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# A study of the interaction of tetracycline with human serum lipoproteins and albumin

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The interaction of 7-[$^3$H]tetracycline at therapeutic concentrations with human serum proteins has been studied by dialysis and by molecular exclusion chromatography. In serum, 53% of tetracycline is in the form of complexes with protein: 54% with albumin; 13% with low density lipoprotein; 19% with high density lipoprotein; 6% with very high density lipoprotein and 8% with other serum proteins. Albumin possesses two binding sites for tetracycline, one a high affinity, low capacity site and the other a low affinity, high capacity site. At therapeutic concentrations three quarters of the tetracycline bound to albumin is associated with the low affinity site. Tetracycline appears to dissolve in the lipophilic portion of the lipoprotein molecule, rather than to be associated with specific binding sites.

It is well documented that the tetracycline group of antibiotics binds to serum proteins with a consequent reduction in its biological potency (Abraham, 1951; Kivman, 1959; Sirota & Saltzman, 1950). Whilst some workers consider that the primary binding of tetracycline is to albumin (Pagnini, Mollo & others, 1966; Sirota & Saltzman 1950), others have reported that tetracyclines form complexes with all protein fractions except $\gamma$-globulin (Kivman, Porfir'eva & Kosolapova, 1966). Wittenau & Yeary (1963), on the other hand, have reported that tetracycline binds with equal facility to both $\gamma$-globulin and albumin. Previous investigations have usually been of a qualitative nature and often carried out at concentrations of tetracycline in excess of those found under therapeutic conditions.

The present investigation is a quantitative study of the interaction with human serum proteins of 7-[$^3$H]tetracycline at therapeutic concentrations.

## METHODS

The binding of tetracycline by whole serum was studied by dialysing 1 ml aliquots of fresh human serum (taken from normal subjects, fasted overnight) in sacs of Visking dialysis tubing, inflated diameter 6·4 mm (Scientific Instrument Centre), against 500 volumes of Krebs phosphate buffered saline, pH 7·4 (Krebs, 1933), containing 7-[$^3$H]tetracycline (Radiochemical Centre, Amersham) diluted with unlabelled tetracycline to give a concentration of 1 $\mu$g ml$^{-1}$, at 10° for 24 h. Aliquots of serum and of a similarly treated saline blank were taken for liquid scintillation counting. The amount of tetracycline bound was determined by a comparison of the concentration of tetracycline in free solution with its concentration in serum. A correction was made for any dilution of the serum proteins during the dialysis. In studies to investigate some of the characteristics of the interaction between tetracycline and lipoproteins and the binding to crystalline human albumin (Miles Laboratories), solutions of the

proteins (10 mg $ml^{-1}$) were dialysed against tetracycline in concentrations ranging from $10^{-8}$ to $10^{-3}$ M.

The interaction between tetracycline and the various serum proteins was studied by applying 5 ml pooled fresh serum to a 3·5 × 100 cm column of Sephadex G-200 (Pharmacia Fine Chemicals) previously equilibrated with Krebs phosphate buffered saline, pH 7·4, containing 7-[$^3$H]tetracycline, 1 $\mu$g $ml^{-1}$. The proteins were eluted with the same buffer at a flow rate of 30 ml $h^{-1}$ and at 10°, collecting 5 ml fractions of the eluate. Samples of the eluate were taken for liquid scintillation counting and for the determination of protein. Bound tetracycline appeared as peaks of increased radioactivity over the background activity.

Lipoproteins were prepared from pooled fresh human serum by preparative flotation as described by Hatch & Lees (1968) and washed once. The fractions prepared were very low density lipoprotein (VLDL), density $<$1·006 g $ml^{-1}$, low density lipoprotein (LDL), density 1·006–1·062 g $ml^{-1}$ and high density lipoprotein (HDL), density 1·062–1·20 g $ml^{-1}$. A very high density lipoprotein fraction (VHDL), density 1·20–1·25 g $ml^{-1}$, was prepared as described by Alaupovic, Sanbar & others (1966). The interaction between tetracycline and lipoprotein was studied by applying the individual lipoprotein fractions, obtained from 6 ml serum, to a 2·5 × 90 cm column of Sepharose 2B (Pharmacia Fine Chemicals), previously equilibrated with Krebs phosphate buffered saline, pH 7·4, containing 7-[$^3$H]tetracycline 1 $\mu$g $ml^{-1}$, and eluted with the same buffer at a flow rate of 20 ml $h^{-1}$ and at 10°.

When the radioactivity of samples was determined by liquid scintillation counting, unlabelled tetracycline, 10 $\mu$g $ml^{-1}$, was added to the scintillant to prevent errors in counting due to the adsorption of the labelled tetracycline to the walls of the counting vial (Allison, Monro & Offerman, 1972). Protein was determined by the method of Lowry, Rosebrough & others (1951). Crystalline human serum albumin was used as the standard for the determination of protein concentrations in whole serum and in eluates after the chromatography of whole serum. The appropriate lipoprotein, determined gravimetrically, was used as the standard for the determination of lipoprotein concentrations.

## RESULTS

### *Interaction between tetracycline and serum proteins*

Tetracycline was bound to serum to the extent of 1·128 $\pm$ 0·023 $\mu$g $ml^{-1}$ (n = 22) when in equilibrium with a free concentration of tetracycline of 1 $\mu$g $ml^{-1}$. There was no significant difference between the binding to sera from males 1·108 $\pm$ 0·028 $\mu$g $ml^{-1}$ (n = 12) and from females 1·153 $\pm$ 0·036 $\mu$g $ml^{-1}$ (n = 10, $P > 0{\cdot}05$). Children exhibited a slightly higher binding 1·415 $\pm$ 0·024 $\mu$g $ml^{-1}$ (n = 19, $P < 0{\cdot}05$).

Krebs phosphate buffered saline, pH 7·4, was chosen as a convenient buffer with an ionic composition similar to that of serum. The replacement of the natural bicarbonate/$CO_2$ buffer by phosphate buffer had no effect upon binding of tetracycline to serum, binding in the presence of 25 mM bicarbonate, pH 7·4, 1·138 $\pm$ 0·025 $\mu$g $ml^{-1}$ (n = 6, $P > 0{\cdot}05$).

The binding of tetracycline to the various serum proteins when fractionated by molecular exclusion chromatography upon Sephadex G-200 is shown in Fig. 1. A total of 33·06 $\mu$g tetracycline was bound by 5 ml serum; 20·8% was associated with the first protein peak eluted, containing lipoproteins (VLDL & LDL) and $\gamma_1$- and $\alpha_2$-

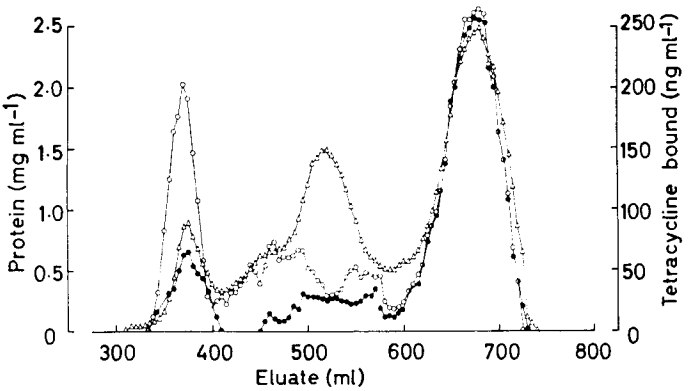


FIG. 1. Binding of tetracycline (○) to human serum proteins (△). Human serum 5 ml, applied to a 3·5 × 100 cm column of Sephadex G-200 and eluted with Krebs phosphate buffered saline, pH 7·4, containing 7-[$^3$H]tetracycline 1 $\mu$g ml$^{-1}$, at a flow rate of 30 ml h$^{-1}$, and at 10°. The binding of tetracycline to 5 ml human serum with lipoproteins, density <1·20 g ml$^{-1}$, removed is also shown (●).

macroglobulins (Fireman, Vannier & Goodman, 1964; Glomset, Janssen & others, 1966; Killander, Bengtsson & Philipson, 1964). A further 25·2% was associated with the middle protein peak and the greater fraction of that bound was eluted early in the peak, together with lipoprotein (HDL) (Glomset & others, 1966), ceruloplasmin (Fireman & others, 1964) and $\gamma$-globulin (Flodin & Killander, 1962). The final protein peak eluted contained albumin, transferrin and $\alpha$-globulin (Flodin & Killander, 1962) and 54·0% of the bound tetracycline.

The extent of the binding of tetracycline to crystalline human serum albumin applied to a column of Sephadex G-200 and eluted in a similar manner, closely resembled the binding to the final protein peak of whole serum. It is thus reasonable to conclude that the binding in this final protein peak occurs almost exclusively to albumin.

Removal of the lipoproteins, density <1·20 g ml$^{-1}$, from serum before fractionation on Sephadex G-200 led to a 31·5% reduction in the binding of tetracycline, mostly associated with the early protein peaks (Fig. 1).

The application of the individual lipoprotein fractions to a column of Sepharose 2B failed to reveal any interaction between tetracycline and VLDL (Fig. 2). Tetracycline was, however, found associated with the other lipoprotein fractions, 0·88 $\mu$g to 3·56 mg LDL prepared from 1 ml serum, 1·28 $\mu$g to 2·60 mg HDL and 0·38 $\mu$g to 0·27 mg VHDL. Thus the binding to albumin and to lipoproteins accounted for 92% of the tetracycline bound in whole serum.

*Characteristics of the interaction*

The interaction between tetracycline and albumin and the two major classes of lipoprotein, LDL and HDL, is shown in Figs 3 and 4 respectively, plotted according to the method of Scatchard (1949), where r = number of moles of tetracycline bound per mole of protein, and $D_f$ = free concentration of tetracycline. Curvature of the plot indicates more than one set of binding sites and obeys the general equation,

$$r = \sum_{i=1}^{i} \frac{n_1 K_1 D_f}{1 + K_1 D_f}$$

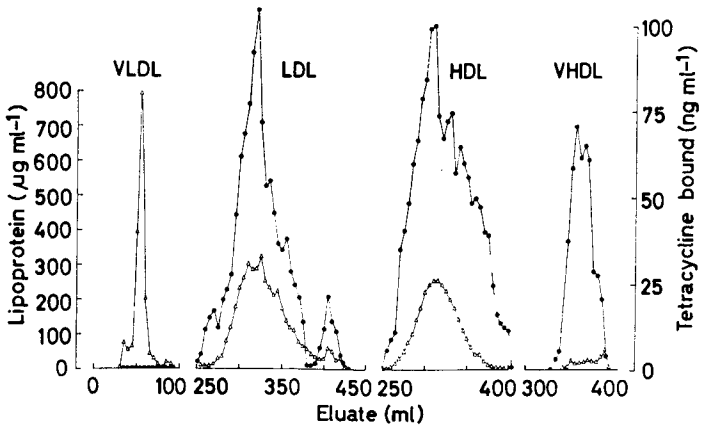


FIG. 2. Association of tetracycline (●) with human serum lipoproteins (△). Lipoproteins prepared from 6 ml serum were applied individually to a 2·5 × 90 cm column of Sepharose 2B and eluted with Krebs phosphate buffered saline, pH 7·4, containing 7-[$^3$H]tetracycline 1 μg ml$^{-1}$, at a flow rate of 20 ml h$^{-1}$ and at 10°.

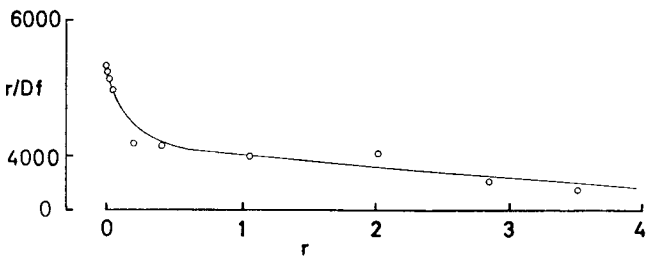


FIG. 3. Binding of tetracycline to human serum albumin, plotted according to the method of Scatchard (1949). Points determined experimentally (○). The continuous line represents a computer plot of the data as described in the text. r = number of moles of tetracycline per mole of albumin; $D_f$ = free concentration of tetracycline in Krebs phosphate buffered saline, pH 7·4.

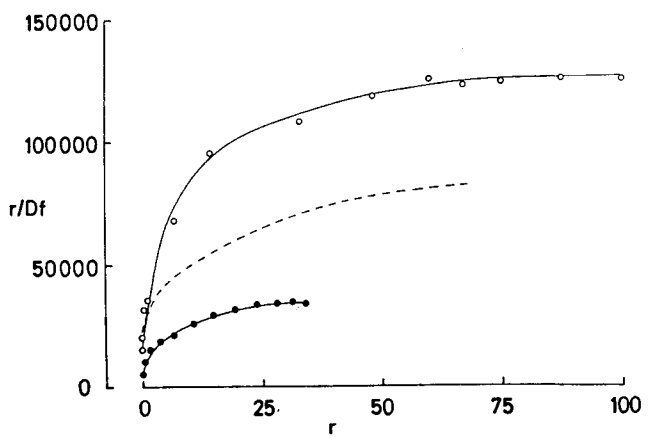


FIG. 4. Interaction between tetracycline and human serum low density lipoprotein (○) and high density lipoprotein (●), plotted according to the method of Scatchard (1949). r = number of moles of tetracycline per mole of lipoprotein; $D_f$ = free concentration of tetracycline in Krebs phosphate buffered saline, pH 7·4. The dotted line represents the solubility of tetracycline in chloroform; r = number of moles of tetracycline per $10^5$ litres chloroform.

(Meyer & Guttman, 1968), where $n_1$ is the total number of sites of type i with an intrinsic association constant of $K_1$. The equation was converted to a rational function and fitted to the data in Fig. 3, for the binding of tetracycline to albumin, using a rational approximation to a discrete function in the least squares sense on an IBM 370/155 computer. Assuming two mutually independent sites this approach yields the constants $n_1 = 0{\cdot}032$, $K_1 = 4{\cdot}38 \times 10^4$, $n_2 = 39{\cdot}50$, $K_2 = 1{\cdot}01 \times 10^2$. The molecular weight of albumin is taken as 69 000 (Scatchard, Batchelder & Brown, 1949).

The interaction between tetracycline and the lipoproteins showed none of the characteristics of the binding of a small molecule to a fixed number of sites on the protein. There was instead, at high tetracycline concentrations, a linear relation between the free concentration of tetracycline and the number of moles of tetracycline associated with lipoprotein. Below concentrations of around 300 $\mu$g tetracycline $ml^{-1}$, for both LDL and HDL, the amount of tetracycline in association with the lipoprotein became relatively less as the concentration of tetracycline decreased. The distribution of tetracycline between chloroform and Krebs phosphate buffered saline, pH 7·4, is also shown in Fig. 4, plotted for convenience as moles of tetracycline dissolved in $10^5$ litres of chloroform. The form of the relation between $r/D_f$ and r was similar to that for the lipoproteins.

## DISCUSSION

The use of radioactively labelled tetracycline in the present investigation has allowed the interaction of tetracycline to be studied quantitatively at therapeutic concentrations, around 1 $\mu$g $ml^{-1}$ (Finland, Purcell & others, 1954).

Tetracycline binds to two sites on human serum albumin, a high affinity, low capacity site and a low affinity, high capacity site. At therapeutic concentrations three quarters of the tetracycline bound to albumin is associated with the low affinity site.

In serum, around 40% of the tetracycline forming a complex with protein is held in association with the lipoproteins LDL, HDL and VHDL. The ability of tetracyclines to precipitate lipoproteins at high $Ca^{2+}$ concentrations has been previously reported (Kivman & others, 1966; Lacko, Kořinek & Burger, 1959). Rather than being bound to specific sites, the tetracycline appears to be associated with the lipoprotein in a manner similar to its distribution between Krebs phosphate buffered saline, pH 7·4, and an organic solvent such as chloroform. It is thus reasonable to consider the tetracycline as being dissolved in a lipophilic portion of the lipoprotein molecule.

The form of the relation indicates that at low concentrations relatively less tetracycline is extracted into the lipophilic phase. This is probably a consequence of the formation of a complex of tetracycline with $Ca^{2+}$ ions (Kohn, 1961). It has been reported by Oxford (1953) that chlortetracycline at low concentrations, in the presence of $Ca^{2+}$ ions, is less efficiently extracted into an organic phase than at higher concentrations.

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