugates

was measured by UV absorbance at 376 nm in 0·1 M NaOH with reference to MTX standards.

Discontinuous SDS polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE was performed according to standard techniques (Laemmli 1970) using a BioRad R150 rod gel apparatus (12 × 0·5 cm internal diameter tubes) with a Raven Electronics power supply. Buffers. Upper tank buffer; Tris 0·052 M, glycine 0·053 M, sodium dodecyl sulphate (SDS) 0·1% w/v. Lower tank buffer; Tris:HCl 0·1 M pH 8·1, SDS 0·1% w/v. Running gel buffer; Tris:HCl 1·5 M pH 8·9, SDS 0·4% w/v. Stacking gel buffer; Tris:HCl 0·5 M pH 6·7, SDS 0·4% w/v. Sample buffer; stacking gel buffer 25% v/v, mercaptoethanol 25% v/v, bromophenol blue (1% w/v) 40% v/v, glycerol 10% v/v, SDS 10% w/v.

Sample preparation. The sample was mixed with the sample buffer in the ratio 5:1 (v:v) to give a final protein concentration of 1 mg mL^{-1}. Samples were then heated in a boiling water bath for 3 min and allowed to cool before application to the gels. Gels. Stock acrylamide solution; acrylamide 28·5% w/v, bisacrylamide 1·5% w/v. Stacking gel; stock acrylamide solution 16·5% v/v, stacking gel buffer 25% v/v, TEMED 20 μL, ammonium persulphate (10% w/v) 300 μL. Running gel; stock acrylamide solution, 18·5% v/v, running gel buffer 25% v/v, TEMED 20 μL, ammonium persulphate (10% w/v) 300 μL (T 5·5%, C5. 0%).

Running conditions. Approximately 250 mL of buffer was placed in each tank, and the sample (20 μL) applied using a micropipette; the gels were run at around 1 mA per tube until the sample had fully entered the stacking gel and then at 2 mA per tube until the dye front was approximately 1 cm from the bottom of the tube. The gels were removed from the tube, washed with distilled water and stained. Gels were calibrated using standard marker proteins.

Staining. Coomassie Brilliant Blue R250 0·2% w/v in MeOH–H_2O–CH_3COOH (50:50:7) was used for 2h. Destaining was with MeOH–H_2O–CH_3COOH (50:880:70).

Gel Scanning. Gels were scanned using a Gilford Instruments Spectrophotometer fitted with a rod gel scanning apparatus, the instrument was set to scan the gels at 1 cm min^{-1} at 570 nm. A chart recorder provided a trace of absorbance against distance along the gel.

Determinaton of apparent specific volume (v)

The apparent specific volume was calculated from density measurements of the conjugate solutions in pH 7·4 buffer at 37°C ± 0·01°C using a 0·5 mL Lipkin pycnometer. Four measurements of the solution under test were taken and the average value used to calculate v (Hunter 1967).

Determination of the diffusion coefficient (D)

Diffusion coefficients were measured using a photon correlation spectrometer (Malvern Instruments, Type 7027) with 60 channels, in conjunction with a He/Cd laser (Linconix) operating at 441·6 nm with a power output of approximately 10 mW. The conjugates were measured in pH 7·4 buffer at 37°C ± 0·1°C and at an angle of 90° to the incident beam. All samples were filtered (0·22 μm) before measurement; occasionally samples required filtration through a 0·1 μm filter to reduced background noise levels.

Analysis of PCS data

For monodisperse systems of small particles the experimental data obtained is the second order autocorrelation function $g^{(2)}(t) = 1 + e^{-2DK^2}$ t, where $k = (4\pi n/\lambda)\sin(\theta/2)$, n is the refractive index of the solvent, θ the measurement angle, λ the laser wavelength and t is the delay time. Thus $(g^{(2)}t - 1)$ is an exponential curve and $\ln(g^{(2)}t - 1)$ is a straight line with a slope of $(-2DK^2)$ from which D is readily obtained. In polydisperse systems a family of exponentials will be obtained and plots of $\ln(g^{(2)} - 1)$ vs t fitted to quadratic functions which provide a coefficient of t which is equal to the z average diffusion coefficient (Koppel 1972). The z average diffusion coefficient is defined as $D = \sum n_i M_i D_i / \sum n_i N_i$, n_i being the number of particles of molecular mass M_i with diffusion coefficient D_i.

Calculation of the Frictional Ratio (f/f_o)

The frictional ratio is defined as f/f_o where $f = kT/D$ k is the Boltzman constant, T the absolute temperature and $f_o = \eta(162\pi^2 Mv/N)^{1/3}$, η is the viscosity of the medium, M the molecular weight, v the partial specific volume and N Avogadro's number (Edsall 1953). For the conjugates the molecular weight was substituted with a molecular weight average $(\sum c_i M_i / \sum c_i)$ derived from the molecular weight of BSA plus the average number of molecules of MTX attached (Halbert et al 1987) and allowing for the polydispersity demonstrated by the SDS PAGE experiments.

Results and Discussion

SDS PAGE was carried out on all the conjugates prepared (30 in total; Halbert et al 1987) as a standard technique and the results presented are representative of those experiments. The SDS PAGE of native BSA (Fig. 1A) produced a profile exhibiting one main band due to the albumin monomer molecule with trace contaminating bands apparently due to albumin polymers and globulins. The profile, however, demonstrated that the albumin used contained 94% of albumin monomer. The SDS PAGE profile of a low strength conjugate (Fig. 1B) exhibits subtle differences from native BSA with a slightly broader main band which is shifted towards a higher molecular weight and extra bands related to the presence of BSA polymers. High strength conjugates (Fig. 1C) continue this trend with again a slightly broader main band shifted to even higher molecular weights due to the increased MTX attachment and the presence of similar quantities of albumin polymers. BSA C (result not shown) produced an electrophoresis profile similar to that of native BSA but with the same quantities of polymeric protein as found in the conjugates. No evidence of an increase in molecular weight or band spreading could be detected in this material.

The shifting and expansion of the main band can be ascribed to changes in the molecular weight induced by the covalent coupling of varying quantities of MTX (molecular weight 454) to each protein molecule. This would appear to

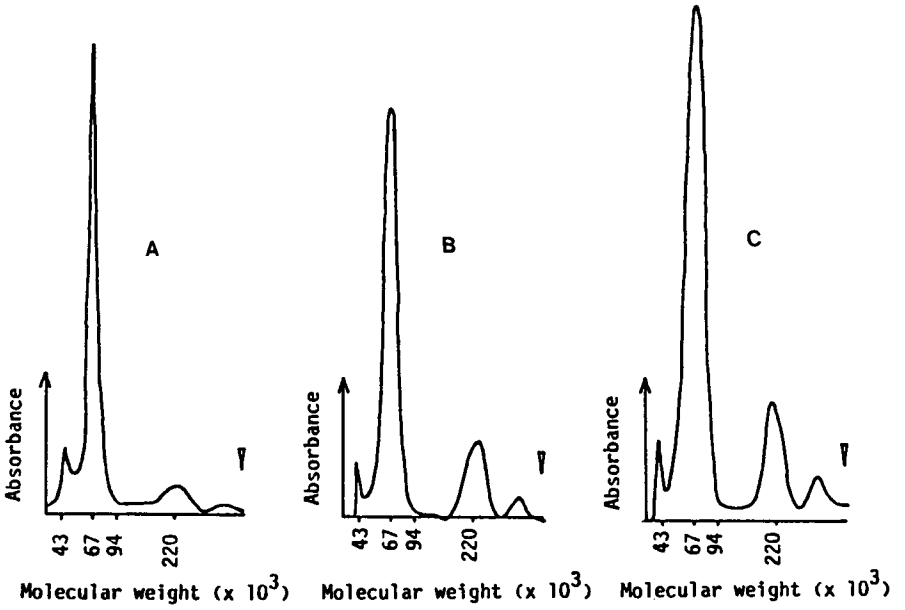

FIG. 1. SDS PAGE Profiles. A. BSA, B. Low Strength Conjugate, C. High Strength Conjugate. ▽ Origin.

be more prevalent in the high strength conjugates indicating a greater degree of variation in drug attachment. These effects have also been noted when MTX was covalently bound to fibrinogen using a water soluble carbodiimide (Dyr et al 1983). The increased presence of albumin polymers is related to the well known ability of carbodiimide to cross link proteins in solution (Timkovich 1977), and this effect was also present in the results of Dyr et al (1983). The quantities of polymers produced in both sets of conjugates were similar, with the monomer reduced to approximately 80 percent of the total protein content, a fact which may reflect that the quantity of protein in each reaction was the same. These results illustrate that although the coupling method used will attach MTX to BSA, the conditions produce a heterogeneous material in relation to the quantity of MTX attached and the presence of polymers.

Apparent specific volumes (v) are given in Fig. 2. A value of $0{\cdot}742 \pm 0{\cdot}03$ mL g^{-1} was obtained for BSA and this agrees with published data (Bull & Breese 1973). There is a significant fall in v on the attachment of MTX, all the conjugates having similar values of around 0·5 mL g^{-1}. This value is below that normally expected of protein molecules in solution and would appear to be a consequence of the attachment of MTX as BSA C shows no change in v.

The measured diffusion coefficients (D) of the conjugates and native BSA are presented in Fig. 3 along with the results for BSA C. The value of D obtained for BSA of $7{\cdot}8 \pm 0{\cdot}2 \times 10^{-7}$ cm^2 s^{-1} is in agreement with literature values (Dubin et al 1967); the results obtained for the conjugates and BSA C are generally lower than for the native protein.

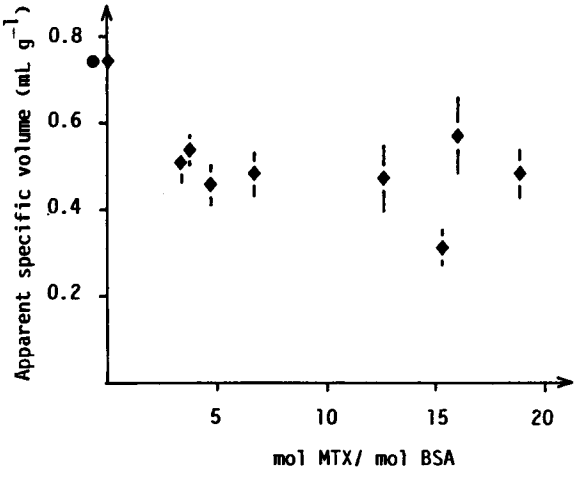

FIG. 2. Plot of apparent specific volume against quantity of MTX attached. ● BSA, ◆ BSA C, ◆ BSA-MTX conjugates, (mean ± s.d., n = 4).

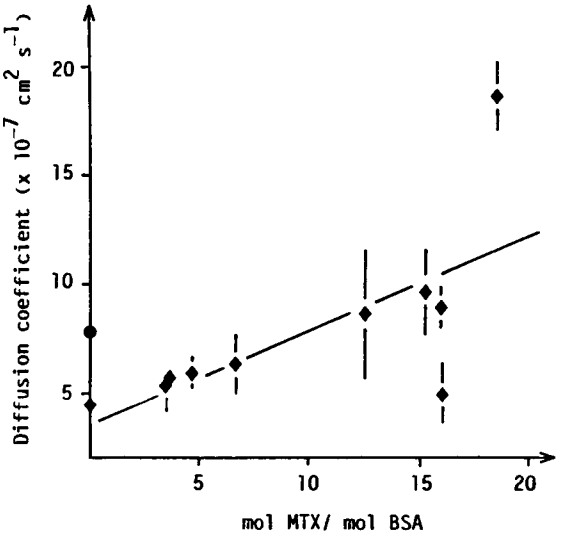

FIG. 3. Plot of diffusion coefficient against quantity of MTX attached. ● BSA, ◆ BSA C, ◆ BSA-MTX conjugates, (mean ± s.d., n = 10).

The measured value for BSA C was for example $4{\cdot}5 \times 10^{-7}$ $cm^2\ s^{-1}$. The results for the conjugates appear to increase from this value in a linear manner (correlation coefficient significant at the 2·5 percent level) as the quantity of MTX attached increases. The decrease in diffusion coefficient found for BSA C can be related to the presence of polymers of BSA which have been reported to have D values less than that of the native protein and dependent on the number of monomer molecules joined together. Bovine serum albumin dimer for example has a measured D of $4{\cdot}5 \times 10^{-7}$ $cm^2\ s^{-1}$ (Harvey et al 1979). The initial fall in D values of the conjugates may also be attributed to the presence of polymers since BSA C also exhibits this change. The subsequent rise of D due to MTX attachment could be related to either changes in size or asymmetry of the molecules. Similar effects have been noted during the physical binding of salicylates by BSA (Harvey et al 1979), when an increase in D was linked to increasing physical binding by the salicylate. However, with the information available in this study it is impossible to construct a precise hypothesis relating to the alterations found. Two factors are evident: the initial alteration in D appears to be due to the effects of cross linking by carbodiimide and subsequent changes related to the action of the bound MTX in perturbing the protein structure in solution.

Using the data previously presented it is possible to calculate for the conjugates a frictional ratio (Fig. 4) that can be used to assess the changes occurring during the coupling reaction. The calculated value for BSA (1.22) corresponds closely with the values reported in the literature (Edsall 1953) but the value for BSA C is very much higher at 2·25. There is a definite trend in the conjugate results with the low strength conjugates having high values at around 2·0 which decrease as the amount of MTX attached increases. The high strength conjugates have calculated values close to the figure obtained for BSA.

The interaction of the BSA with carbodiimide causes a rapid increase in f/f_o as noted with the BSA C result. Since the carbodiimide does not markedly alter the molecular weight of the BSA monomer, this result is probably related to the formation of BSA polymers in the system. The subsequent covalent binding of MTX reduces this high initial result in a linear manner to around 1·2. The large values of f/f_o obtained for the low strength conjugates must also be linked to the presence of polymers as simply increasing the BSA molecular weight (in the calculation of f/f_o) to account for the attachment of three or four extra MTX molecules gives an f/f_o ratio of around 1·4, well below the measured value.

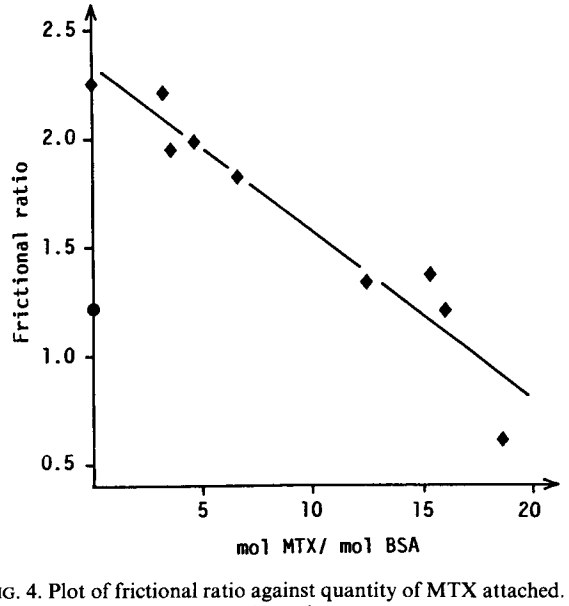

FIG. 4. Plot of frictional ratio against quantity of MTX attached. ● BSA, ◆ BSA C, ◆ BSA-MTX conjugates.

Changes in f/f_o can be related to alterations in the shape and hydration of the molecule (Edsall 1953). In this study no attempt was made to measure the state of hydration of the proteins but a standard value of 0·2 g H_2O (g protein)$^{-1}$ may be assumed (Tanford & Buzzell 1956). Using this value the results obtained for the conjugates provide axial ratios that range from 16:1 (low MTX attachment) to 3:1 (high MTX attachment) for a prolate ellipsoid. If hydration remains constant during these changes, this would appear to represent a rounding of the molecular shape with increasing MTX attachment. The calculated axial ratios for the low strength conjugates are improbable and complex changes must be taking place in the system. The shape and hydration of the BSA monomer may change and these alterations will be compounded by the introduction of cross linking into the system.

The frictional ratio is based on mathematical calculations that rely upon the behaviour of monodisperse ellipsoids of revolution under ideal conditions. The application of f/f_o to real situations is therefore not perfect and this is amplified by the inherent polydispersity of the conjugates, which will upset the measurements and the subsequent calculation of f/f_o. It is therefore impossible to attribute the changes to specific modifications of protein structure. However, trends are evident in the results.

The data demonstrate that the coupling of drugs to proteins using an intermediate coupling agent will induce several changes in the properties of the protein. In this case the changes appear to be attributable to two major factors. The first is the use of the carbodiimide coupling agent which has the ability to cross link the protein producing polymers. The actual attachment of drug to the protein also induces a degree of change that is not as severe as that produced by the coupling agent alone but in this study is appreciable. The results suggests that the use of specialized coupling agents (Trouet et al 1982) will only reduce any effect on protein structure and that drug attachment itself will have a significant contribution to make. Assessment of the suitability of the drug and method of coupling for each carrier should therefore be undertaken to provide an insight into the potential for the coupling procedure to disrupt the function of the carrier molecule. Such studies will allow the carrier to be loaded with the maximal quantity of drug without inflicting detrimental effects upon any targeting specificity it may possess.

Acknowledgements

We acknowledge the support of Professor K. C. Calman formerly of the Department of Clinical Oncology, University of Glasgow for his invaluable assistance during this work, and the SERC studentship to GWH.

References

Bull, H. B., Breese, K. (1973) Temperature Dependence of Partial Volumes of Proteins, Biopolymers 12: 2351–2358

Dubin, S. B., Lunacek, J. H., Benedek, B. (1967) Observation of spectrum of light scattered by solutions of biological macromolecules. Proc. Nat. Acad. Sci. USA. 57: 1164–1171

Dyr, J. E., Slavik, K., Vodrazka, Z. (1983) Chemical binding of Folic Acid and Methotrexate to Bovine Fibrinogen. Thromb. Res. 31: 737–746

Edsall, J. T. (1953) The size, shape and hydration of protein molecules, H. Neurath and K. Bailey (eds.), The Proteins, Volume 1, Academic Press, New York, pp. 549–726

Garnett, M. C., Baldwin, R. W. (1986) An improved synthesis of a Methotrexate-Albumin-791T/36 Monoclonal Antibody Conjugate cytotoxic to Human Osteogenic Sarcoma Cell Lines. Cancer Res. 46: 2407–2412

Goldberg, E. P. (1983) Targeted Drugs in L.G. Donaruma and O. Vogl (eds.) Polymers in Biology and Medicine Volume, 2. John Wiley and Sons, New York

Halbert, G. W., Florence, A. T., Stuart, J. F. B. (1987) Characterization of in vitro drug release and biological activity of methotrexate-bovine serum albumin conjugates. J. Pharm. Pharmacol. 39: 871–876

Harvey, J. D., Geddes, R., Wills, P. R. (1979) Conformational studies of BSA using Laser Light Scattering, Biopolymers 18: 2249–2260

Hunter, M. J. (1967) Partial Specific Volume of Bovine Plasma Albumin in presence of Potassium Chloride. J. Phys. Chem. 71: 3717–3721

Koppel, D. E. (1972) Analysis of Macromolecular Polydispersity in Intensity Correlation Spectroscopy- Method of Cumulants. J. Chem. Phys. 57: 4814–4820

Kulkarni, P. N., Blair, A. H., Ghose, T. I. (1981) Covalent binding of Methotrexate to Immunoglobulins and the effect of antibody-linked drug on tumour growth in vivo. Cancer Res. 41: 2700–2706

Laemmli, U. K. (1970) Cleavage of Structural Proteins during assembly of head of Bacteriophage-T4. Nature 227: 680–685

Marriott, R. J., Pouton, C. W. (1986) Hydrolytic degradation of methotrexate-albumin conjugates in the presence and absence of trypsin. J. Pharm. Pharmacol. 38 (Suppl): 62P

Poznansky, M. J., Cleland, L. G. (1980) Biological Macromelecules as Carriers of Drugs and Enzymes, R.L. Juliano (ed.), Drug Delivery Systems: Characteristics and Biomedical Applications. Oxford University Press, Oxford, pp 253–315

Tanford, C., Buzzell, J. G. (1956) The viscosity of aqueous solutions of Bovine Serum Albumin between pH 4·3 and 10·5. J. Phys. Chem. 60: 225–331

Timkovich, R. (1977) Polymerisation side reactions during protein modifications with carbodiimide. Biochem. Biophys. Acta. 74: 1463–1468

Trouet, A., Masquelier, M., Baurain, R., Campaneere, D. D. (1982) A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases as required for a lysosomotropic drug carrier conjugate; in vitro and in vivo studies. Proc. Nat. Acad. Sci. USA. 79: 626–629