The Study of an Equilibrium Ultrafiltration Procedure for the Measurement of Steroid-Protein Binding Constants

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Abstract

A new procedure of equilibrium ultrafiltration has been developed for the study of steroid-protein interactions. Its validity in the determination of binding parameters in the interaction of testosterone and bovine serum albumin has been examined by a comparison with the classical method of equilibrium dialysis. Values for the association constant and maximum number of binding sites for bovine albumin-testosterone complex have been calculated at different protein concentrations using both methods. The binding affinity values derived from this method have been found to be consistently higher than those obtained by the equilibrium dialysis method although the value for the number of binding sites is the same. These higher values have been attributed to a higher accuracy obtainable by this method, although the possibility that structural alterations in the protein molecule are responsible cannot yet be ruled out.

Using both methods a dependence of the binding affinity on protein concentration has been demonstrated directly for the first time in the case of testosterone-bovine serum albumin interaction.

Section I

General Introduction

For the past three decades the interactions which occur between various small molecules and serum proteins have been the subject of an active area of interest on the part of many investigators. Interactions such as those of the steroid hormones with serum proteins appear to be of major importance for the transport and distribution of these compounds (1). The existence in plasma of specific and non-specific binding systems for these biologically significant substances has always been explained in terms of adaptation for transport. This seems to be true in such examples as the transport of oxygen by hemoglobin and the transport of non-esterified fatty acids by albumin (2). The thyroid hormone and the steroid hormones are also known to circulate bound to a considerable extent to the plasma proteins (1).

In the case of the thyroid hormone it has been suggested (3) that the metabolic activity of the body is determined by the minute concentration of the unbound thyroxin rather than by the total thyroxin of circulating plasma, and this appears also to be true in the case of steroids. Binding to plasma proteins is hence of considerable physiological significance in understanding the meaning of plasma hormone levels. It also partly explains the limited filtration of steroid hormones across the glomerular membrane resulting in a low clearance rate (2). On the other hand the metabolic products

of the steroid hormones (4) and their conjugates with glucuronic acid, being much less strongly protein bound, exhibit relatively high rate of glomerular filtration (5 & 6).

A similar important physiological function has been reported recently (7) for the protein-protein interaction between prealbumin and retinol binding protein, an interaction which appears to prevent the glomerular filtration of the relatively small protein molecule which binds retinol and hence prevents the loss of the retinol binding protein and vitamin A in the urine.

Binding proteins in serum :-

Intensive investigation over a number of years have revealed that, of the plasma proteins, serum albumin appears to have a high binding capacity for many different types of small molecules, a property which endows serum albumin with an important transport function in the circulating blood (8). The extraordinary high capacity of serum albumin in this respect is seen in the wide range and variety of substances which it binds e.g.; azobenzenes (9), adenosine (10), sulfonamides (11), dinitrophenyl compounds (12), thiocyanates (13), certain metallic cations such as cupric and zinc ions (14), long chain fatty acid anions (15), sulfabromophthaline (16), penicillins (17), long chain alkyl sulfates and sulfonates (18), the anticoagulant drug warfarin and its metabolites (19), digitoxin (20), fluorescein (21). Studies on drug-protein interactions are reviewed by Goldstein (22).

Steroid hormone - protein interactions

Probably the first interaction between a steroid hormone and a protein was that recognized by Brunelli (23) who was able to demonstrate thirty five years ago, in a simple dialysis experiment, that estrogenic hormones are bound to serum proteins. At that time the concept of the transport function of the blood had just been developed by Bennhold (8). Steroids being available in pure form and exhibiting a wide range of variation in structure represent, from a physicochemical viewpoint, a particularly suitable class for the study of the phenomenon of small molecule-protein interactions (24). The biological significance of such interactions, i.e. the formation of steroid-protein complexes, has been emphasized by Ryan (25), who pointed out their importance not only with respect to the transport properties of serum proteins but also to the possibility of providing a better understanding of the problem of hormone action at the molecular level.

In this context Talwar et al (26) have studied the factors influencing the binding of oestradiol-17-β to serum albumin and also to a receptor protein, "a uterine macro-molecule fraction". They have reported the high affinity of this receptor protein for binding oestradiol, a feature which contrasts with the behaviour of serum albumin. Gorski and co-workers (27,28) have studied the relationship between the

oestrogen binding activity of the material found in the "cytosol" and that of nuclear fractions of the rat uterus and have reported that 17-β-oestradiol is first bound to a 9 S receptor protein in the "cytosol". They suggest that association causes a conformational change resulting in its subsequent movement into the nucleus where it becomes bound to a 5 S protein. It would seem very likely that this material plays a definite role in the action of oestrogens in the uterus.

Binding of steroids to serum albumin :-

Although it seems likely that the binding of steroids can occur to practically any protein of high molecular weight, it appears that the transport of steroids in human plasma can be attributed mainly to albumin, α- and β-globulins (29-32). For steroids other than corticosteroids, albumin has been considered until very recently to be the most important of these. Since it is readily available in relatively pure crystalline form, it is understandable that most of the information on interactions between proteins and steroid hormones has been obtained from the study of serum albumin of either bovine or human origin (33-36). According to Bischoff and Stauffer (37) albumin is the most important element in the transport of oestrogens in serum.

Corticosteroid-Binding Globulin :-

The participation of globulins in steroid interactions was early indicated; for oestrogens by Brunelli (23) for corticosteroids by Roberts and Szego (38) and for 17-β-ketosteroids by Gardner (39). Such studies culminated in the discovery of the corticosteroid-binding globulin by Daughaday (40) and Bush (41). This globulin was later named transcortin by Slaunwhite et al (42). The discovery of transcortin appears to demonstrate clearly the importance of a trace protein for specific interactions with one class of steroid hormones.

Non-Albumin Testosterone Binding Protein :-

Earlier indications of the presence in plasma of a non-albumin testosterone binding protein were reported by Chen et al (29) and De Moor et al (43). The former authors have observed that, at a concentration between 20 and 200 μg/100 ml and at 37°C, testosterone was bound to the extent of 88% to a 5% albumin and 95% to plasma. Pearlman and Crépy (36) and Pearlman et al (44), using the equilibrium dialysis technique involving sephadex G-25, have also demonstrated the existence in human plasma of a macromolecular factor other than albumin for the binding of testosterone. This protein has the properties of a β-globulin. It has high binding affinity but limited binding capacity for testosterone and, as with transcortin (45), its level increases during pregnancy.

Vermeulen and Verdonck (46) have observed its specificity for 17-β-OH group and also have demonstrated its affinity for oestradiol. Studies on the sex distribution and specificity of this testosterone binding protein in human plasma have been reported recently by Rosner and Deakins (47). In spite of the existence of such high affinity steroid binding globulins in plasma, albumin plays a very significant quantitative role. It is thus clear that the phenomenon of steroid protein binding is one of expanding physiological significance.

Experimental investigation of steroid-protein interactions :-

The physicochemical techniques which have been used for the experimental demonstration of the reversible combination of steroids and proteins are shown in Table 1. These methods have been classified into two groups; true equilibrium methods as e.g. the equilibrium dialysis procedure and non-equilibrium procedures as e.g. electrophoresis. In the non-equilibrium methods the interaction is characterized after a greater or lesser distortion from the equilibrium state has developed. Nevertheless, it is now generally agreed that essentially the same binding information can be obtained by any of these methods, providing that conditions of temperature and electrolyte concentrations are kept constant.

Table 1

Methods used for the study of steroid-protein interactions

Method	Reference
Dialysis	23
Ultrafiltration *	29, 33, 48
Solvent partition *	34, 49, 50
Ultracentrifugation	51
Paper chromatography	52
Equilibrium paper electrophoresis	53
Spectrophotometry	54, 55
Continuous flow electrophoresis	32, 56
Equilibrium dialysis; (with visking membranes) *	34, 35, 57-60
Equilibrium dialysis; (with sephadex G-25) *	36
Multiple equilibrium dialysis *	61
Ultraviolet difference spectroscopy *	25
Fluorescence quenching	62

* methods used for the determination of binding parameters.

Binding Parameters :-

The description of the interaction according to the exact physicochemical treatment of Klotz (63) employs the concept of "binding sites" of equal binding affinity. Klotz has derived, from the mass action law, the following simple expression for the interaction between small molecules and proteins;

$$\frac{1}{r} = \frac{1}{nk} \times \frac{1}{s} + \frac{1}{n}$$

Where:

r represents moles of small molecule bound per mole protein.

n represents the maximum number of binding sites per mole of protein.

k is the association constant for the interaction at each site.

s is the concentration of substrate molecules remaining free in solution, when equilibrium between bound and unbound forms is established.

Reciprocal Plot :-

Using the above expression, it can be seen that a plot of 1/r versus 1/(s) yields a straight line from which values of nk and n may be calculated. The intercept on the ordinate axis gives the value for 1/n and 1/nk is given by the slope of the line.

The Scatchard Plot :-

Such data obtained from measurements of r & s can also be represented in the manner derived by Scatchard (64), from data obtained by the equilibrium dialysis procedure. The above equation can be written as;

$$\frac{r}{s} = k \quad (n-r)$$

Where a plot of r/(s) as the ordinate against r as the abscissa gives a straight line. The intercept on the abscissa gives n, the maximum number of binding sites. The intercept on the ordinate gives nk. In the Scatchard plot, values for n are obtained more accurately than in the case of the reciprocal plot, since the extrapolation to the abscissa is very easily measured. It must be noted that there are many situations where binding data do not fit such a simple model involving homogeneity of sites and data have to be fitted to a model involving two or more classes of sites. Furthermore, as some of our results tend to show, the value of intrinsic site parameters are not necessarily constant. However, the value for nk is a true measure of the total binding affinity, whether the sites are homogeneous or heterogeneous. It can be seen that at low values or r;

$$nk = \frac{r}{s}$$

$$= \frac{(1 - \alpha)}{(\alpha)} \quad x \quad \frac{1}{P}$$

Where $\alpha = \frac{S_{unbound}}{S_{total}}$

S_{bound} and $S_{unbound}$, may be given either as percentage of total steroid or as molar concentrations. P is the concentration of the protein in gm/liter. Hence from a single measurement of the percentage steroid bound at low steroid concentration one can arrive at an approximation of the binding affinity of protein for steroid which is useful in comparing binding affinity of different steroids to a given protein. However, in the work described here complete binding curves have been determined giving true values for nk.

Section II

The Binding of Testosterone to Bovine Albumin and the Measurement of the Binding Parameters by Means of the Classical Equilibrium Dialysis Method.

Binding of testosterone to bovine albumin

Part A

I N T R O D U C T I O N

The reversible binding of testosterone to bovine albumin has been studied by several investigators (34,36,49, 50,57,65,66). Probably the most intensive investigation was that of Schellman et al (49) who have demonstrated a linear relationship between the reciprocal of the moles of bound testosterone per mole of albumin and the reciprocal of the free testosterone concentration, giving an estimate of the maximum number of binding sites on the albumin molecule available for testosterone binding as 5-10, depending on the preparation of albumin used. These investigators have found that binding increased with the purity of the preparation, with temperature and with pH. The increase in binding with increase in pH has also been demonstrated by Levedahl and Perlmutter (50). In studying the binding of testosterone to bovine albumin, Schellman et al (49) have also shown that the presence of thiocyanate and methyl orange anions as well as zinc and cupric cations decreases the binding affinity. This has been explained as being due to a direct competition for the sites on the protein molecule. Levedahl and Bernstein (57) have presented evidence showing that blocking of ε-amino groups does not influence the ability of the protein to bind

the steroid. Alfsen (34) has found a value of 4 for the number of binding sites at 25°C in phosphate buffer using the equilibrium dialysis technique and a value of 7 at the same temperature using the solvent partition procedure. This author has also shown that the removal of the fatty acids from the albumin resulted in no difference in its binding characteristics. However, the values published for nk for testosterone-bovine albumin interaction vary from 1.90 to 4.00 (Table 6), at pH 7.4.

Measurement of binding :-

In view of the variability in the published values of nk for BSA-testosterone interaction, it was necessary for us to establish the value for our own batch of bovine albumin for the purpose of work described in a later section. We chose to do this using the classical equilibrium dialysis procedure, employing visking membranes. This is the most widely used technique for the measurement of the binding of small molecules (ligands) by macromolecules and has been accepted as a reliable and accurate method. It has, however, the disadvantage of requiring a long time for completion (24-72 hours). There are also some possible sources of error as, for example, the membrane adsorption of the ligand, denaturation of the protein, alteration in the membrane pore size and also the interference of material shed by unwashed membranes, particularly significant if the steroid concentration determinations are to be performed by measurements in the

ultraviolet region. In this method, as described in the experimental section, a solution containing a macromolecule is separated by the semipermeable membrane from another solution containing only the solvent and small molecule or ion, which can pass through the membrane and whose binding is under study. The two solutions are shaken at constant temperature until analysis shows that equilibrium has been established on both sides of the membrane. The concentration of steroid on each side of the membrane is then measured and from this values for r are obtained.

Part B

1- E X P E R I M E N T A L

a) Material and Methods

Bovine serum albumin

This was obtained from The Armour Laboratories, lot number T 68204. It was used without any further purification, such as that of deionization or extraction. Some experiments were carried out using crystalline bovine albumin obtained from Pentex, lot number 15. One experiment was done with albumin dialyzed for 48 hours against phosphate buffer at $4^{o}C$, changing the buffer at least 6 times during that period. The protein concentrations were always determined by absorbance measurements, using the value of 6.67 for absorbance of 1% solution at 279 mμ and taking the value of 69,000 for the molecular weight.

Phosphate buffer

Phosphate buffer of pH 7.6, μ 0.1 prepared from reagent grade sodium salts and deionized water was used in preparing all the solutions of albumin and steroid.

Testosterone

Testosterone was obtained from Mann Research Laboratories, New York, and was recrystallized from acetone. The melting point was 153-154^{o}C. Testosterone-4-C^{14} of better than

97% radiochemical purity was obtained from New England Nuclear Corp., Boston, Mass. The molecular extinction coefficient for testosterone in phosphate buffer was 15,700 at 249 mμ. The absorbances were measured in a Bausch and Lomb Precision Spectrophotometer using silica cells of 1 cm path length. Stock solutions in phosphate buffer were prepared by evaporating a measured volume of a 1-5 mM solution in ethanol under a stream of nitrogen in an Erlenmeyer flask. The fine film so obtained was dried for about one hour at 60°C in a vacuum oven, dissolved with shaking in buffer and filtered through a fine-porosity sintered glass filter. When lower concentrations were needed, stock aqueous solutions were diluted with buffer. The labeled testosterone solutions were prepared by evaporating a known amount of the methanol-benzene solution of testosterone-4-C^{14} under a stream of nitrogen; the residue was dissolved in the aqueous unlabeled testosterone solutions.

b) Equilibrium dialysis procedure

Early in the present studies it was found that the use of untreated membranes in phosphate buffer resulted in leaching an ultraviolet absorbing material from the tubing. Since the steroid used in this study contains an α,β-unsaturated ketone chromophore group in ring A, spectrophotometric assay was originally contemplated for the measurement

of free steroid, as in the method of Westphal (58). However, this ultraviolet absorbing material shed by the membrane produced anomalous results. Table 2 shows the absorbance at different wave lengths of phosphate buffer pH 7.6, μ 0.1, (15 ml) in which a visking membrane of 4 inch length was placed for the indicated periods of time.

Table 2

Absorbance at different wave lengths of phosphate buffer pH 7.6, μ 0.1, (15 ml) in which a visking membrane of 4 inch length was placed.

Wave length mμ	Absorbance			
	4 hr	6 hr	24 hr	48 hr
210	0.162	0.174	0.208	0.214
215	0.139	0.152	0.198	0.199
220	0.121	0.133	0.177	0.181
230	0.089	0.097	0.162	0.134
240	0.058	0.067	0.126	0.130
250	0.037	0.046	0.090	0.095
260	0.029	0.035	0.068	0.073
270	0.026	0.032	0.057	0.063

Although the equilibrium dialysis method using visking membranes has been used by many investigators in studying the binding of various steroids to proteins, it is only in recent years that investigators have started to refer to this problem of the ultraviolet absorbing material shed by the dialysis membranes. Various ways for washing and cleaning the membranes have been described. In one of his recent papers Westphal (67) claimed to have cleaned the visking membranes by washing the tubing in distilled water followed by the treatment with a solution of 0.002 M disodium EDTA and 0.002 M sodium ascorbate in 0.05 M phosphate buffer pH 7.6 for 24 hours, using continuous shaking and followed by rinsing the tubing with six changes of water over a 24-hour period. Kripalani and Sorby (55) reported that they cleaned the membranes by heating for 12 hours in distilled water, changing the water three times during this period. The membranes were then rinsed in distilled water and finally for 6 hours with phosphate buffer. Reynolds et al (18) reported cleaning of the membranes by boiling for one hour in distilled water followed by repeated washing with distilled water. Zimmering et al (68) using special membranes of controlled pore size employed a method for removal of the ultraviolet absorbing material involving washing in 20% ethanol for four days followed by distilled water for 10-20 days, while Craig (69) has suggested the use of dilute acetic acid for this purpose. Attempts were made in the present work to

clean the membranes using these various procedures. It was found that practically none of these methods was efficient in significantly reducing the absorbance of the phosphate buffer in which a sample of membrane, which had been washed by means of these procedures, was shaken for different periods of time. This is illustrated in Tables 3(a), 3(b) which may be compared with the results of unwashed membrane shown in Table 2. We have also tried to set up a device to clean the dialysis membranes from outside as well as from the inside by continuous rinsing in a recycling bath of deionized distilled water for periods up to three days. Visking membranes of approximately forty inch in length were attached by the ends to polyethylene tubing and placed without stretching inside a glass tube of about forty eight inch in length and one and half inch in diameter. Distilled water was circulated at a fairly slow rate for a period of three days after which the membranes were washed with phosphate buffer for about 6 hours. The buffer was changed at least three times during this period. McMenamy (70) has recently described a similar device for cleaning the membranes. However, this did not give results significantly better than with the other procedures. In the present studies it was found that the best results were those obtained by soaking the membranes in 0.1 M nitric acid for one day, 0.01 M nitric acid for two to three days (71) followed by the repeated washing with distilled and deionized water. These results are illustrated in Table 3(c), which may be compared

with Tables 2, 3(a) and 3(b). This washing procedure was adopted in all later experiments in which steroid concentration was measured with the aid of testosterone-4-C^{14}. The Westphal method (58), involving measurement of steroid concentration using ultraviolet absorbance, had to be abandoned for the above reasons as is well illustrated in Table 4. This table shows the results of equilibrium dialysis experiments on testosterone-albumin interaction, performed using dialysis bags made from visking membranes which were untreated and also treated with the various methods of washing indicated. Binding to albumin within the bag would be exhibited by absorbance values of the external solution lower than that anticipated by dilution. It can be seen that in all cases these values are much higher.

Table 3(a)

Absorbance of phosphate buffer pH 7.6, μ 0.1, (15 ml) in which a 4 inch length of visking membrane was placed for 24 hours, after washing by the indicated procedures.

Wave length mμ	Washing Procedure			
	20% alcohol, then water		Circulating deionized distilled water and phosphate buffer	
	I	II	I	II
220	0.086	0.098	0.090	0.088
230	0.067	0.077	0.088	0.084
240	0.051	0.061	0.084	0.082
245	0.051	0.054	0.082	0.079
250	0.050	0.053	0.082	0.078
255	0.050	0.051	0.078	0.076
260	0.050	0.050	0.078	0.076

Table 3(b)

Absorbance of phosphate buffer pH 7.6, μ 0.1, in which membranes previously washed by the method of Westphal (67) were placed for 24 hours; 4 inch length of visking membrane in 10 ml buffer.

Wave length mμ	I	II	III	IV
230	0.130	0.080	0.135	0.115
240	0.100	0.060	0.101	0.085
245	0.095	0.055	0.092	0.080
249	0.090	0.050	0.088	0.078
260	0.085	0.048	0.080	0.074

Table 3(c)

Absorbance of phosphate buffer pH 7.6, μ 0.1, (10 ml) in which a 4 inch length of membrane, previously washed with nitric acid was placed for 24 hours.

Wave length mμ	I	II	III	IV
240	0.028	0.029	0.029	0.030
245	0.027	0.020	0.029	0.026
249	0.008	0.011	0.010	0.010

Table 4

Absorbance of testosterone solution from outside dialysis bags after 24 hours shaking in an equilibrium dialysis experiment. The bags were previously treated as indicated. Each bag contained 5 ml of 4×10^{-5} M bovine albumin in phosphate buffer. The bags were immersed each in 10 ml testosterone solution of absorbance 0.356 at 249 mμ.

Method of washing the membranes	Absorbance at 249 mμ
Membranes washed with 5% acetic acid for 20 hours, then distilled water, deionized water several times and finally with phosphate buffer	0.328
Membranes washed with the method of boiling for one hour, then washing with distilled and deionized water	0.311
Membranes washed with 20% alcohol for 24 hours	0.332
Membranes washed with distilled water for 24 hours	0.388
Unwashed membranes	0.475
Expected absorbance, allowing for dilution and assuming no binding	0.237

i) Preparation of dialysis bags and equilibration :-

Dialysis bags were made from Nojax casing 20/32" diameter. The membranes were washed using the nitric acid procedure mentioned earlier and then were stored at $4^{o}C$. Just before use the membranes were rinsed several times with deionized water followed by phosphate buffer. The membranes were freed from water but were not allowed to dry. The bags were made by first tying a double knot at one end. Then 3 ml of albumin solution in buffer was introduced into the bag as well as two small glass beads in order to ensure efficient mixing. The bags were tied at the other end without tension. The bags were placed in 50-ml stoppered Erlenmeyer flasks in which 10 ml of the testosterone-4-C^{14} in phosphate buffer had been introduced. To keep the system free of mold, streptomycin sulfate was added to the outside solution to give a final concentration in the dialysis system of 10 μg/ml. Each steroid concentration was set up either in duplicate or in triplicate. Blanks were prepared with only buffer inside the bags, so as to check attainment of equilibrium. The flasks were placed in a shaking water bath at a constant temperature of $25^{o}C$ for 48 hours. When the dialysis was complete, duplicate 1/2 ml samples were withdrawn both from the external and internal solution in each flask and were added to the counting vials. Triplicate samples of each stock solution of testosterone-4-C^{14} together

with an appropriate number of toluene-C^{14} standards were also counted. To each vial, containing 0.5 ml sample 18.5 ml of the scintillation solution was added. The scintillation solution consisted of 12.5 ml toluene + 6.0 ethanol + 0.6% PPO and 0.01% POPOP. All radioactive samples were counted in Nuclear Chicago Liquid Scintillation Counter. Background counts were about 50 cpm and efficiency was 75% and was checked in each experiment using the appropriate toluene-C^{14} standards.

ii) Calculation of binding values :-

The concentration of free steroid is given by counting activity of the external solution. The concentration of the bound steroid was obtained as the difference between the total steroid concentration inside the bag and the concentration of free steroid. A typical set of such data is presented in Table 5. Such data from equilibrium dialysis studies of protein-steroid interactions are plotted either as reciprocal plots or Scatchard plots in figures 1-8.

2- D E S I G N O F E X P E R I M E N T S

The original aim of the studies reported in this section was to establish values of the binding parameters of the albumin to be used in studies reported in a later section and at a concentration which is lower than that at which most reported studies have been made. Our initial values for the protein concentrations 3.55 and 10.72 x 10^{-5} M (0.245% and 0.739%) reported in Table 10, when seen in the light of the variation of the published values (Table 6), led us to suspect a dependency of the binding parameters on protein concentration and binding level. We therefore decided to study this problem, which has hitherto been scarcely recognized, in greater detail. In order to investigate whether or not there is such a dependence it was decided to carry out an equilibrium dialysis experiment with only one concentration of testosterone and with different concentrations of bovine albumin, together with other experiments in each of which there were four or more concentrations of steroid and with the protein concentration constant in a given experiment but varied from one experiment to the other. The results of these experiments are found in Tables 8 and 10 and in figures 1-8.

3- R E S U L T S

Table 5 shows the results of an equilibrium dialysis experiment performed with five different concentrations of testosterone and with 3.55×10^{-5} M bovine albumin at $25^{o}C$ and pH 7.6. These results are represented graphically in fig. 3. From this plot values for n and nk x 10^4 were found to be 5 and 2.97 respectively. When this value for binding affinity is compared with the published values shown in Table 6, it may be seen that our value agrees well with that obtained by Levedahl (65) and with one of those obtained by Pearlman and Crépy (44), but differs significantly from those of other investigators. A further equilibrium dialysis experiment was carried out with the same range of testosterone concentrations but increasing the albumin concentration three fold i.e. 10.72×10^{-5} M. The results of this experiment are shown in Table 7 and graphically represented by means of the reciprocal plot in fig. 6. It may be seen that this three fold increase of the albumin concentration resulted in decreasing the value for nk from 2.97 to 1.96 using the same albumin preparation under the same experimental conditions, as is evident in Table 10. This is further borne out when measurements were extended to other protein concentrations. It is also seen in the results of the experiment where total testosterone concentration was held constant and albumin concentration varied (fig. 8). It must be emphasized that

almost all published studies on BSA-testosterone interaction have been performed at protein concentrations which are essentially held constant and which are much higher than the concentrations we have used in the present work. The Scatchard plot of these results in fig. 7 exhibits a non rational positive slope. This is similar to that obtained by Brunkhorst and Hess (35) for serum albumin-cortisol interaction. This positive slope suggests a change in the values of binding parameters with the change in protein concentration and seems to imply an increase in the value of n or k, or possibly both parameters, as the protein concentration decreases. Our results for this experiment are also represented by means of the reciprocal plot in fig. 8 and may be compared with a similar one in fig. 9 obtained from data taken from the results of Brunkhorst and Hess (35) for BSA-cortisol interaction and shown in Table 9. Both reciprocal plots exhibit non rational characteristics.

Part C

D I S C U S S I O N

As has been stated previously binding of testosterone to bovine serum albumin has been studied by Schellman et al (49), Alfsen (34), Levedahl and co-workers (50) and Pearlman and Crépy (36). The values of nk x 10^{-4} M^{-1} as found in the literature vary from 1.90 to 4.00 at 25°C and values for n vary from 4 to 10 (Table 6). In the present work, values for nk were found to fall in the range between 1.96 and 4.71 but under conditions in which the concentration of the same protein preparation was varied quite widely at the same conditions of temperature, buffer and pH. As has been stated previously, the published values of nk and n for bovine serum albumin-testosterone interaction have been measured in experiments performed with limited range of steroid concentration and with the albumin concentration essentially held constant. In most cases the latter was approximately 1% (49,50,65) or 2% (34,49). The variability of the value of nk obtained by other investigators is probably due to the use of different albumin preparations. Thus for example; Schellman et al (49) using the techniques of equilibrium dialysis and the solvent partition have published three different values for nk: 1.90, 2.30 & 4.00 for the same concentration of three different preparations of bovine albumin, the values for n being 10, 6 & 5 respectively. These authors

have thus suggested that the increase in the value of nk is a function of the purity of the preparation. Alfsen (34), using one and the same preparation of bovine albumin found values of 1.95 and 4 for nk and n respectively, using the equilibrium dialysis technique with visking membranes and values of 1.97 and 7 using the solvent partition method. The equilibrium dialysis values obtained by Alfsen agree very closely with those of ours for 10.72×10^{-5} M.

In the present studies, using the same albumin preparation, different values for nk have been obtained when the protein concentration was varied from 0.438 to 10.72×10^{-5} M with pH 7.6 and at 25^{o}C. This observation provides evidence for a dependence of the binding affinity on protein concentration for BSA-testosterone interaction. Very few studies have been carried out to investigate the relationship of the ligand binding to protein concentration and this is particularly true in the case of steroids. Klotz and Urquhart (9) have examined the effect of varying bovine albumin concentration on the binding of methyl orange and demonstrated that the binding is dependent upon protein concentration. Ray et al (72) have reported a similar phenomenon for the binding isotherms of two long-chain ligands, dodecyl sulfate and dodecanol. In the case of the interaction of steroids, Brunkhorst and Hess (35) provide apparently clear evidence for such a phenomenon in the case of serum albumin - cortisol

interaction. This has recently been confirmed by Westphal and Kerkay (73) who have also demonstrated an opposite effect of protein concentration in the case of the binding affinity of α_{-1} acid glycoprotein (orosomucoid) for cortisol and progesterone. On the other hand, in the case of HSA-progesterone interaction, the same authors found no change in the binding affinity values when the protein concentration was varied by 100-fold. A dependence of the binding affinity on protein concentration has been demonstrated indirectly by Attallah and Lata (62) in their studies on the BSA-testosterone interaction using the fluorescence quenching technique. These authors found that such a dependence becomes more significant in very dilute protein solutions (0.002%). They have interpreted their results as an increase in the value of n. The few above mentioned observations are the only published studies on such an effect. As has been stated before, virtually all other studies of the binding between steroids and proteins have been carried out with fairly limited ranges of steroid concentrations and with a single fixed high concentration of protein.

In the present studies, it was of interest to observe that despite the significant variation in the value obtained for nk with varying protein concentration, the value for n, within the limits of error of such determinations, was constant. This is contrary to the results of Attallah and Lata (62) mentioned above. It may be noted that the

fluorescence method used by these authors does not give a direct measure of bound steroid. The increase in the value of nk with decreasing protein concentration must be therefore due to an increase in the value of the intrinsic association constant of the binding site. From a physicochemical viewpoint, this appears to be different from the case where the increase in the value of nk with decreasing protein concentration might be due to increase in the number of binding sites. The latter could arise as a result of a configurational change of the albumin molecule. Our results also suggest a conformational change of the albumin molecule accompanying an increase in the apparent affinity of the binding site. This probably would require a much less extensive change than one associated with increasing n.

Brunkhorst and Hess (35) have suggested that the decrease in the binding affinity of cortisol for serum albumin associated with the increase in protein concentration might be due to the transformation of the albumin from the N to the F form, the N form having a higher binding affinity for cortisol than the F form. Ray et al (72) in their studies on the binding of dodecyl sulfates and dodecanol, have suggested that the binding of one ligand to more than one BSA molecule may account for the lower binding observed at higher protein concentrations.

Very recently Cassel et al (74) re-examined the apparent dependence of the binding of methyl orange to BSA on protein concentration, as reported by Klotz and Urquhart (9), and also the similar observations of Ray et al (72) for the binding of dodecyl sulfate and dodecanol. They have suggested that these two observations were experimently invalid and have shown that such an observed effect on protein concentration was an experimental artifact due to a delayed attainment of equilibrium at high protein concentration. They further attributed the low binding at high protein concentration to the possibility that the protein in high concentration may coat the dialysis membrane in such a way as to reduce the permeability to the ligand. This may be true for the two above reported observations of the protein concentration effect. On the other hand, Keen (17) in his studies of binding of penicillins to bovine serum albumin has demonstrated experimently that the 18-hour period used for attainment of equilibrium with albumin concentration up to 4% was quite adequate. Brunkhorst and Hess (35) performed their equilibrium dialysis for only three hours, a length of time which they claimed to be sufficient for reaching equilibrium. It is quite possible that their observation is invalid, since they have used albumin concentrations up to 4%. However, as stated earlier, Westphal and Kerkay (73) have confirmed their finding using a 48-hour dialysis time, which they have

found to be more than sufficient for attainment of equilibrium. It should be noted that Westphal and Kerkay (73) did not measure the true binding parameters but rather the values of r/s at low values of r.

Our observations, indicating by direct measurement a protein concentration effect for BSA-testosterone interaction, have been made using equilibration time of 48 hours and with a protein concentration range which is much lower than protein concentrations used by other investigators. Furthermore Alfsen (34) using bovine albumin concentration of 2% (28.9×10^{-5} M) found a value of 1.95×10^{4} for nk for BSA-testosterone complex using a 48-hour equilibrium dialysis technique, a length of time which she found to be sufficient for attainment of equilibrium. This is very close to the lowest value observed by us using a protein concentration of 10.72×10^{-5} M (0.73%).

Thus, it seems that our results indicate unequivocally and in a manner not subject to the criticism quoted above a clear dependence of the value of the binding parameters for testosterone-BSA interaction on protein concentration. In this, it seems that the value of k rather than n which varies. It will be seen that the results of section III using a new method in which criticisms as to non-attainment of equilibrium are not applicable, further support this.

Table 5

Typical data for BSA-testosterone binding by equilibrium dialysis. Bovine albumin (Armour), 3.55×10^{-5} M (0.245%). Testosterone concentrations used; $1.43 - 9.17 \times 10^{-5}$ M. Phosphate buffer, pH 7.6, $\mu = 0.1$, 25°C.

Average cpm/$\frac{1}{2}$ ml				conc. of bound steroid $\times 10^5$ M	r	1/r	$1/(S) \times 10^{-4}$ M^{-1}
outside the bag	inside the bag	added	bound steroid				
6780	13853	10856	7073	0.932	0.262	3.809	11.19
6593	12294	10870	5701	1.446	0.407	2.460	5.95
6798	12856	10761	6058	2.720	0.766	1.305	3.26
6862	12793	10767	5931	3.370	0.949	1.053	2.57
6879	12573	10869	5694	5.060	1.425	0.701	1.72

$nk \times 10^{-4}\ M^{-1} = 2.97$

$n = 5$

Table 6

Published values for n and nk for BSA-testosterone interaction, 25°C.

pH	n	nk x 10^{-4} M^{-1}	Method	Protein conc.	Reference
7.4	5	2.97	Solvent partition	1 %	65
7.5	4	2.84	Solvent partition	1 %	50
7.4	10	1.90	Equilibrium dialysis	1 %	49
7.4	6	2.30	Solvent partition	2 %	49
7.4	5	4.00	Solvent partition	2 %	49
7.4	4	1.95	Equilibrium dialysis	2 %	34
7.4	7	1.97	Solvent partition	2 %	34
7.4	-	2.78	Equilibrium dialysis with sephadex G-25	0.4 %	36
7.4	-	3.08	Equilibrium dialysis with sephadex G-25	0.4 %	36

Table 7

BSA-testosterone binding by equilibrium dialysis.

10.72×10^{-5} M BSA, testosterone concentrations as indicated. Phosphate buffer pH 7.6, $\mu = 0.1$, 25°C.

Conc. of Testosterone used	Conc. of bound steroid	Conc. of free steroid	r	1/r	$1/(S) \times 10^{-4}$ M^{-1}
x 10^5 M					
2.77	2.73	1.30	0.25	3.92	7.69
4.58	4.32	2.34	0.40	2.48	4.27
6.11	5.80	2.93	0.54	1.84	3.41
8.47	8.80	4.10	0.82	1.21	2.43

$nk \times 10^{-4}\ M^{-1} = 1.96$

$n = 5$

Table 8

BSA-testosterone binding by equilibrium dialysis. Testosterone concentration fixed (4.685×10^{-5} M). Albumin concentrations as indicated. Phosphate buffer pH 7.6, $\mu = 0.1$, 25°C.

Protein conc.	Conc. of free steroid	Conc. of bound steroid	1/(S) x 10^{-4}	r	1/r	r/(S)
x 10^5 M						
1.810	2.917	1.501	3.42	0.829	1.206	2.84
3.620	2.649	2.663	3.77	0.735	1.360	2.78
7.240	2.298	4.306	4.35	0.594	1.683	2.58
10.860	2.011	5.319	4.97	0.489	2.044	2.43
14.480	1.793	6.226	5.57	0.429	2.331	2.39

Table 9

Data used to represent the results of Brunkhorst and Hess (35) by means of the reciprocal plot (shown in fig. 9), for the interaction of cortisol with bovine serum albumin at 25°C, at varying protein concentration and fixed steroid concentration.

r	$r/s \times 10^{-3}$	1/r	$1/s \times 10^{-3}$
2	2.50	0.500	1.250
3	3.30	0.333	1.099
4	3.75	0.250	0.938
5	4.50	0.200	0.900
7	5.70	0.144	0.810
8	6.30	0.125	0.787

Table 10

Values for nk and n for BSA-testosterone binding; obtained from 48 hours equilibrium dialysis experiments. Each experiment was performed with different albumin concentration as indicated. Phosphate buffer pH 7.6, 25°C.

Exp.	Protein conc. x 10^5 M	Steroid conc. used x 10^5 M	nk x 10^{-4} (M^{-1})	n	Fig.
1	0.438	0.127-0.923	4.71	5	1
2	0.898	1.43 -9.17	3.06	5	2
3	3.550	1.43 -9.17	2.97	5	3
4	6.865	1.29 -8.88	2.39	5	4
5	9.720	2.52 -8.47	1.97	5	5
6	10.720	2.77 -8.47	1.96	5	6

Fig. 1

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6, 25°C.

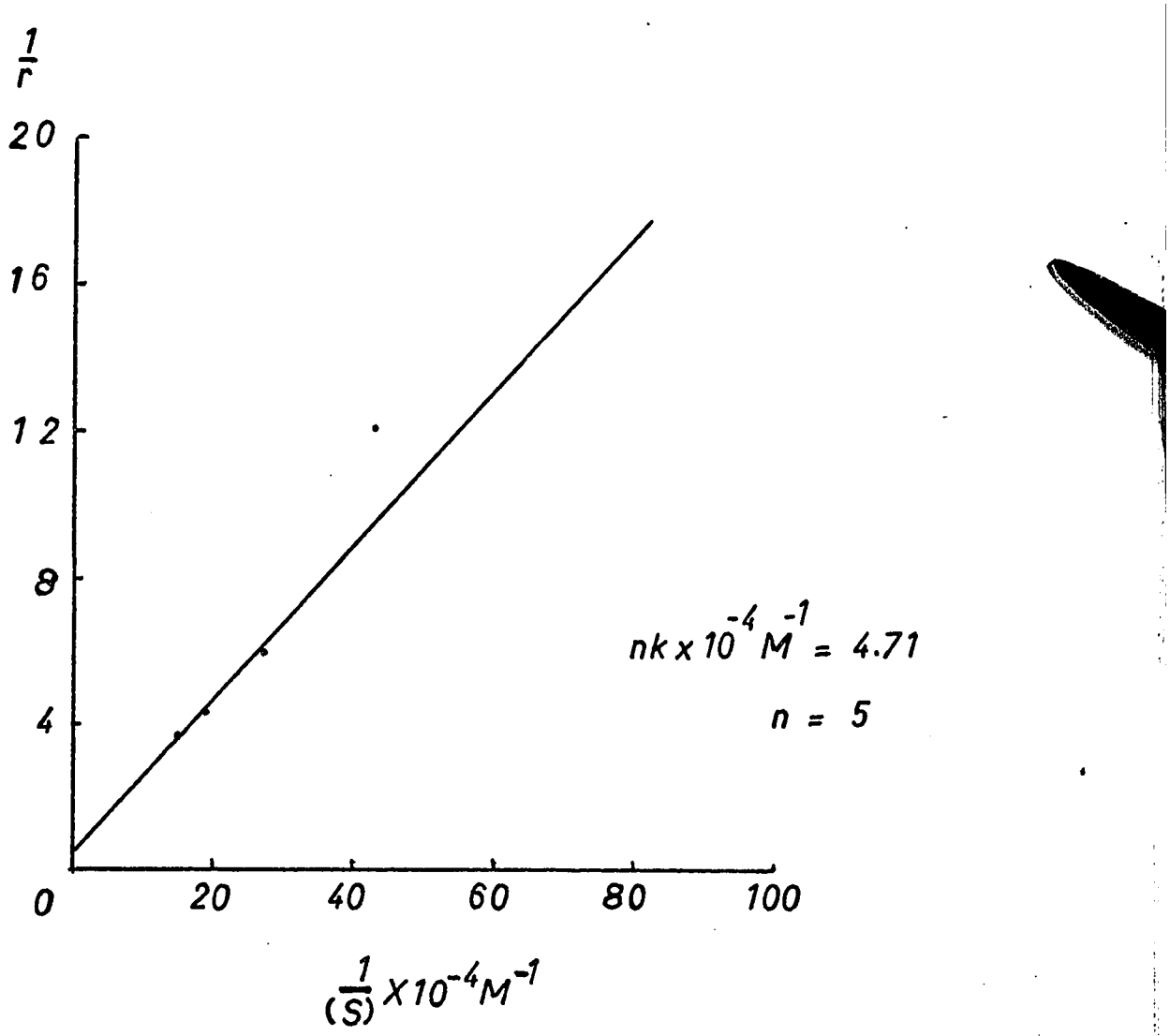


Fig.2

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6 , 25 °C.

0.898×10^{-5} M BSA

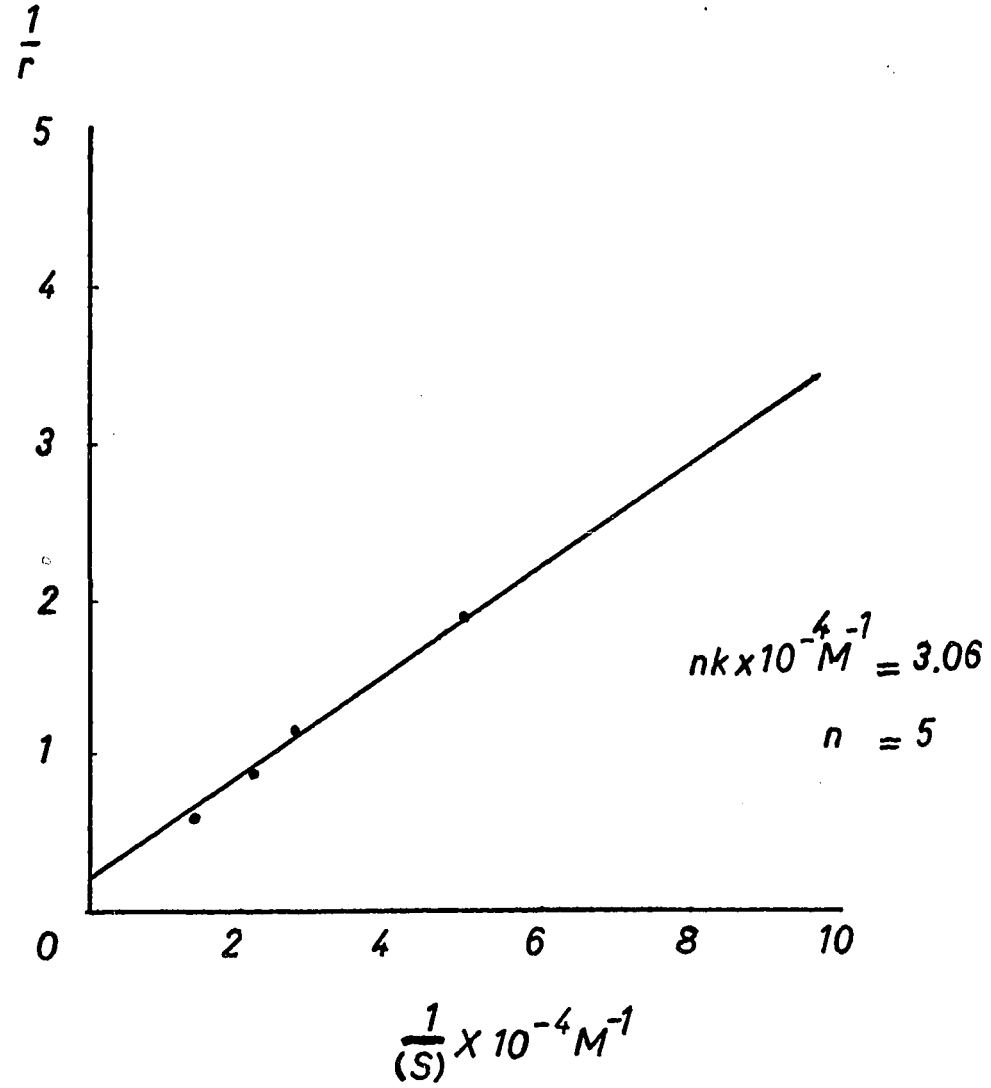


Fig. 3

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6 , 25 °C.

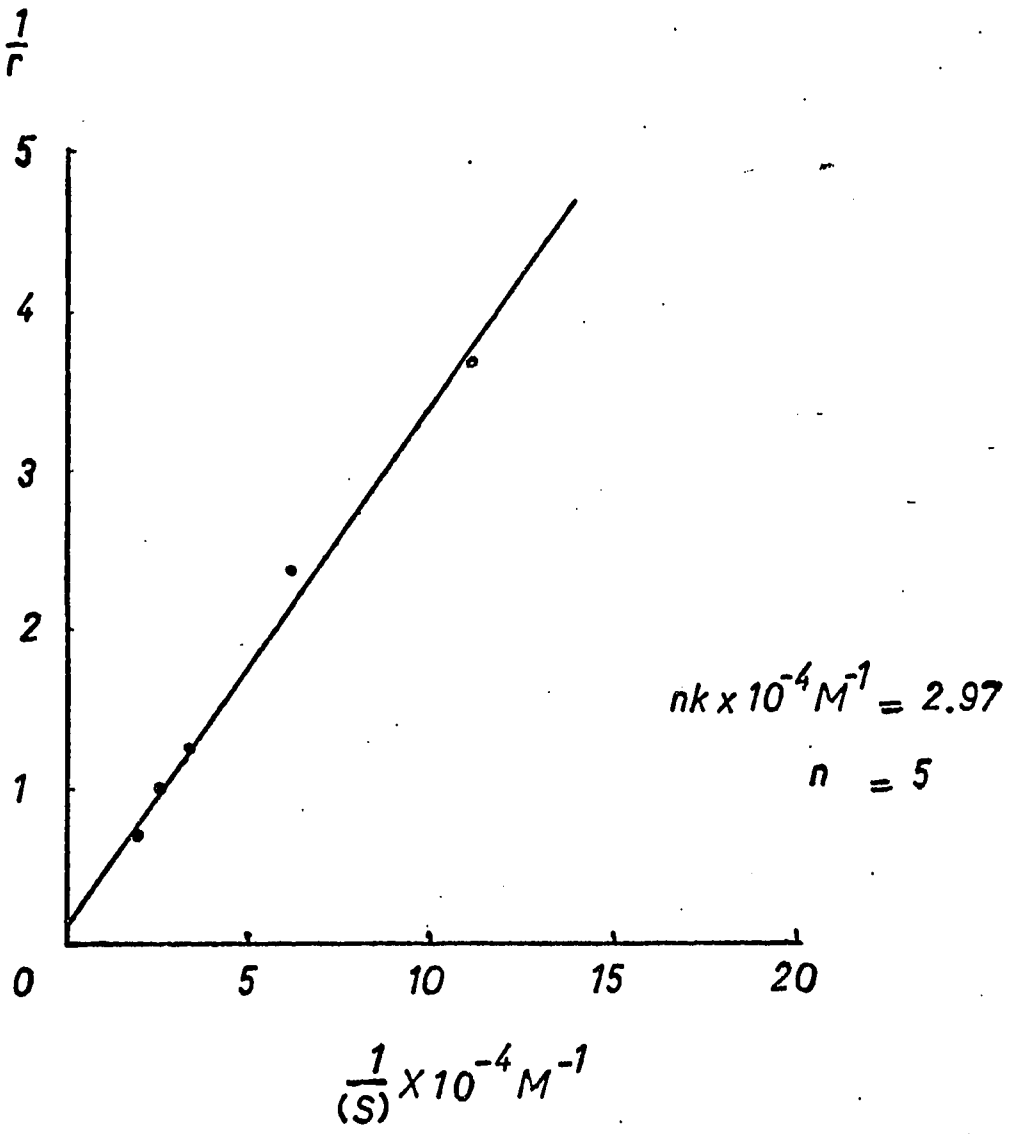


Fig. 4

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6, 25°C.

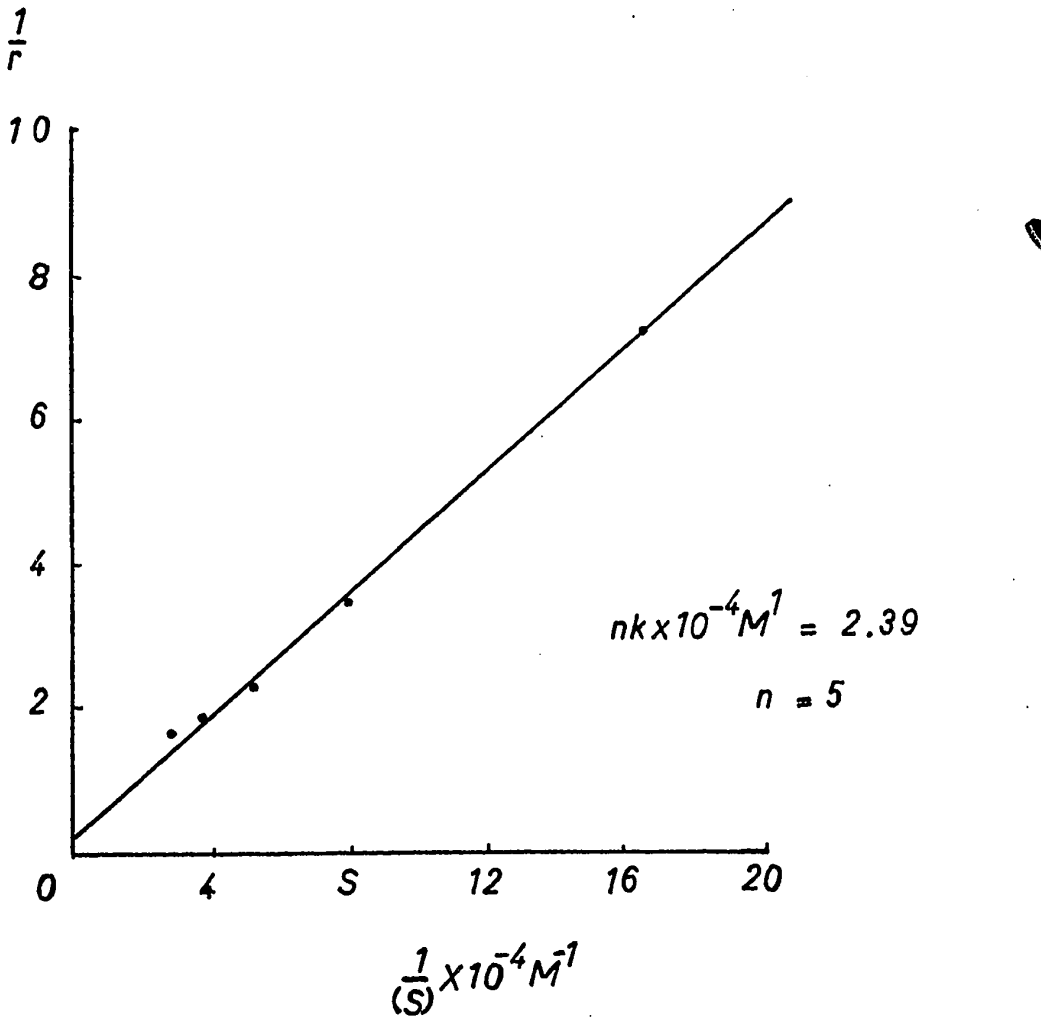


Fig. 5

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6, 25°C.

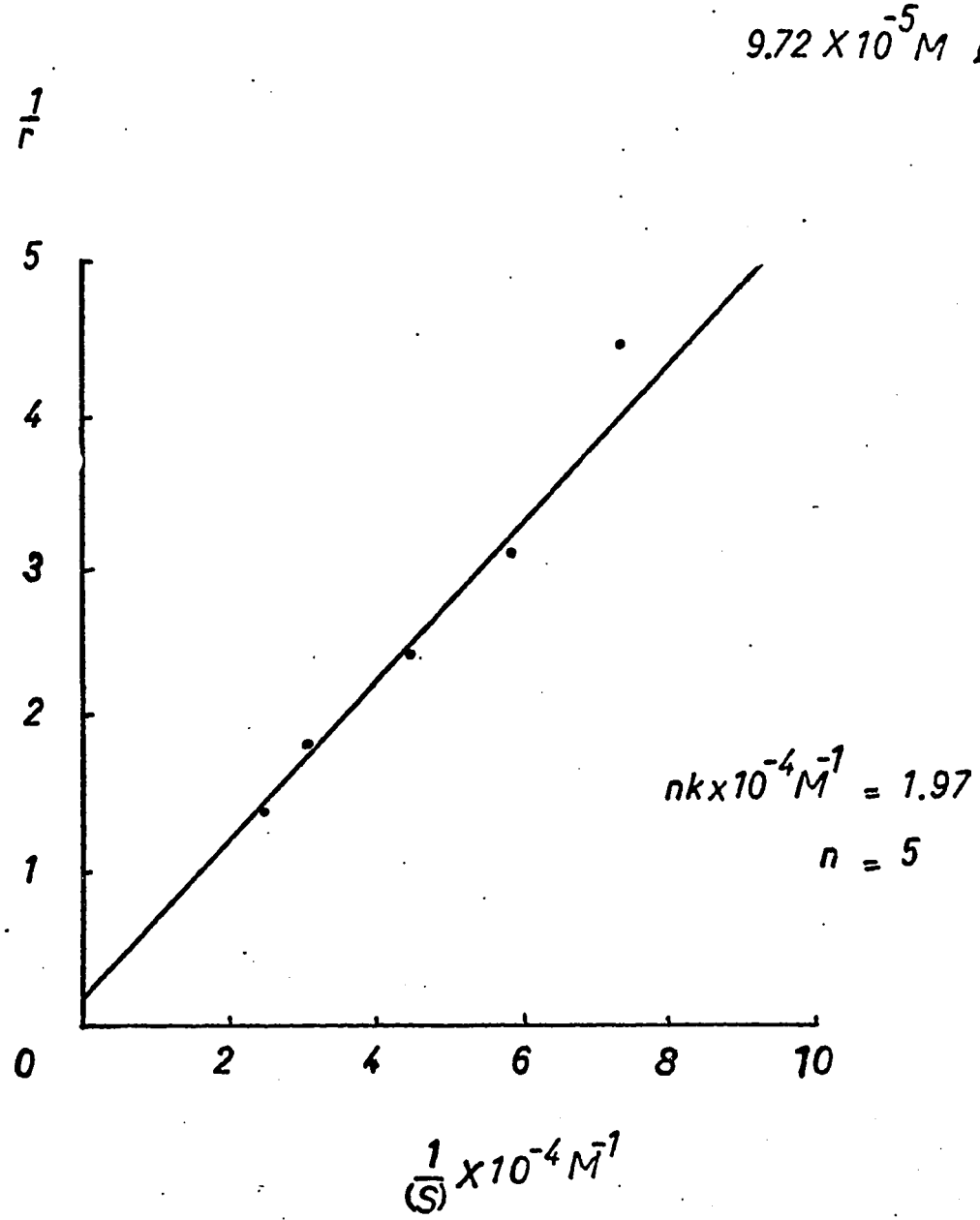


Fig. 6

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6 , 25 °C.

10.72×10^{-5} M BSA

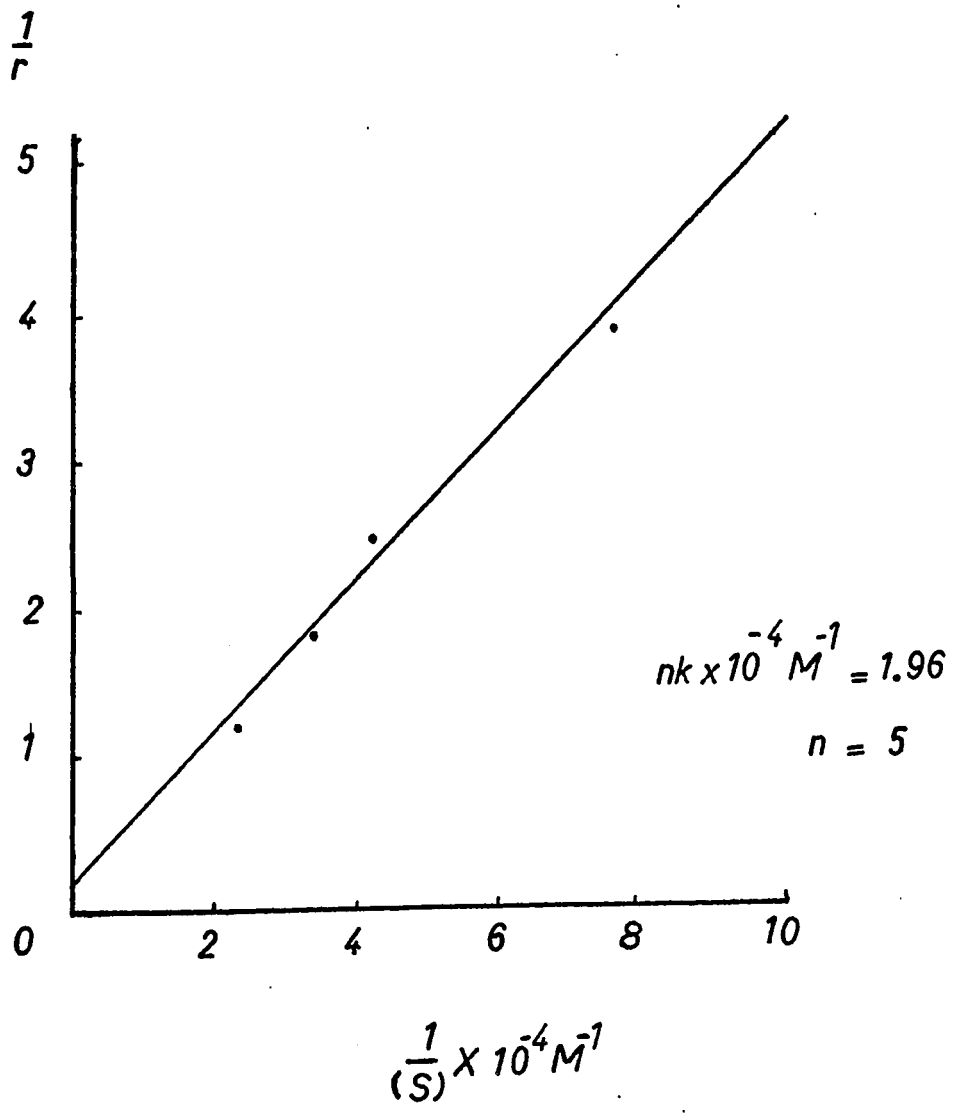


Fig. 7

Scatchard plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6, 25°C. The albumin concentration was varied from 1.81×10^{-5} M to 14.48×10^{-5} M, testosterone concentration fixed: 4.685×10^{-5} M.

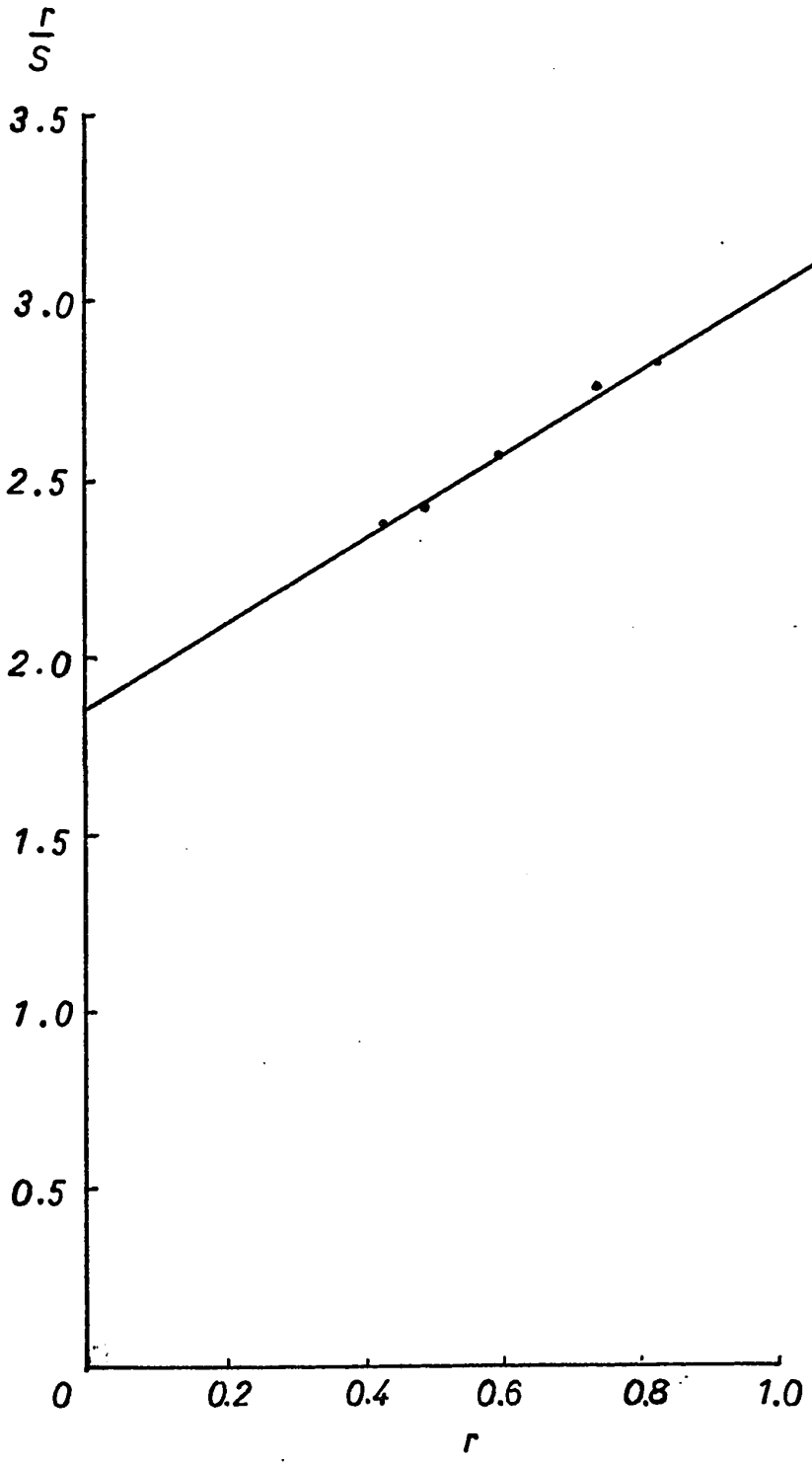


Fig. 8

Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6, 25°C. The albumin conc. was varied from 1.81×10^{-5} M to 14.48×10^{-5} M, testosterone conc. fixed: 4.685×10^{-5} M.

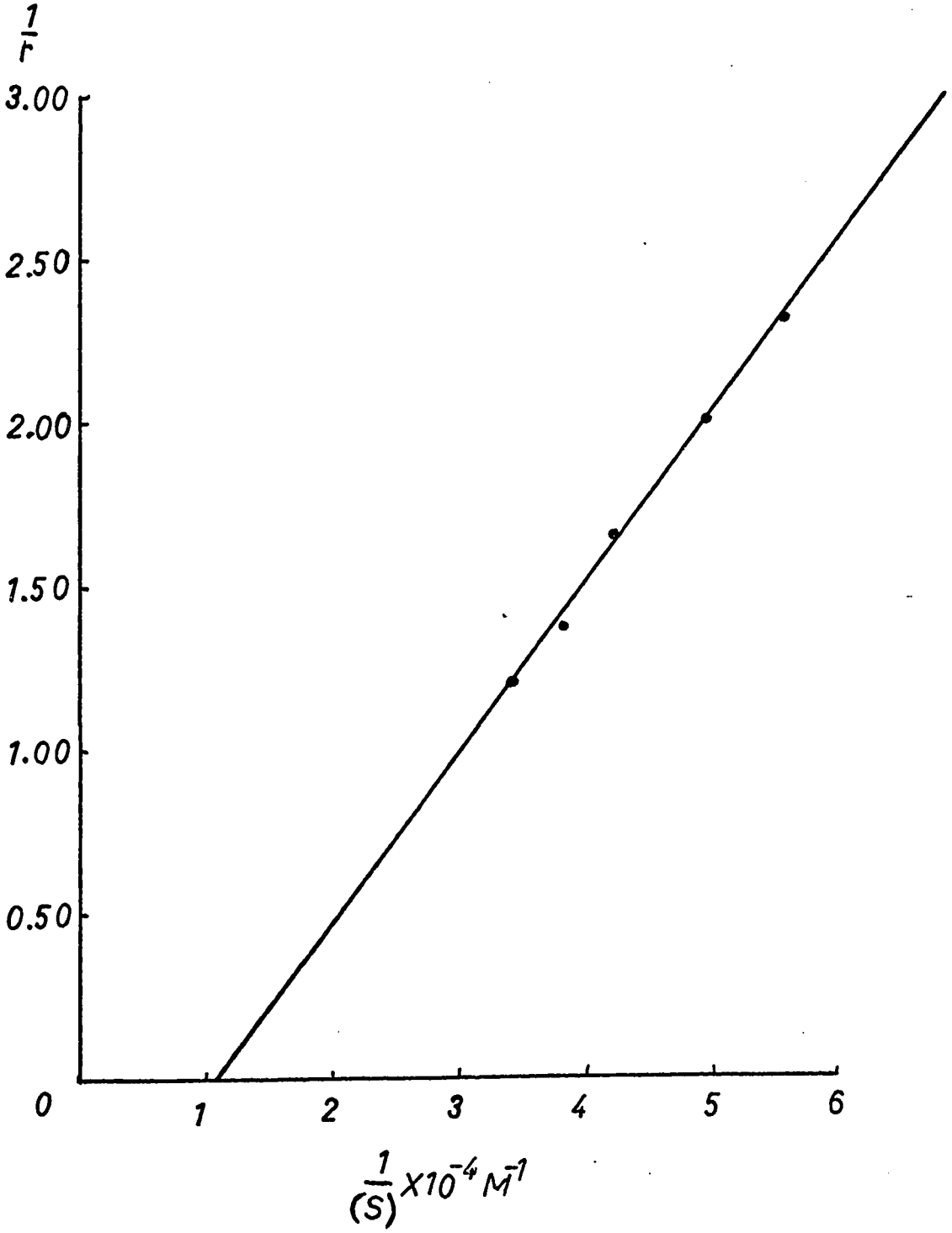
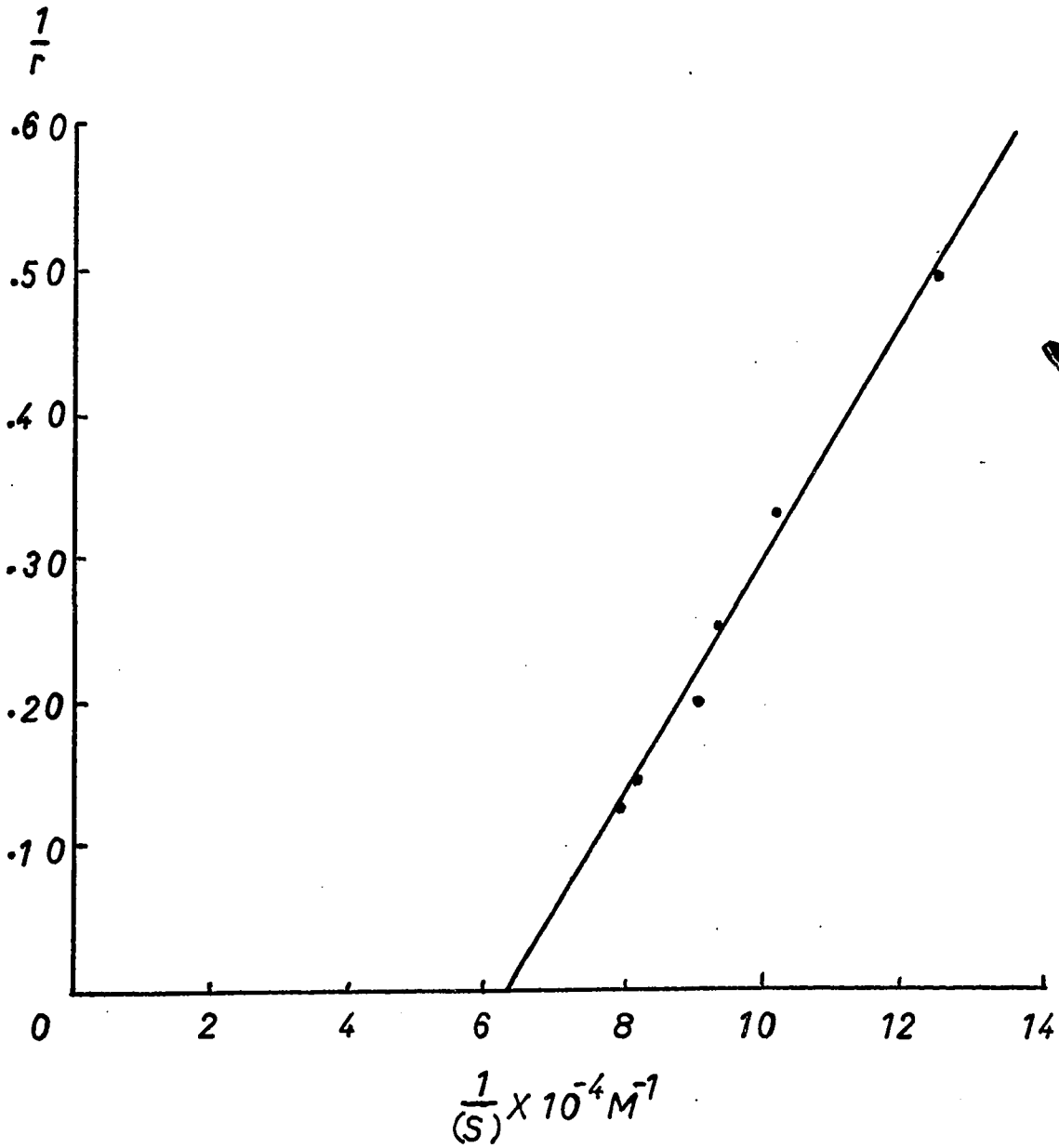


Fig. 9

Reciprocal plot for BSA testosterone binding by equilibrium dialysis. Adapted from the results of Brunkhorst and Hess (35). The albumin conc. was varied from 0.5% to 4.00%, cortisol conc. (constant) = 1×10^{3} umoles/ml.



Section III

The examination of a novel equilibrium ultrafiltration procedure for the measurement of binding parameters of BSA-testosterone interaction.

Part A

I N T R O D U C T I O N

Most of the techniques which have been used for studying steroid-protein interactions have disadvantages. The classical equilibrium dialysis technique, which is considered to be the most accurate and reliable method for such studies, has the disadvantage of requiring 24-72 hours for attainment of equilibrium and the consequent possibility of protein denaturation. Other disadvantages in this procedure are the interference of ultraviolet absorbing material shed by the dialysis membranes, even when using the most careful cleaning and washing, and the possibility of a change in the pore size of the membrane. In the method developed by Pearlman and Crépy (36), which is based on the equilibrium dialysis principle but using sephadex G-25, there are some advantages, e.g. it is not time consuming and thus avoids the possibility of denaturation of the protein and requires only very small amounts of protein and steroid. Despite these advantages, this technique has some limitations not encountered in conventional dialysis with visking membranes, e.g. the internal phase cannot be sampled and there is a discrepancy between the experimental and theoretical values of the partition factor. Westphal (58), in his spectrophotometric technique for studying the interaction of Δ^4-3-ketosteroids with proteins, has claimed to have avoided disadvantages encountered in other methods as e.g., the several hours required

in the classical equilibrium dialysis, the interference of membranes or of a third phase such as of paper in paper chromatography or of solvents in the solvent partition technique. Alfsen (34), using the solvent partition technique for studying testosterone binding to bovine albumin, has found that heptane used as a solvent increased the levo-rotation of the albumin and also produced an increase in the molar ratio of bound steroid.

Recently Attallah and Lata (62) have reported a fluorescence quenching technique and reported some advantages in the use of this technique in studying steroid-protein interaction, e.g. it is a rapid and sensitive method and only microquantities of materials are needed. They have also used the solvent partition method and indicate that their method is preferable because it avoids the problem referred to above of interaction of the solvent with the protein. However, this is an indirect method and since it cannot lead directly to values for r it may lead to erronous conclusions, as is indeed indicated in Section II.

In a non-equilibrium method such as electrophoresis, the interaction between the steroid and the protein is characterized after a greater or lesser distortion from the equilibrium state has been produced. In this the relation-ship between bound steroid and free steroid may be disturbed since the bound steroid moves in a steroid-free medium.

According to Daughaday (53), such a method, although useful in cases where the bond between steroid and protein is as strong as in the case of cortisol and the cortico-steroid binding globulin (transcortin), it is not suitable for the study of weaker protein-steroid interactions.

In the present work the validity of a novel equilibrium ultrafiltration technique for the study of the binding of testosterone to bovine albumin has been investigated for the first time and the results are compared with those obtained with the standard equilibrium dialysis procedure. This technique was made possible by the appearance on the market of a new high flux semipermeable membrane. When a solution containing a ligand is fed continuously through a cell bounded by such a membrane and containing a non-diffusible protein, a curve of ligand concentration in the effluent versus effluent volume can be constructed in a short time. The characteristics of this curve reflect the binding phenomenon.

In the present work, in order to observe the behaviour of the ultrafiltration membrane in the absence of the protein, we have used the following equation for plotting a theoretical dilution curve. Comparison of this curve with an experimental one provides a test as to whether the membrane is behaving ideally or not.

$$C = C_o [1 - (1-\alpha)^n]$$

where C is the ligand concentration for a given effluent fraction.

C_o is the concentration of the feed ligand solution.

α is the fractional volume = $\frac{\text{fraction volume}}{\text{cell volume (65 ml)}}$

n is the fraction number.

For any given steroid feed concentration C_o, a theoretical curve can be constructed by plotting C against n as in fig. 16.

Ultrafiltration procedure :-

This consists of two steps:

a) measurement of an experimental dilution curve which can be obtained by performing an ultrafiltration experiment (see below and fig. 16) in the absence of protein, values for C being obtained from absorbance measurement on the spectrophotometer while values for n are obtained from the number of fractions collected by means of a fraction collector.

b) measurement of the "ultrafiltration curve"; this is obtained by repeating the above experiment in the presence of a protein solution inside the cell. A similar plot of C against n can be constructed, and will be observed to be shifted if binding occurs. This gives not only a very rapid

qualitative demonstration of binding (see fig. 16) but provides also the basis for a quantitative measurement of bound steroid. Fig. 16 shows such a set of curves.

The following equation, derived from a consideration of total steroid fed into the cell, total steroid collected and total unbound steroid in the ultrafiltration cell has been used to calculate total bound steroid at any given fraction number (75).

$$S_b = F\ (C_o) - V\ (C) - A$$

Where S_b total moles of bound steroid at a given value of C

F number of fractions (in ml) collected to this point

V the volume of the ultrafiltration cell

A the area under the curve which represents number of moles of steroid passed out from the cell

C concentration of steroid in the effluent solution

C_o concentration of steroid in the feed solution

See for example Table 14 and fig. 16.

Part B

E X P E R I.M E N T A L

In this section the equilibrium ultrafiltration procedure for the binding of testosterone to the same bovine albumin preparation used in the preceeding section is described in detail, together with a direct comparison of the values for binding parameters as measured in this procedure with those obtained with the equilibrium dialysis technique. Some measurements have also been made using the method of Ryan and Gibbs (77) involving ultraviolet difference spectroscopy.

1- Materials and methods

A schematic diagram of the experimental set up of the apparatus used is shown in fig. 10. The apparatus used consists of a pressurized reservoir (B) made of fiberglass (75) and fitted with a pressure relief valve which opens at a pressure of 115-145 lbs. The reservoir is connected through its inlet by "polyflo" tubing (76) to a nitrogen tank (A) and through its outlet to a model 50 ultrafiltration cell (D) of 65 ml capacity (75) fitted with a pressure releave valve. On the connecting tubing between the reservoir and the ultrafiltration cell there is a needle valve (C) to control the flow to the cell. The ultrafiltration cell is designed to accomodate a 43 mm diameter membrane which is supported by a porous disc.

The cell is fitted with a teflon-coated magnetic stirring bar which barely clears the membrane surface during filtration and with adequate stirring polarization, or "caking", of the high molecular weight solute is prevented. The cell is immersed on its side in a water bath (F) maintained at 22°C and clamped to a submersible magnetic stirring table (G). Early in the present studies, it was found necessary to elongate the filtrate port of the ultrafiltration cell so that it projects above the surface of the water in order to avoid leaking from or to the cell while submersed. For this purpose, a $3\frac{1}{2}$ inch length of stainless steel tubing, 1.2 mm I.D., was fitted to the filtrate port. This stainless steel connection was ground to fit a Touhy luer adaptor (E) connected to 1.2 mm teflon spaghetti tubing. The latter is connected by means of a specially made adaptor (H) to a flow cell (Hellma) (I) of 0.6 ml capacity and 1 cm light path. The outlet of the flow cell was connected by means of teflon tubing to the fraction collector (K) with the aid of another teflon adaptor. The absorbance of the filtrate passing through the flow cell is measured in a Bausch and Lomb Precision Spectrophotometer (J) and recorded on a Sargent model SRL Recorder (L) in log mode. The fractions collected are marked by means of fraction marker pen fitted on the recorder and activated by the fraction collector.

2- Factors considered so as to ensure adherence to the theoretical dilution equation

a) The effect of the reservoir material on the buffer and the steroid used :-

Prior to the performance of actual binding experiments, it was found necessary to check the effect of the reservoir material on the steroid used and also on the phosphate buffer. It appeared also necessary to check whether or not the steroid binds to the reservoir material. For this purpose a definite volume of phosphate buffer was placed in the reservoir and the absorbance was checked at different intervals of time at the wave length used for measuring the steroid concentration. Even with the most exhaustive washing of the reservoir with ethyl alcohol followed by repeated washing with distilled and deionized water and finally with buffer, it was found that the absorbance of the phosphate buffer in the reservoir was significantly increased. When testosterone in the same buffer was introduced in the reservoir and the absorbance at 249 mμ checked at intervals, it was observed that the reservoir material binds the steroid and that the binding increased with time. It was then decided to use a glass bottle of 375 ml capacity which was placed inside the reservoir, Later, when a larger volume of the feed solution was needed, polyethylene bags were used instead of the glass bottle. These bags, when checked for the

possibility of leaching ultraviolet absorbing material or for steroid binding, were found to be inert over a period of 24 hours. The absorbance of the steroid solution in the bag was checked at the end of every experiment and was found to be exactly the same as at the beginning.

b) Ultrafiltration membranes used :-

The ultrafiltration membranes, ultrafiltration cell and the reservoir, were obtained from the Amicon Corp., Cambridge, Mass. Two kinds of membrane were tested in the present work; Diaflo XM-50 and UM-10. In addition some work was done on the PM-10, which has become available during the progress of this investigation.

i) Membrane Retentivity

We decided to examine the possibility of using the UM-10 and XM-50 membranes. The nominal cut-off value of UM-10 (10,000) seemed to make it the membrane of choice. While retentivity tests, in which the effluent from a buffer washed protein solution is monitored (fig. 13), confirmed the results of Blatt et al (78) in this respect, we encountered other difficulties discussed below which precluded its use without further investigation. The cut-off value of the XM-50, which is designated as 60,000 (79), has been found to vary from one batch of membranes to another (fig. 13). In most of the measurements reported in the present work

it was found that, when the ultrafiltration cell was filled with a solution of BSA and buffer fed under 50 lbs pressure from the reservoir, a small amount of ultraviolet absorbing material came out of the cell in the first few fractions and the monitored absorbance then fell to a very low constant value slightly above the instrumental base-line. In some cases, however, there was a considerable leakage through the membrane. Nevertheless it seems still possible to make reliable measurements in such cases, since indications are that corrections for protein thus lost from the cell can readily be made. However, the binding values reported here have been obtained with membranes exhibiting only slight leakage. The results of the preliminary investigation of the PM-10 membrane shown in fig. 11 suggest that it may indeed be the most suitable one for studies with steroids and serum albumin since it has the advantages of low cut-off value (10,000) and of also being inert with respect to the steroid binding as is mentioned below.

ii) Membrane Binding

It was necessary to establish that the steroid used (in our case testosterone) does not bind to the ultrafiltration membranes. Accordingly it was decided to check the binding of testosterone to each of the two kinds of membrane. A new XM-50 and an UM-10 membrane were each put in a beaker containing 32 ml of testosterone of absorbance 0.545 at 249 mμ. The two

beakers were covered and occasionally shaken. Table 11 (a) shows the absorbance of the steroid solution from the two beakers at different time intervals. The binding of testosterone to the two kinds of membrane was also checked using testosterone-4-C^{14}. A volume of 32 ml of testosterone-4-C^{14}, of absorbance 0.850 at 249 mμ, was placed in each of two beakers, one with an XM-50 membrane and the other with a UM-10 membrane. The beakers were covered and occasionally shaken. At the end of the indicated time intervals, 0.5 ml of each of the steroid solutions was withdrawn and counted in a liquid scintillation counter. The results are shown in Table 11 (b). It can be seen that the XM-50 membrane does not significantly bind testosterone and hence could be used for further investigation. The UM-10, on the other hand, exhibits marked binding activity and was not suitable for use without further evaluation of its binding properties. This membrane was investigated further in the hope that suitable blanks for membrane binding could be constructed since the cut-off value of this membrane is more suitable for the study of steroid binding to serum albumin than that of the XM-50.

The possibility of the ligand binding to the two kinds of membrane has also been examined directly by comparing theoretical and experimental dilution curves (figs. 11 & 12). These results also indicate the absence of significant binding

of testosterone to the XM-50 membrane. This also seems to be true in the case of the PM-10 (fig. 11). This figure, however, also indicates that the UM-10 membrane binds not only testosterone but also cortisol.

iii) Membrane Rejection

The binding behaviour of the two kinds of membrane was also examined by performing some experiments where a steroid solution was perfused from the reservoir through the ultrafiltration cell which was filled with the same steroid solution. In the case of the UM-10 membrane, we encountered in this way not only the phenomenon of steroid binding to the membrane but also that of the rejection of the steroid by the membrane. This has been also observed by Blatt et al (78) in the case of methyl orange. This is illustrated in fig. 14 for the results of an experiment where a testosterone solution of absorbancy 0.700 at 249 mμ was placed both in the reservoir and ultrafiltration cell and the absorbancy of both cell contents and effluent was monitored over a 100-fraction period. The absorbancy of the cell contents is higher at all points than that of the effluent, and terminates in a stable value above that of the feed solution. In the case of XM-50, on the other hand, it was found that the steroid is only insignificantly bound to the membrane at the beginning of the experiment until a stage is quickly reached after which the absorbance of the effluent solution becomes exactly the

same as that of the feed solution and then stays constant for a period of time as long as ten hours. Simultaneous measurement of effluent concentration and the concentration inside the cell have shown that they are exactly the same during the whole time period with this membrane. It would seem still possible to use the UM-10 membrane for binding measurements since the values for the reflection coefficient which, as listed in Table 12, are seen to be reasonably constant for varying testosterone concentration. This would require applying the appropriate corrections both for the phenomenon of rejection and that of steroid binding, based on the constancy of these values. In the present work the UM-10 membrane has been used in some experiments employing different protein and steroid concentrations. The results of these experiments are expressed without applying corrections and in all cases good reciprocal plots were obtained. Moreover these plots exhibited the same value of 5 for n which has been obtained in all experiments reported in the present studies, using either the classical equilibrium dialysis technique or the equilibrium ultrafiltration procedure employing the XM-50 membranes. On the other hand, the values for nk obtained using the UM-10 membrane were always much higher than those obtained using the XM-50 membranes, as for example in the results of fig. 15 showing one of the experiments using the UM-10 membrane for the binding of testosterone to 69.9 μM BSA. Further investigation is thus necessary to

provide the basis for applying appropriate corrections for binding and rejection in the case of UM-10. For this reason, we decided to concentrate our studies on the use of the XM-50 membrane.

c) Volume of cell contents and void volume :-

In order to avoid fluctuations of the cell volume, the cell was filled with 65 ml of buffer or protein solution. All measurements reported here were based on experiments which have been referred to by Blatt et al (78) as the "Wash-in" type, in which the sign of change in [C] was positive. Corrections for the "Void Volume" were made by extrapolating the initial linear portion of the dilution and ultrafiltration curves back to the absorbancy base-line of the recorder chart. The apparent void volume obtained in this way differed only slightly from the measured value of 3.2 ml in the case of the XM-50 and PM-10 membranes, whereas with the UM-10 there was an increase in the void volume indicating significant binding and also membrane rejection (see fig. 11).

d) Checking the whole apparatus for binding of the steroid and for leakage of the ultrafiltration cell :-

It was also found necessary to check whether the steroid binds to the connecting tubing or to the inner wall of the ultrafiltration cell which might contribute to the void

volume observed. This could be tested by the procedure described previously in which a testosterone solution of known absorbance was perfused from the reservoir to the ultrafiltration cell containing the same steroid solution and was then passed to the flow cell in the spectrophotometer and the absorbance was recorded on the recorder at 249 mμ for a period of ten hours. It was found that the absorbance was constant over this period of time. The same experiment was repeated three times while immersing the ultrafiltration cell completely on its side in a water bath at 22°C, to check if there was any leakage from or to the cell. As has been mentioned earlier, it was found necessary to attach the stainless steel tubing to the filtrate port of the ultrafiltration cell in order to avoid the entry of the water on the effluent side of the cell. With this attachment, it was found that the absorbance of the solution perfused through the cell immersed in water was constant over a period of as long as ten hours, when using the XM-50 membrane.

e) Fraction volume :-

Early in the present work it was observed that the fraction cutting by means of the drop counter was not precise, due either to drops adhering to the drop counter head of the fraction collector or drops not being counted. This caused a significant error in the fraction volume, an error which accumulated as the number of fractions collected increased and

consequently lead to great errors in the calculation. This has been eliminated by discarding the drop nozzle from the drop counter head of the fraction collector and carefully centering the end of the capillary teflon tubing coming from the flow cell outlet above the photocell so that drops are counted without touching the drop counter head. By doing this, the adherence of the drops to the drop counter head and the consequent error in counting was avoided. The time-flow collection could not be used due to variation in the flow rate. It was necessary to determine the fraction volume with a high degree of accuracy in several experiments. The results in Table 13 illustrates one of the experiments performed for such a determination. In this 168 drops were collected in each fraction since it was found that when the drop selector was set at 170 drops this number was collected only in the first fraction and succeeding fractions consisted of 168 drops. Two drops were missed as the tube-rack moves from one fraction to the other. The value of the fraction volume used in calculation in all experiments was 5.41 ml with 1.1% standard error.

3- Procedure

In view of the results quoted above, the XM-50 membrane, which has a cut-off value of 60,000, was chosen in the present work in the evaluation of a proposed equilibrium ultrafiltration

procedure for the determination of binding parameters for BSA-testosterone interaction. For this purpose the ultrafiltration cell was filled with 65 ml of phosphate buffer pH 7.6, μ 0.1, and the reservoir was filled with testosterone in the same buffer. In connecting the tubing from the reservoir to the ultrafiltration cell the steroid solution was allowed to fill the whole length of the tubing before attachment to the inlet of the ultrafiltration cell. The volume between the supporting disc in the cell and the end of the spaghetti tubing leading to the fraction collector was ensured to be empty so that the first fraction collected is the actual first fraction coming out from the ultrafiltration cell. Measurement of the experimental dilution curve was performed by opening the needle valve. From 70 to 100 fractions were collected in a period of 8-10 hours, a time in which the concentration of the solution in the effluent reaches that of the feed solution. In order to ensure the zero stability of the spectrophotometer and the recorder, a blank cell containing buffer as well as another cell containing a solution of known testosterone concentration were mounted with the flow cell in the Fisher cell carrier of the spectrophotometer. The programmer was set to cycle between the three cells so as to give a long dwell time for the flow cell and a short dwell time for the others. In almost all the experiments reported here, it was found that the zero was remarkably stable over the whole length of time

required for completion of the experiment. The ultrafiltration curve is obtained in a similar way to that mentioned above. The ultrafiltration cell is first filled with the protein solution and the reservoir and connecting tubing filled with buffer. The protein is washed till the absorbance of the effluent reaches a low constant value very close to the base-line. This usually takes 5-10 fractions in case of the membrane that behaves 'ideally'. In cases where there was significant protein leakage the membrane was rejected. The pressure is released and the contents of the reservoir and the connecting tubing to the ultrafiltration cell is exchanged for the testosterone solution. Ultrafiltration is then initiated and continued until the concentration in the effluent reaches a value equal to or very close to that of the feed solution.

Measurement of binding parameters :-

When it became possible to eliminate difficulties which were encountered in attempting to set up the ultrafiltration procedure (i.e. the fraction volume, leakage of the ultrafiltration cell under water, binding of the steroid to the reservoir material, the ultraviolet absorbing material shed by the inner walls of the reservoir before employing the polyethylene bags and the behaviour of the membrane towards the steroid), experiments were carried out employing the same albumin preparation used in the preceding section, where binding constants has already been determined by the equilibrium

dialysis method. Albumin concentrations similar to those used in the equilibrium dialysis experiments were used. The measurements of areas required for determination of bound steroid S_b and values for r has been made using a Gelman planimeter and also by the weighing method using graph paper. For this purpose, rectangles of different areas, cut by a sharp knife from different parts of the graph paper, have been accurately weighed and the mean weight per square inch determined. This process has been repeated occasionally to check the uniformity of thickness of the graph paper and was always found to agree well with that using the planimeter. In fact, it was much simpler and more accurate - particularly with very small areas.

For the sake of clarity the following details of a typical equilibrium ultrafiltration experiment are given as a model (see Table 14 and fig. 16).

Membrane used	Diaflo XM-50
Bovine Albumin	Armour lot T 68204 Conc. used 3.55×10^{-5} M; "as measured by absorbance at 279 mμ".
Testosterone	Conc. of feeding solution; 3.318×10^{-5} M (absorbance 0.521 at 249 mμ).
Buffer	The protein and the steroid solutions were made with phosphate buffer pH 7.6, μ 0.1
C_o	33.18×10^{-3} μmole/ml

C	was measured at fractions :- 10, 20, 30, 40, 50, 60, 70
Fraction Volume	5.41 ml
A	was represented by :- A_1, A_2, A_3, A_4, A_5, A_6, A_7

Where ,

$A_1 = a_1$

$A_2 = a_1 + a_2$

$A_3 = a_1 + a_2 + a_3$ and so on.

Moles of protein in the ultrafiltration cell	35.50 x 65 = 2307.5 nanomoles

4- Spectrophotometric method

In the present work the difference spectroscopy method of Ryan and Gibbs (77) has been used as a tool for comparing the testosterone binding properties of an albumin solution which has been continuously perfused in an ultrafiltration set-up with phosphate buffer with a freshly prepared solution of the same concentration.

All absorbance values were determined at room temperature in a Bausch and Lomb Precision Spectrophotometer. Depression of absorbance at 259 mμ was measured using tandem cell technique (25) and employing rectangular cells of 4.5 mm light path per chamber. This permits the most precise measurement of the change at 259 mμ, since total steroid and protein concentration are the same in both the reference and the

sample cells. The cells were cleaned before and between measurements by soaking in a mixture of concentrated nitric and sulfuric acid (3:1), washing with distilled and deionized water. They were then washed with alcohol and dried in the oven under vacuum (25). The concentration of both untreated albumin and that which had been washed in the ultrafiltration cell was adjusted so that on dilution it was 3.55×10^{-5} M. In the reference cell the steroid and the albumin solutions were mixed in one compartment and the other compartment contained only buffer. In the sample cell the steroid and the albumin solutions were in separate compartments but at the same concentrations as in the reference cell. Four testosterone concentrations were used in the case of both albumin samples. These concentrations are shown in Table 15. Composition of solutions in both the reference and sample cells are shown in Table 16.

5- Results and Discussion

Figure 18 represents a reciprocal plot of data shown in Table 14 for protein concentration of 3.55×10^{-5} M. This gives values for nk and n of 3.68×10^{4} and 5 respectively. When these values are compared with those obtained from the equilibrium dialysis experiments in the preceding section for the same albumin preparation at the same protein concentration, it can be seen that despite the good linearity obtained by the two procedures the value for nk is almost 25% higher in the

ultrafiltration method than that obtained by the equilibrium dialysis procedure (2.97×10^4). The plots together with the agreement in values for n seem to establish the validity of equilibrium ultrafiltration as an experimental procedure for measuring binding parameters, but of course raise the question of the significance of the higher values for nk. In view of the results in section II, it was decided to perform equilibrium ultrafiltration experiments at different protein concentrations, but still comparable to those used in the preceding section. Before doing this, it was found necessary to check the reproducibility of the data obtained by this procedure. For this purpose, it was decided to perform three ultrafiltration experiments at the same albumin concentration of 6.99×10^{-5} M and using a testosterone feed solution of 5.01×10^{-5} M in phosphate buffer pH 7.6, μ 0.1. These results are shown in fig. 19 and exhibit values of 3.29, 3.28 and 3.22 for nk and a value of 5 for n. This is an example of the remarkable precision possible under ideal conditions with this method. Comparison with the value of nk obtained by the equilibrium dialysis method for the same protein concentration (fig. 4) reveals again that it is significantly higher than that for the latter (2.39).

It was then decided to make a more comprehensive study over a wider protein concentration range (2 to 16 $\times 10^{-5}$ M). These results, presented in Table 17 and figs 17-21, reveal not only the same dependency of nk on protein concentration

already observed when using the equilibrium dialysis technique but also that the equilibrium ultrafiltration values are consistently higher than those for equilibrium dialysis at all protein concentrations used in these studies. However, it is significant that, as can be seen in Table 17, that the value of n does not change. One can also represent the values for a given protein concentration by means of a Scatchard plot as in fig. 22 for 2.00×10^{-5} M bovine albumin. However, when one selects values for r and s from equilibrium ultrafiltration experiments performed at different protein concentrations and taken at approximately the same free steroid concentration and plots them in a Scatchard plot, one obtains the effect seen in fig. 23 from data tabulated in Table 18. This effect is analogous to that of fig. 7 obtained from equilibrium dialysis experiments. Thus the data represented both by the Scatchard and reciprocal plots support the suggestion made in section II that the binding affinity for BSA-testosterone interaction depends on protein concentration. This is best represented, as also seen in the preceding section, by reciprocal plots for data obtained at a fixed protein concentration and varying steroid concentration, each of which gives very good plot but leads to decreasing values of nk when the protein concentration is increased from one experiment to another.

The significant fact that the value of n is the same in all experiments using both procedures lends further support to the claim that this new procedure is a valid technique for

rapid and convenient measurements of binding parameters. However, the reason for the higher values of nk obtained using this method remains unexplained. It may be noted that a similar behaviour was exhibited by a different batch of albumin (Pentex, figs 24 and 25). There are at least three possible explanations for this dependency; first, that the membrane properties change in the presence of the protein. Although this has not yet been tested it seems that it is unlikely since increasing protein concentration leads to decreasing binding values as is also the case in the use of the equilibrium dialysis method. Secondly, that the conditions of equilibrium ultrafiltration (such as, for example, the continuous perfusion with stirring of the steroid to the ultrafiltration cell) change the binding properties of the protein. Thirdly, that the values for nk obtained by the classical equilibrium dialysis are lower than the true values.

In a tentative attempt to test for the second possibility, it was decided to carry out an equilibrium dialysis experiment using protein solution which had been continuously perfused with phosphate buffer and stirred for a period of 12 hours in the ultrafiltration cell. The albumin solution was taken from the diaflo ultrafiltration cell at the end of such perfusion and its concentration, as determined from its absorbance at 279 mμ, was found to be 5.40×10^{-5} M. An equilibrium dialysis experiment was then performed with this concentration of the albumin solution in the usual manner, using four concentrations of

testosterone-4-C^{14}. Equilibration was performed for the usual period of 48 hours at constant temperature in a shaking water bath, taking all precautions as to keep the system free of mold and using gentle shaking. The results of this experiment are shown in Table 19 and fig. 26, where it can be seen that the percentage of bound steroid was considerably decreased to a value of about 24 as compared to a value of 47 obtained in other equilibrium dialysis experiments. This was accompanied by a decrease in the value of nk to 0.60×10^4 M^{-1}. This is in surprising contrast to the high values obtained above. Other experiments performed on albumin which was merely washed by dialysis, using repeated changes of phosphate buffer at $4^{o}C$, revealed no change in the binding parameters (fig. 5). These results seem to suggest that the conditions of equilibrium ultrafiltration lead to a change in the protein molecule rendering it susceptible to denaturation in subsequent equilibrium dialysis rather than to an increase in the binding affinity, but leave unanswered the question of the significance of the higher values obtained for nk in the actual equilibrium ultrafiltration experiments. Further work would be necessary to elucidate the precise reason for the change, but it is likely that it is related to the stirring conditions and temperature of the experiments. It is possible that it might not be observed at lower temperature. The phenomenon has not been reported by Blatt et al (78) in their studies of the binding of methyl orange and calcium to human serum albumin.

Some tentative attempts have been made to verify the binding behaviour of the protein solution which was washed in the ultrafiltration cell with phosphate buffer and stirred for 12 hours by means of a procedure which avoids the possibility of denaturation on further shaking. For this purpose, it was decided to use difference spectroscopy technique developed by Ryan and Gibbs (77). These authors have re-examined the spectrophotometric method of Westphal (58) and established that if measurement of absorbance depression are made by tandem cell technique at 259 mμ, where a difference peak reflecting the actual phenomenon of binding of certain steroids is observed, there is a linear relationship between ΔA and r. Thus, both an untreated albumin sample as well as one which has been subjected for ten hours to the conditions of the ultrafiltration technique were examined by means of the above difference spectroscopy procedure. These results of the two experiments are shown in Table 20 and represented in fig. 27, where they are expressed by plotting values for ΔA_{259} mμ against values for r in the case of both the washed and unwashed protein. The values of r under these conditions were obtained by applying values for percentage of total steroid which is bound, derived from equilibrium dialysis experiments under the same conditions of total steroid and unwashed protein.

The data obtained from the results of these two experiments tentatively show that, within the experimental error, the

value of the albumin solution which has been washed in the ultrafiltration cell does not differ appreciably in its binding behaviour from the freshly prepared albumin solution. Thus, these preliminary attempts to explain the difference in binding values obtained using the two procedures do not definitely answer the question raised regarding the significance of the higher nk values obtained with the equilibrium ultrafiltration procedure, but do tend to suggest that the third alternative may be correct i.e., the equilibrium dialysis values are lower than the true values and that the values obtained by this new procedure are in fact the correct values and reflect the higher degree of accuracy attainable by the equilibrium ultrafiltration technique.

While the results of this section tend to establish the validity of the equilibrium ultrafiltration procedure as a simple rapid means of measuring binding parameters, its applicability for the study of steroid-protein interactions requires further study, particularly as to the nature of the apparent change in the protein structure which is produced and of possible means of preventing it. There is one further disadvantage which has introduced considerable difficulty into the execution of the work reported here. The above results were obtained with XM-50 membranes which behaved "ideally". Approximately 50 per cent of the membranes received from the company were useless, either because they were completely

"plugged" or else because the cut-off value was much higher than the nominal, resulting in leakage of the protein through the membrane, although, as suggested, the latter difficulty can probably be overcome.

Further studies are necessary to investigate the behaviour of the PM-10, since preliminary testing showed that it neither binds nor rejects testosterone, the possibility of applying appropriate correction to the case of the UM-10 and also the behaviour of other steroids and proteins in the case of these and other membranes.

Part C

G E N E R A L C O N S I D E R A T I O N S

The use of an equilibrium ultrafiltration technique for the measurement of values for nk and n for two preparations of bovine albumin has been described. It has been found that the values of nk as determined by this procedure were consistently higher than those obtained using the classical equilibrium dialysis method at all protein concentrations employed. It was interesting to find that the use of this method also demonstrates that same dependence of binding affinity on protein concentration which has been shown, in the second section, by means of the equilibrium dialysis procedure. It is also significant to note that the value for n obtained by the equilibrium ultrafiltration procedure is the same in all experiments and equal to that obtained using the equilibrium dialysis method.

Both reciprocal and Scatchard plots obtained from the results of the experiments performed by this technique were excellent. Moreover, the technique is characterized by its extreme simplicity, requiring only a few hours to obtain a complete binding curve, from which several values for the bound and free steroid can be obtained. Consequently binding constants can be obtained with great ease. It must be emphasized, however, that careful attention to technical details is necessary as e.g., the value of fraction volume

collected, the binding of the ligand to the reservoir material or to the ultrafiltration membranes. The elimination of these technical problems, together with the proper selection of the membranes, can lead to excellent results. Its use for accurate quantitative measurements needs further investigations. The very low nk value obtained from the results of the equilibrium dialysis method applied to the albumin solution which had been subjected to conditions of ultrafiltration in the absence of steroid appears to be due to the instability of the modified protein. On the other hand the few results obtained from the use of the spectrophotometric method tend to show that there is no loss in binding affinity at the end of the ultrafiltration period, in spite of the marked drop during the subsequent 48 hour dialysis.

Table 11

Binding of testosterone to the XM-50 and UM-10 membranes

(a)

Absorbance of a 34.4 μM testosterone solution (32 ml), containing the XM-50 or UM-10 membrane

Time	Absorbance at 249 mμ	
	XM-50	UM-10
0	0.545	0.545
3 hr	0.524	0.329
4.5 hr	0.522	0.269
22 hr	0.522	0.235
24 hr	0.522	0.230
28 hr	0.522	-----

(b)

Radioactivity of a 54.2 μM testosterone-4-C^{14} solution (32 ml) containing either an XM-50 or UM-10 membrane

Time	c.p.m. per $\frac{1}{2}$ ml	
	XM-50	UM-10
0	1481	1481
0.5 hr	1475	1264
1.5 hr	1467	1206
4.0 hr	1448	1174
10.0 hr	1440	1167

Table 12

Membrane rejection of testosterone and cortisol by the UM-10 membrane, based on simultaneous measurement of effluent (C') and cell (C'') concentration

Feed Conc.	(C')	(C'')	C''/C'	Reflection Coefficient ($\alpha = 1 - \frac{C'}{C''}$)
		Testosterone		
55.4 μM	54.5 μM	66.3 μM	1.20	0.166
54.7 μM	53.0 μM	66.9 μM	1.26	0.207
41.4 μM	41.7 μM	48.1 μM	1.15	0.131
44.6 μM	36.9 μM	47.2 μM	1.27	0.211
44.6 μM	38.3 μM	49.6 μM	1.29	0.230
44.6 μM	40.8 μM	52.2 μM	1.28	0.218
44.6 μM	42.0 μM	53.2 μM	1.27	0.209
44.6 μM	43.6 μM	53.9 μM	1.23	0.186
	43.6 μM	54.4 μM	1.25	0.200
44.6 μM	44.6 μM	54.7 μM	1.23	0.186
		Cortisol		
54.6 μM	54.5 μM	79.2 μM	1.36	0.265
	59.5 μM	83.6 μM	1.40	0.285

Table 13

Values from one of the experiments performed for the determination of the mean value for the fraction volume

Fraction number	Weight of 168 drops	Difference from mean fraction value	$(\text{difference})^2 \times 10^{-3}$
1	5.507	+.094	8.836
2	5.458	+.045	2.025
3	5.464	+.051	2.601
5	5.459	+.046	2.116
10	5.499	+.086	7.396
15	5.494	+.081	6.561
20	5.437	+.024	0.576
25	5.489	+.076	5.776
30	5.402	+.011	0.121
35	5.415	+.002	0.004
40	5.397	-.016	0.256
45	5.373	-.040	1.600
50	5.360	-.053	2.809
55	5.375	-.038	1.444
60	5.388	-.025	0.625
65	5.312	-.101	10.201
70	5.347	-.066	4.356
75	5.350	-.063	3.969
80	5.335	-.078	6.084

Mean value for fraction volume = 5.413 ml

Per cent error = 1.099

Table 14

BSA-testosterone binding by equilibrium ultrafiltration
Concentration of BSA = 3.55×10^{-5} M
Concentration of testosterone feed solution = 3.318×10^{-5} M
Phosphate buffer pH 7.6, μ 0.1, 22°C - XM-50 membrane

Fraction No.	Effluent volume (F) ml	Unbound steroid ("S") µmoles	Area A			FC_o µmoles	VC µmoles	Bound[a] steroid µmoles	r[b]	1/r	$\frac{1}{(S)} \times 10^{-4}$ M^{-1}
			in gm.	in Sq "	µmoles in..						
10	54.1	10.44	0.0450	0.6206	0.2757	1.7917	0.6789	6.8371	0.363	2.757	9.57
20	108.2	18.50	0.1690	2.3310	1.0349	3.5834	1.2006	1.3479	0.584	1.712	5.41
30	162.3	23.25	0.3460	4.7724	2.1188	5.3752	1.5111	1.7453	0.756	1.322	4.32
40	216.4	27.00	0.5625	7.7586	3.4445	7.1669	1.7554	1.9670	0.852	1.173	3.70
50	270.5	29.04	0.7950	10.9655	4.8684	8.9586	1.8878	2.2024	0.954	1.048	3.44
60	324.6	30.57	1.0480	14.4551	6.4180	10.7503	1.9872	2.3451	1.016	0.984	3.27
70	378.7	31.59	1.3065	18.0206	8.0013	12.5420	2.0534	2.4873	1.078	0.927	3.16

a = FC_o - VC - A

b = Moles steroid bound per mole protein

Table 15

Testosterone concentration used in the spectrophotometric method

Sample Number	Untreated albumin	Washed albumin*
	x 10^5 M	
1	1.415	2.290
2	4.680	2.720
3	6.096	3.100
4	8.424	4.930

* Perfused for ten hours with phosphate buffer, pH 7.6 μ = 0.1, in the ultrafiltration cell in a water bath of 22°C.

Table 16

Composition of the mixtures in the reference and the sample tandem rectangular cells used in the spectrophotometric method

Compartment	Reference cell	Sample cell
Front	2 ml albumin soln. 2 ml testosterone soln.	2 ml albumin soln. 2 ml buffer
Back	Buffer	2 ml testosterone soln. 2 ml buffer

Table 17

Values for nk and n for the interaction of testosterone and BSA as measured by equilibrium ultrafiltration and equilibrium dialysis. Each experiment was performed with a fixed albumin concentration.

Concentration of BSA (μM)	Equilibrium Ultrafiltration (XM-50 membrane)			Equilibrium Dialysis		
	n	10^{-4} x nk	fig.	n	10^{-4} x nk	fig.
4.38 (Armour)	-	---	-	5	4.71	1
8.98 (Armour)	-	---	-	5	3.06	2
20.00 (Armour)	5	3.87	17	-	---	-
35.50 (Armour)	5	3.68	18	5	2.97	3
54.00 (Armour)[a]	-	---	-	5	0.60	26
69.90 (Armour)	5 5 5	3.28 3.29 3.22	19 19 19	5	2.39	4
69.90 (Armour)	5	4.37[b]	15	-	---	-
97.20 (Armour)	-	---	-	5	1.97	5
107.20 (Armour)	-	---	-	5	1.96	6
114.20 (Armour)	5	3.11	20	-	---	-
142.00 (Armour)	5	2.84	21	-	---	-
52.00 (Pentex)	5	2.50	25	-	---	-
71.00 (Pentex)	-	---	-	5	1.85	24

a Sample had first been subjected to conditions of equilibrium ultrafiltration for 12 hours, using buffer instead of a steroid feed solution.

b based on values obtained with the use of UM-10 membrane without correction for membrane rejection.

Table 18

Values for r and r/(S) for BSA-testosterone binding obtained from the results of different equilibrium ultrafiltration experiments. These values are taken at approximately the same concentration of free steroid and are represented graphically by means of the Scatchard plot in fig. 23.

Protein conc.	Free steroid conc. x 10^5	r	r/(S)
2.00×10^{-5} M	2.85	0.971	3.41
3.55×10^{-5} M	2.70	0.852	3.15
6.99×10^{-5} M	2.68	0.752	2.80
14.20×10^{-5} M	2.54	0.617	2.42

Table 19

Results of an equilibrium dialysis experiment performed for 48 hours at 25°C, with 5.40×10^{-5} M albumin sample which was previously washed with phosphate buffer pH 7.6, $\mu = 0.1$, in the ultrafiltration cell for ten hours at 22°C.

Testosterone conc. used	Free Steroid conc.	Bound Steroid conc.	r	1/r	$1/(S) \times 10^{-4}$ M^{-1}	Percent steroid bound
x 10^5 M						
2.516	1.686	0.535	0.099	10.10	5.93	24
4.522	2.773	0.841	0.155	6.42	3.60	23
6.854	4.429	1.355	0.250	3.98	2.25	23
8.471	5.658	2.270	0.420	2.38	1.76	28

Table 20

Values for ΔA_{259} mμ obtained by the spectrophotometric method for a fresh bovine albumin sample and for a sample which was previously washed with phosphate buffer in the ultrafiltration cell for ten hours at 22°C. Values for r were calculated from equilibrium dialysis results for unwashed protein.

	Fresh albumin 35.5 μM BSA				Albumin sample which was washed and stirred in the ultrafiltration cell. 39.4 μM BSA			
	I	II	III	IV	I	II	III	IV
ΔA_{259} mμ	.038	.120	.158	.188	.050	.075	.063	.125
r	.187	.632	.810	1.29	.270	.324	.370	.588

Equilibrium ultrafiltration assembly . A nitrogen cylinder (A) supplies pressure to a reservoir (B) containing steroid solution which is fed through a needle valve (C) to an ultrafiltration cell (D) immersed in a water bath (F) and clamped to a magnetic stirrer (G). The effluent is fed through a Touhy luer adapter (E) and teflon spaghetti tubing to a flow cell (I) in a spectrophotometer (J) and thence to a fraction collector (K). Both fraction cutting and effluent concentration are recorded on the recorder(L).

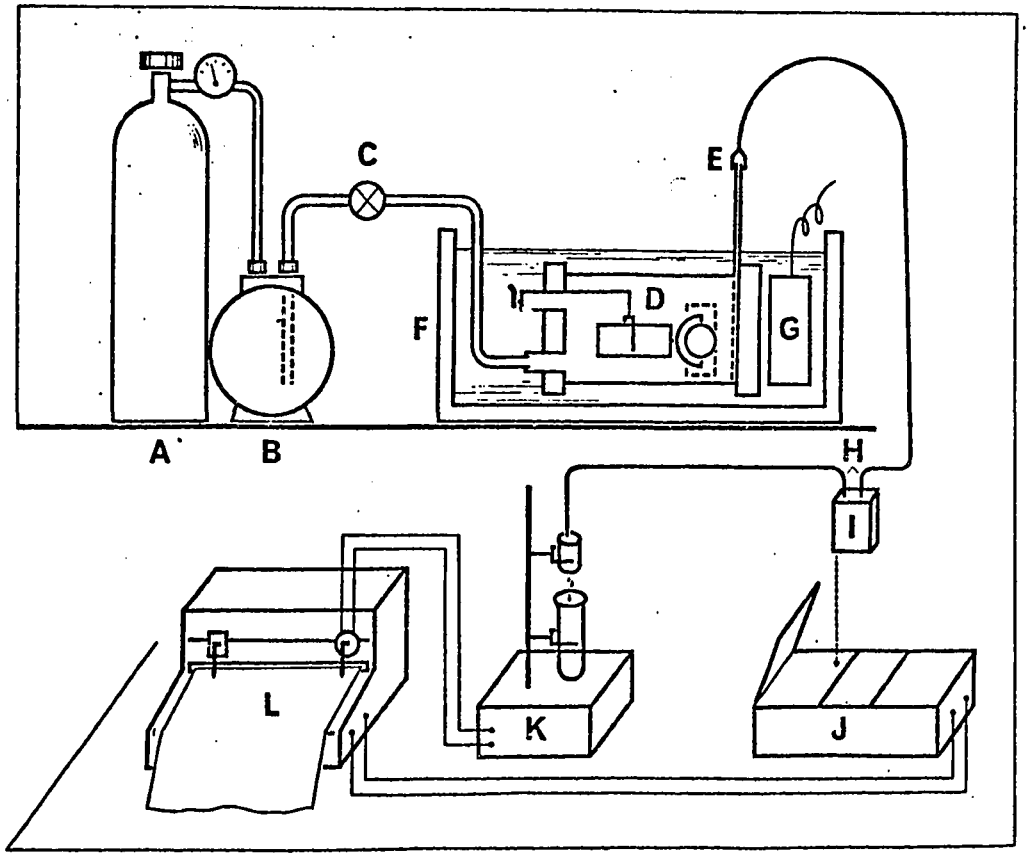


Fig. 11

Comparison of theoretical and experimental curves for UM-10 , PM-10 and XM-50 membranes. ◘, theoretical dilution curve for 59.2 uM testosterone solution (A_{249} = 930 ; as on fig. 12) ▲ , experimental dilution curve for this solution using XM-50 (as on fig. 12) , ▲, experimental dilution curve for 59.2 uM testosterone using PM-10 , ○ , experimental dilution curve for a 60.5 uM testosterone solution using the UM-10 , ● , experimental dilution curve for a 54.7 uM testosterone (A_{249} = 0.860) using the UM-10, ■ , experimental dilution curve for cortisol solution (A_{249} = 0.860) and the UM-10, ◘ curve for ultrafiltration of 54.7 uM testosterone through 69.9 uM BSA using the UM-10, , ◆ curve for ultrafiltration of 54.7 uM testosterone through 14.2 uM BSA using the UM-10 .

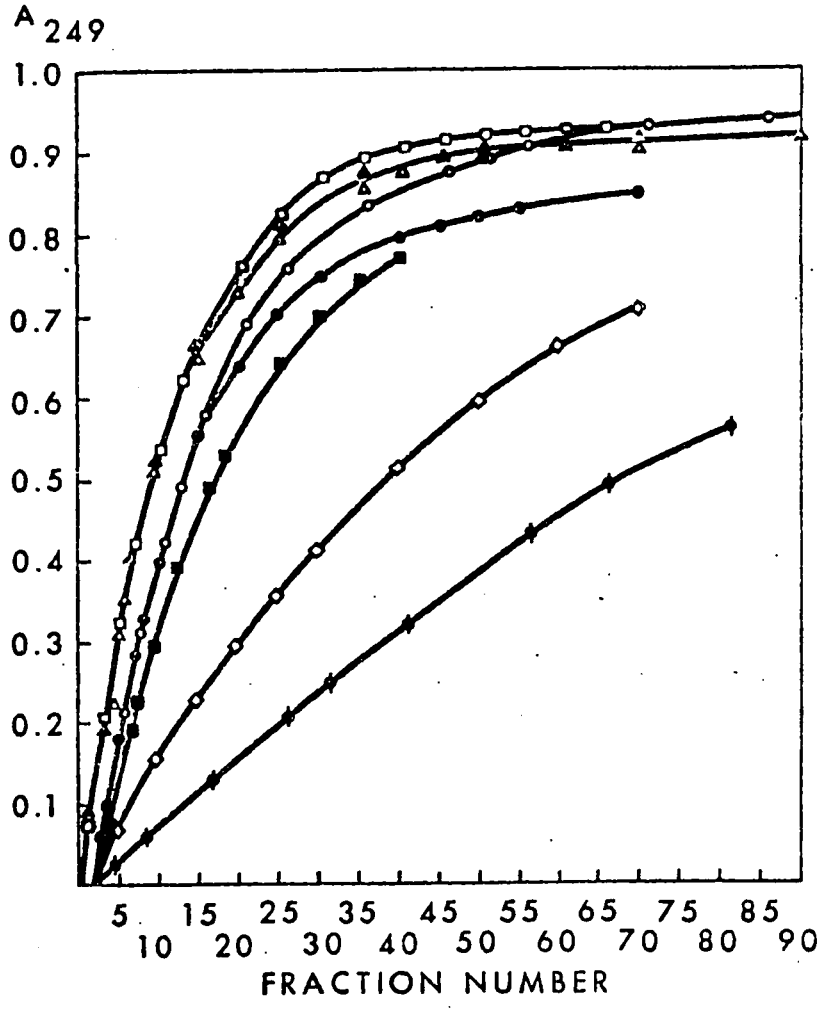


Fig. 12

Theoretical and experimental curves expressing the relationship between effluent concentration (as absorbancy) and effluent volume (as number of fractions) using the XM-50 membrane . ■ , theoretical dilution curve for a 59,2 uM testosterone solution (A_{249} = 0.930) , ● , experimental dilution curve for the 59.2 uM solution, ■ , experimental dilution curve for a 33.18 uM testosterone solution (A_{249} = 0.521) , ▲ , curve for ultrafiltration of 59.2 uM testosterone through 69.9 uM BSA, ◆ , curve for ultrafiltration of 33.18 uM testosterone through 35.5 uM BSA. Areas under the ultrafiltration curve used for calculation are indicated as a_1 , a_2 , etc. (as in fig. 16) .

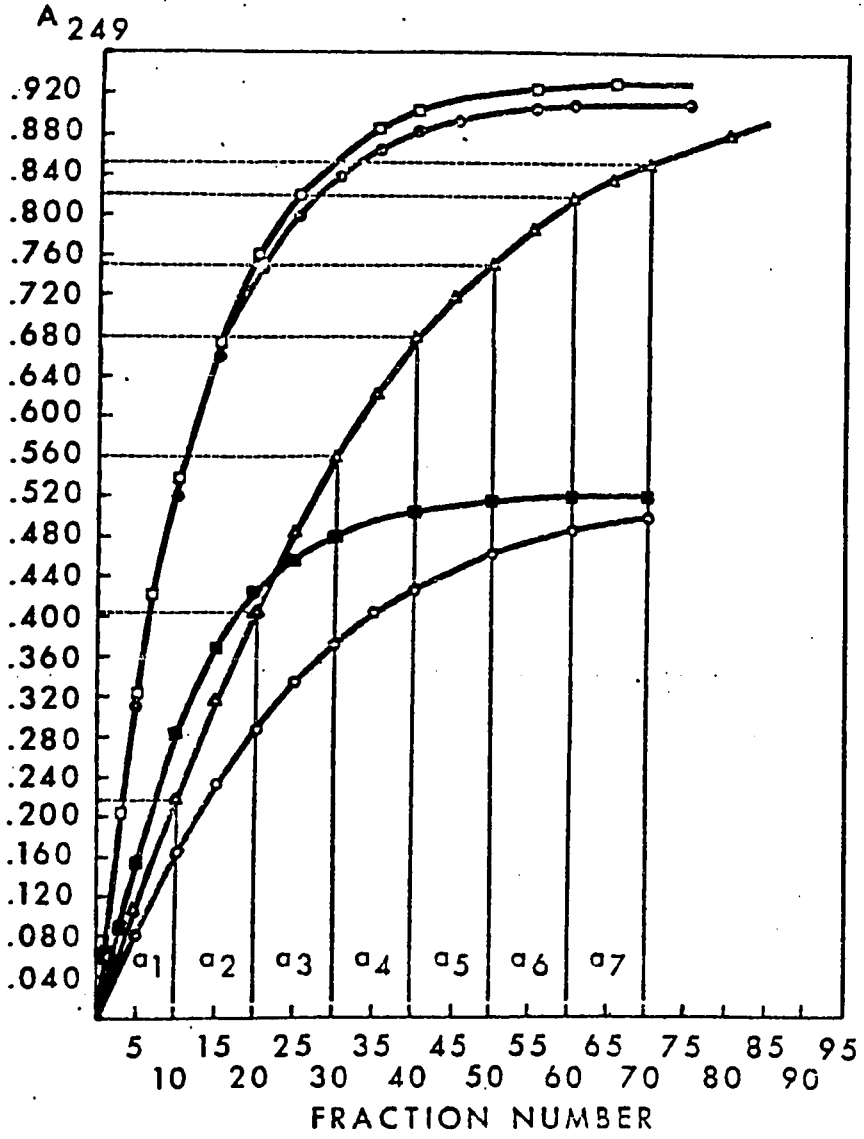


Fig. 13

Retentivity of the diaflo membranes towards BSA. Plot of absorbance of effluent against effluent volume when washing albumin solution in the ultrafiltration cell with buffer ○, XM-50, ●, XM-50, □, XM-50, ▲, PM-10, ■, UM-10.

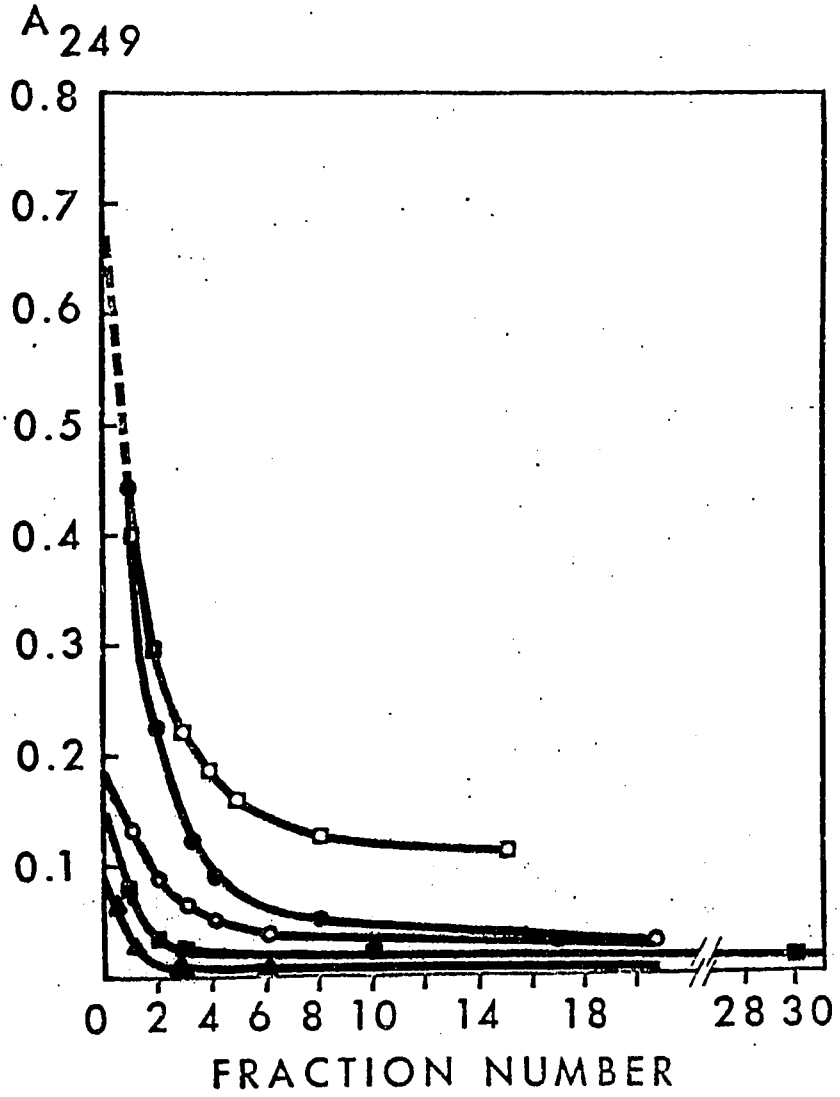


Fig. 14

Binding and rejection of testosterone by UM-10 membrane. A testosterone solution of absorbance 0.700 was placed in the ultrafiltration cell, connecting tubing and reservoir, and the absorbance of the effluent (▫) and cell contents (○) monitored.

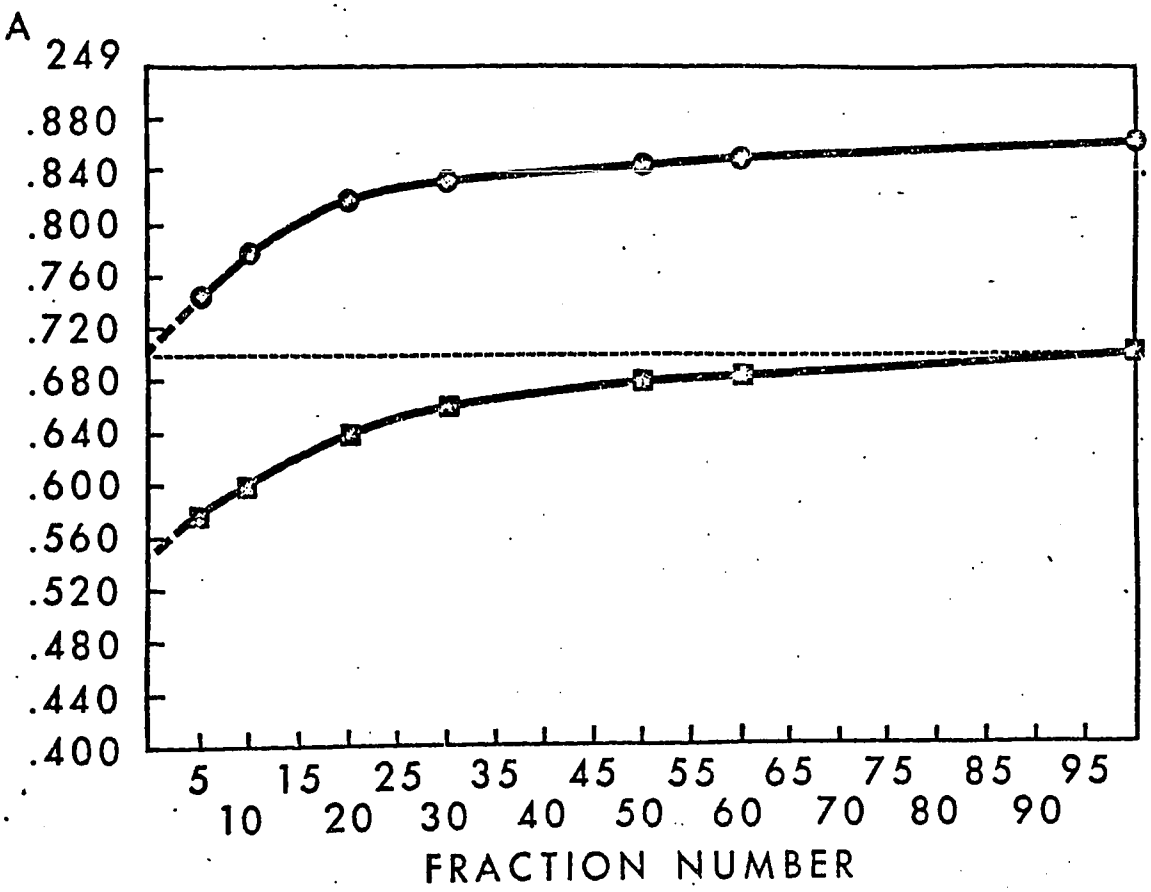


Fig. 15

BSA-testosterone binding by equilibrium ultrafiltration using the UM-10 membrane. Corrections have been applied for saturation binding (manifested in increase of void volume) but not for low affinity binding or membrane rejection.

54.7 μM testosterone
69.9 μM BSA.

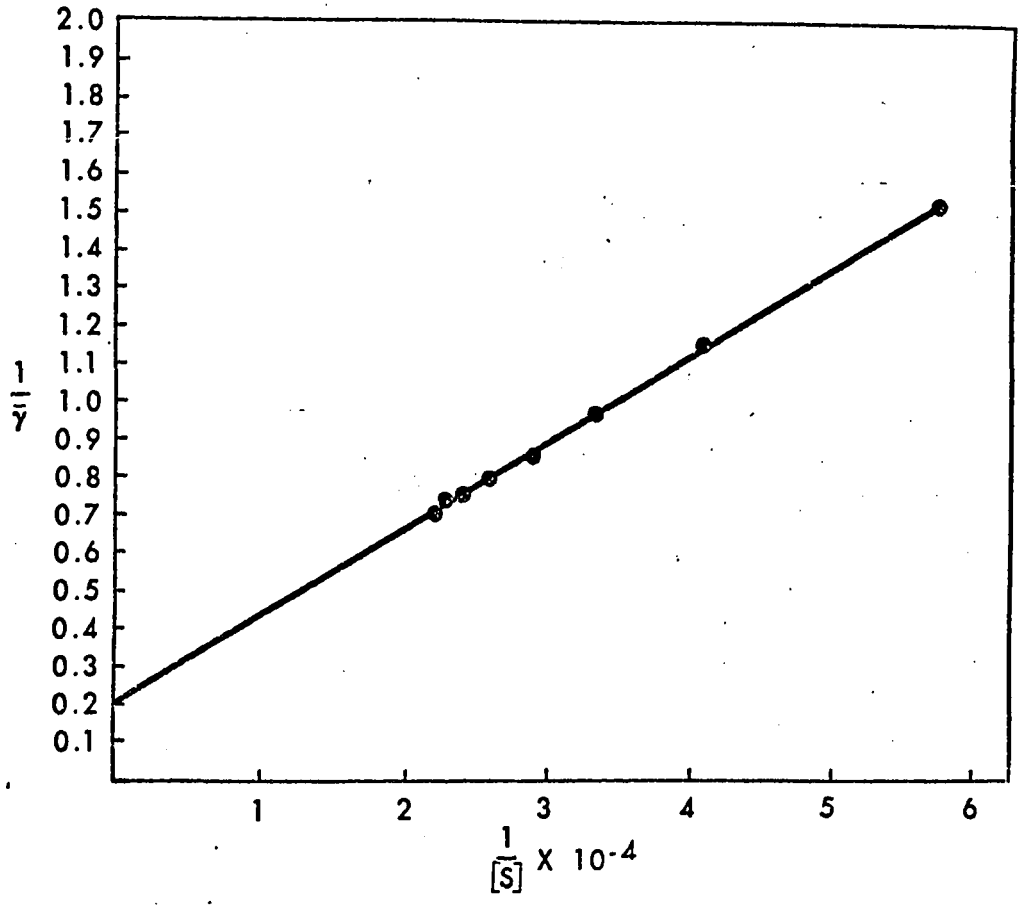


Fig. 16

Equilibrium Ultrafiltration Curves.
Testosterone binding to Diaflo XM-50 Membrane and to bovine albumin

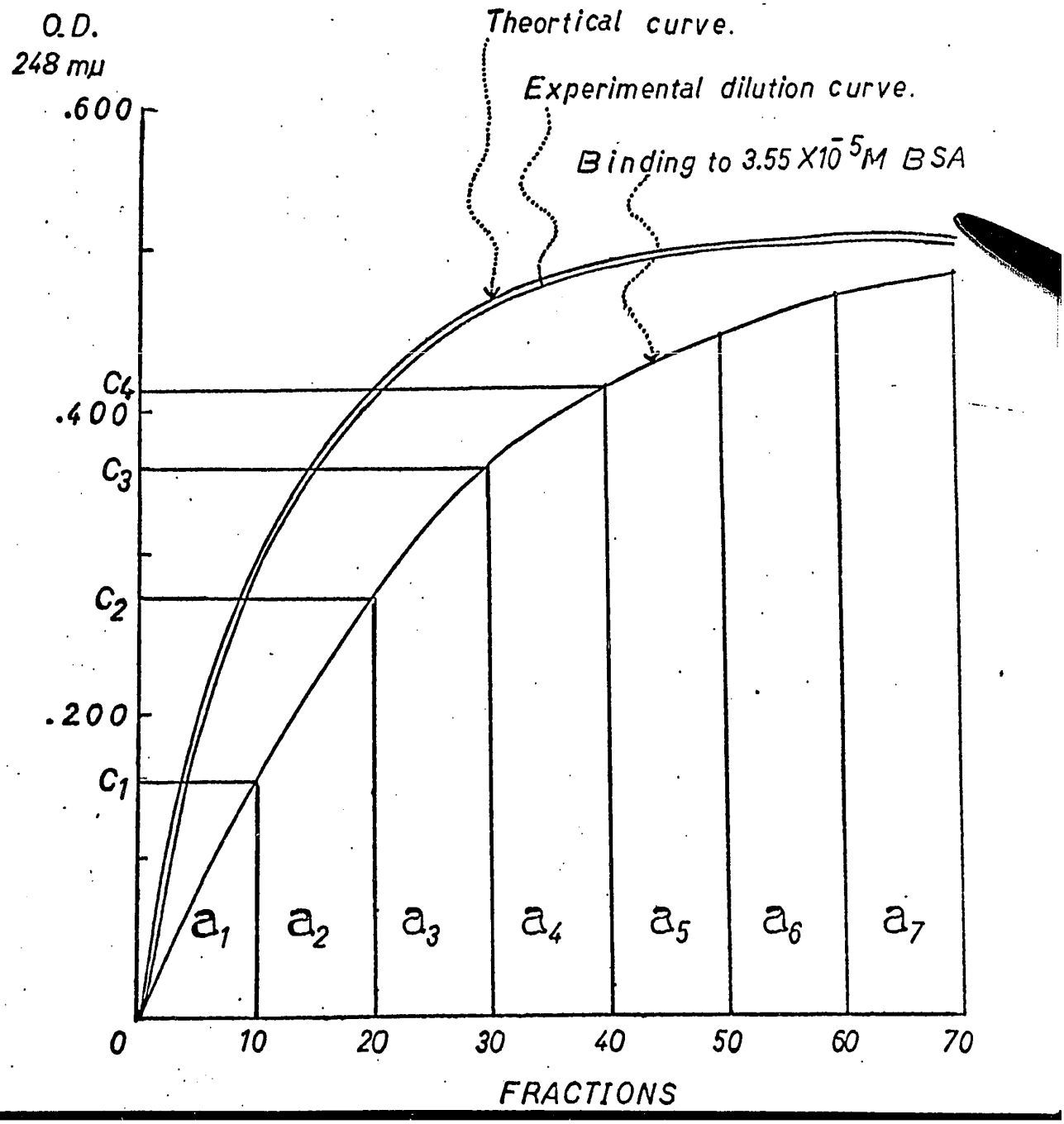


Fig. 17

Reciprocal plot for BSA-testosterone binding by equilibrium ultrafiltration, pH 7.6, 22 °C.

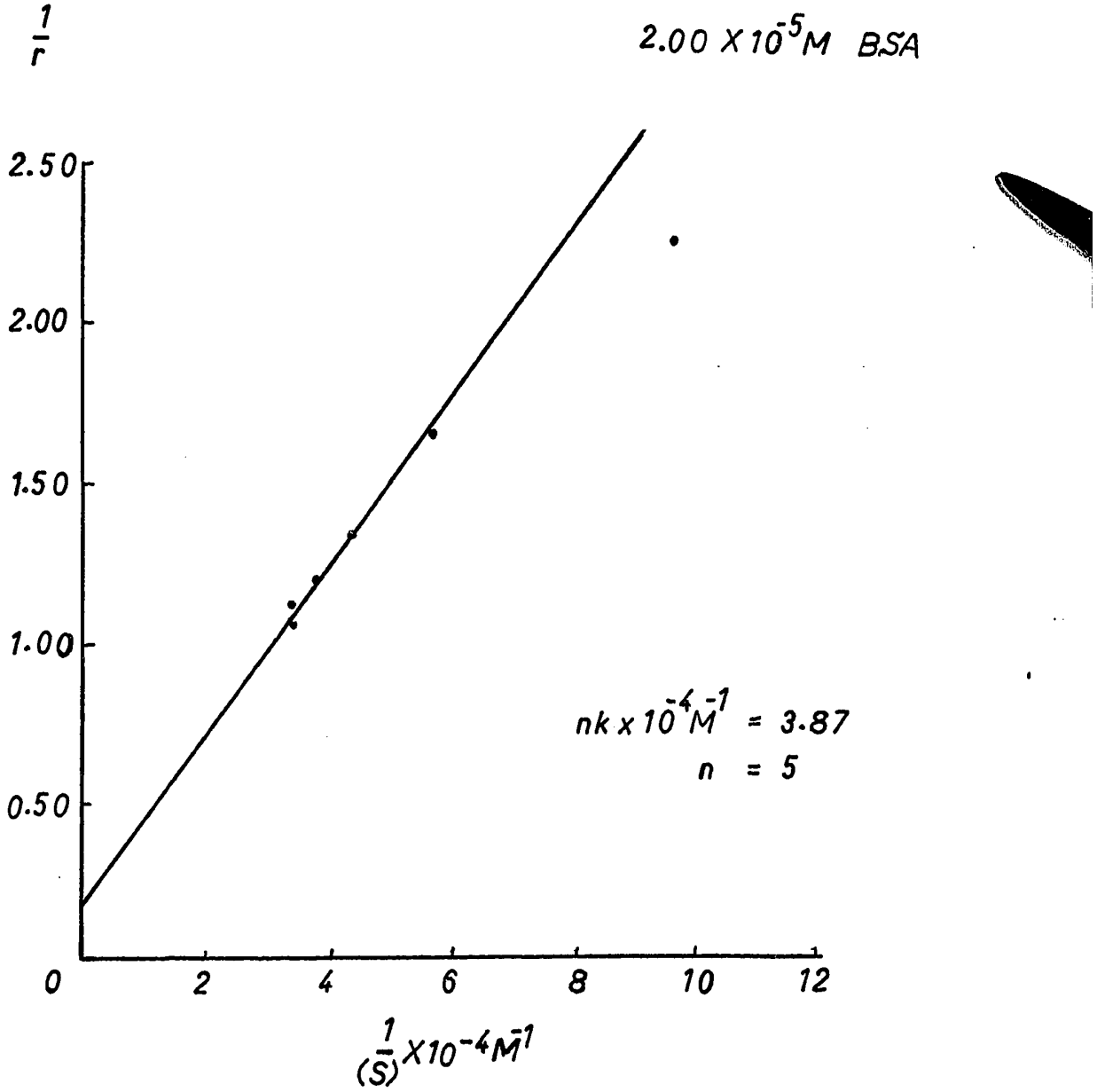


Fig. 18

Reciprocal plot for BSA-testosterone binding by equilibrium ultrafiltration, pH 7.6 , 22 °C.

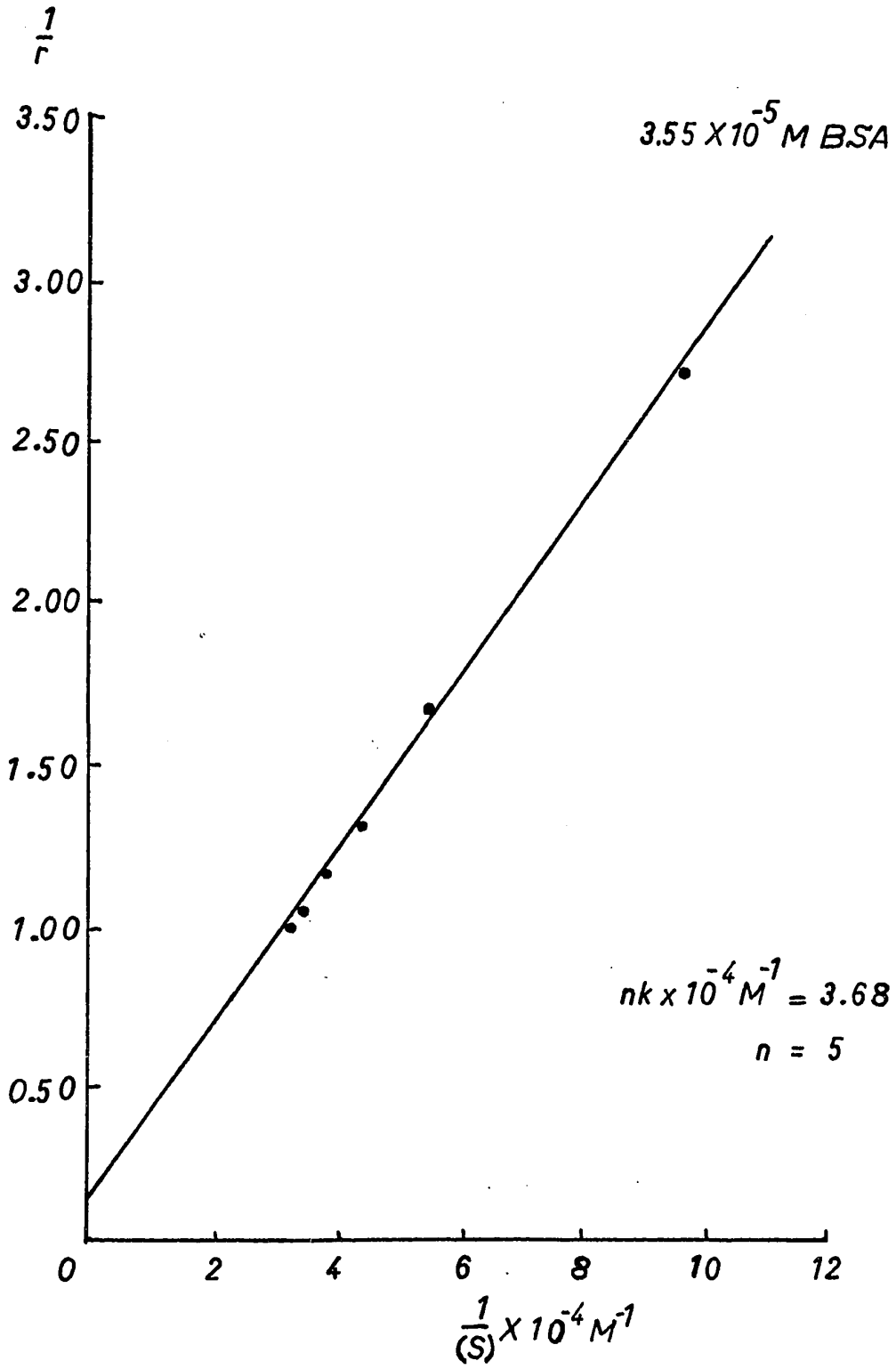


Fig. 19

Reciprocal plots for the binding of testosterone to 69.90 μM BSA by equilibrium ultrafiltration, pH 7.6 , 22 °C.

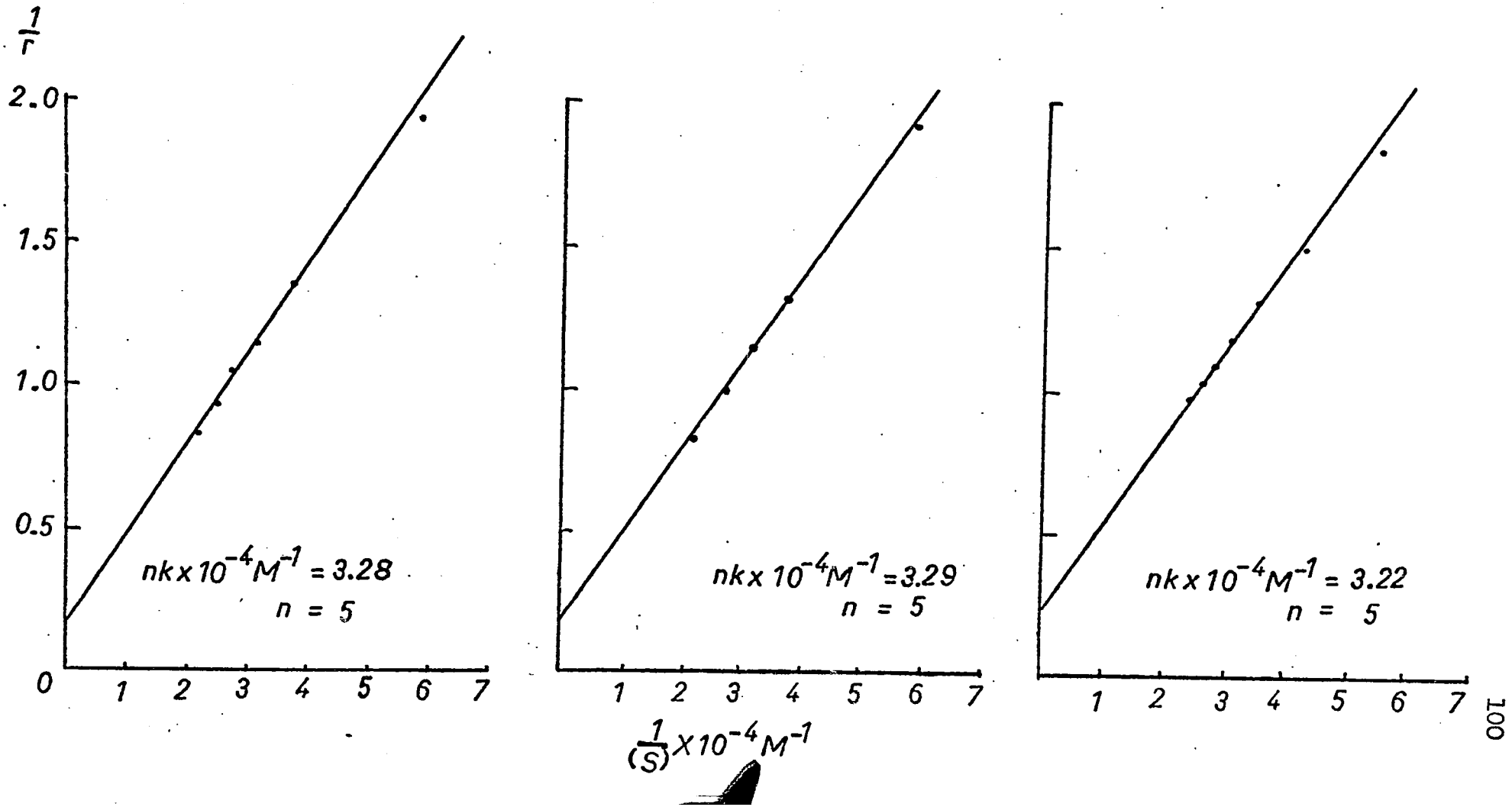
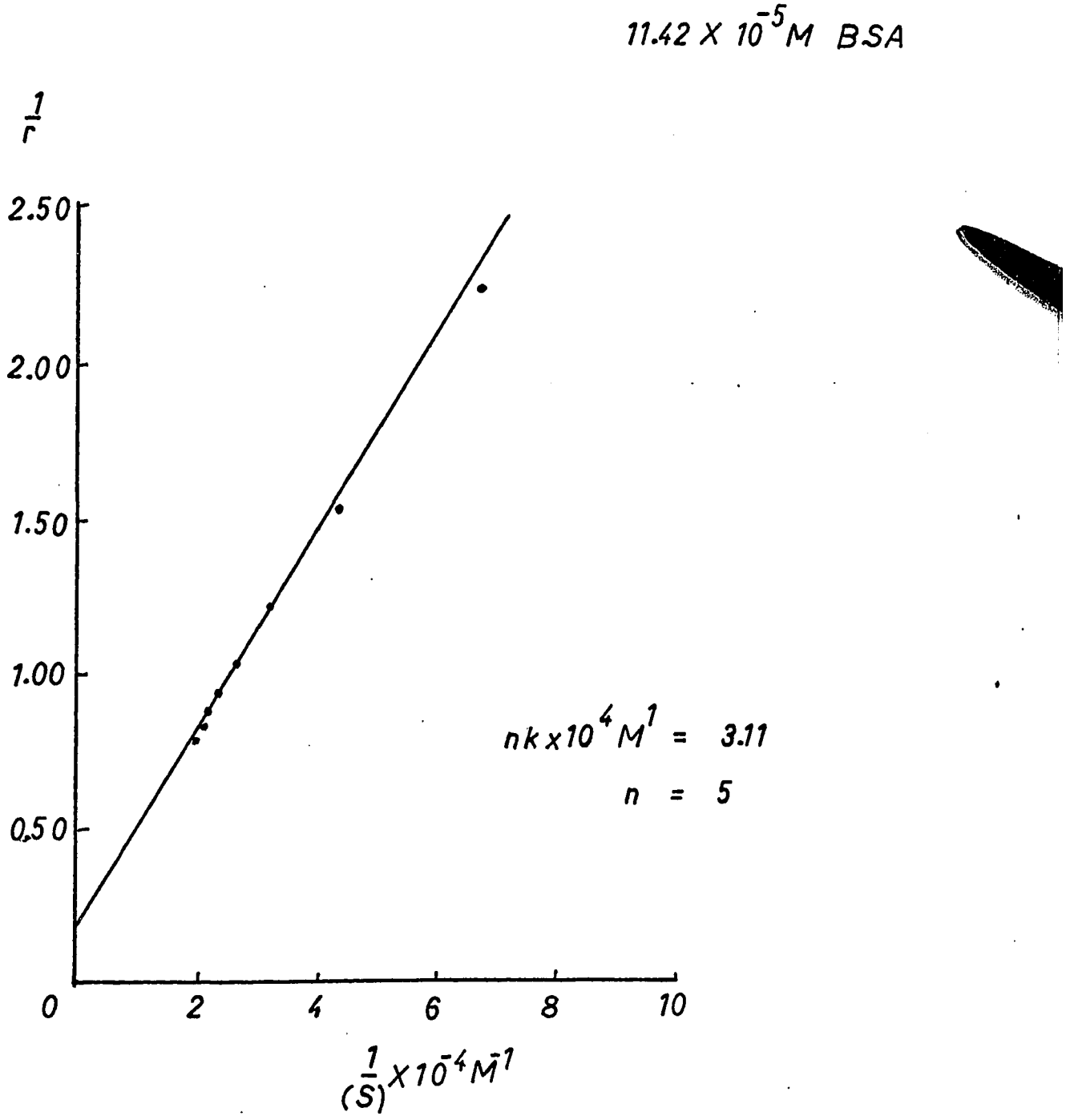


Fig. 20

Reciprocal plot for BSA-testosterone binding by equilibrium ultrafiltration, pH 7.6, 22 °C.



Reciprocal plot for BSA-testosterone binding by equilibrium ultrafiltration, pH 7.6, 22 °C.

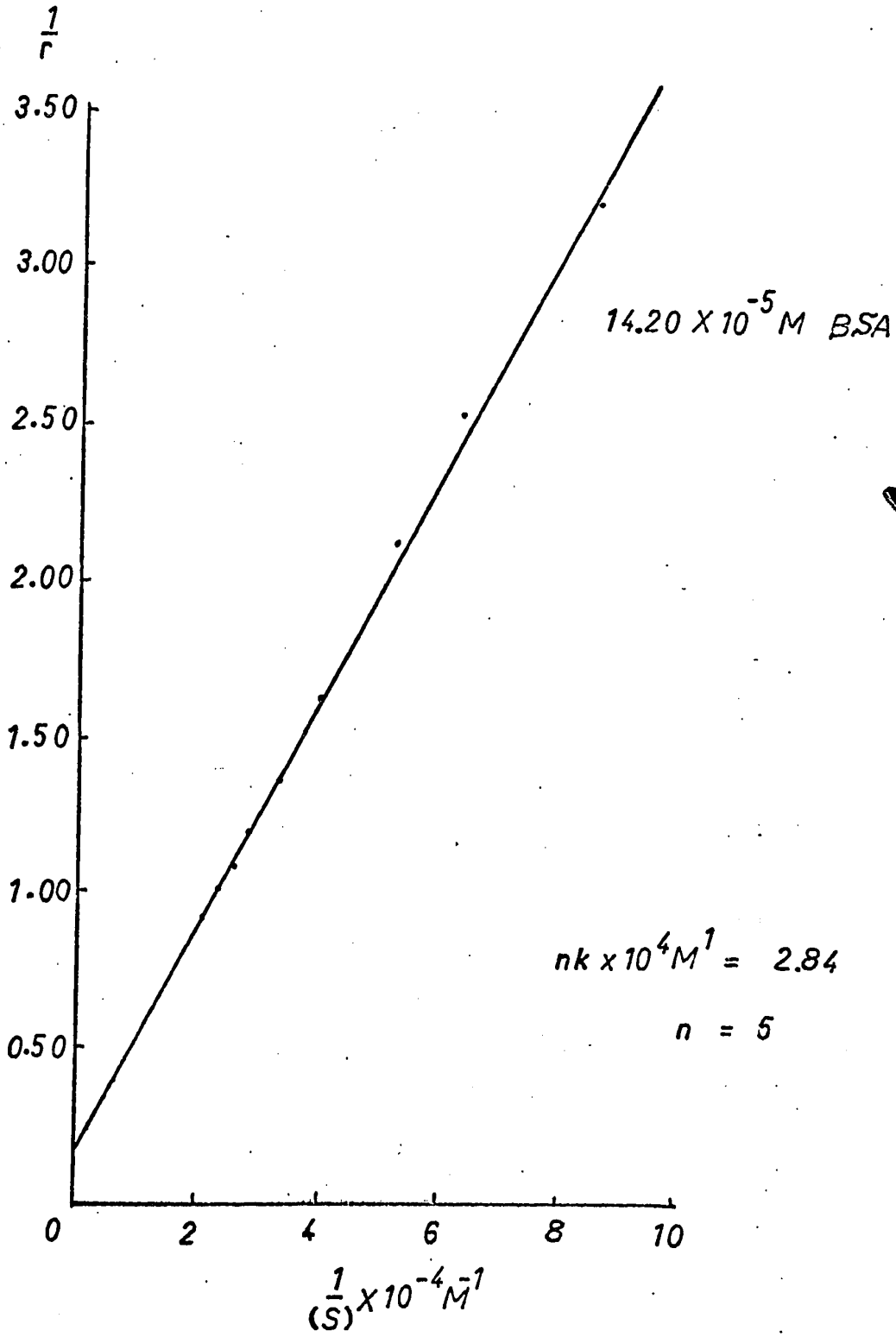


Fig. 22

Scatchard plot for BSA-testosterone binding by equilibrium ultrafiltration, pH 7.6, 22°C.

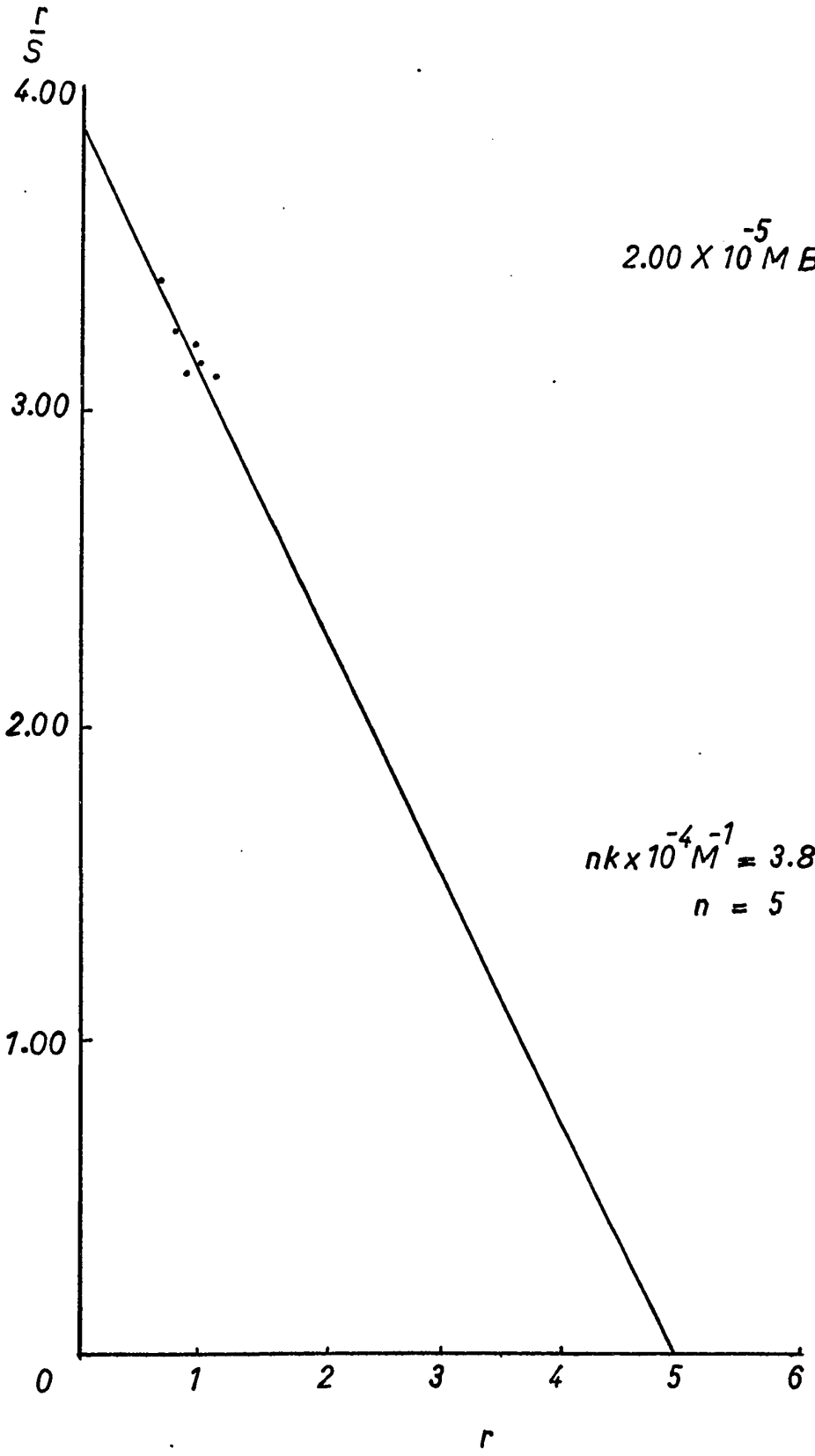


Fig. 23

Scatchard plot for BSA-testosterone binding by equilibrium ultrafiltration, pH 7.6, 22 °C.

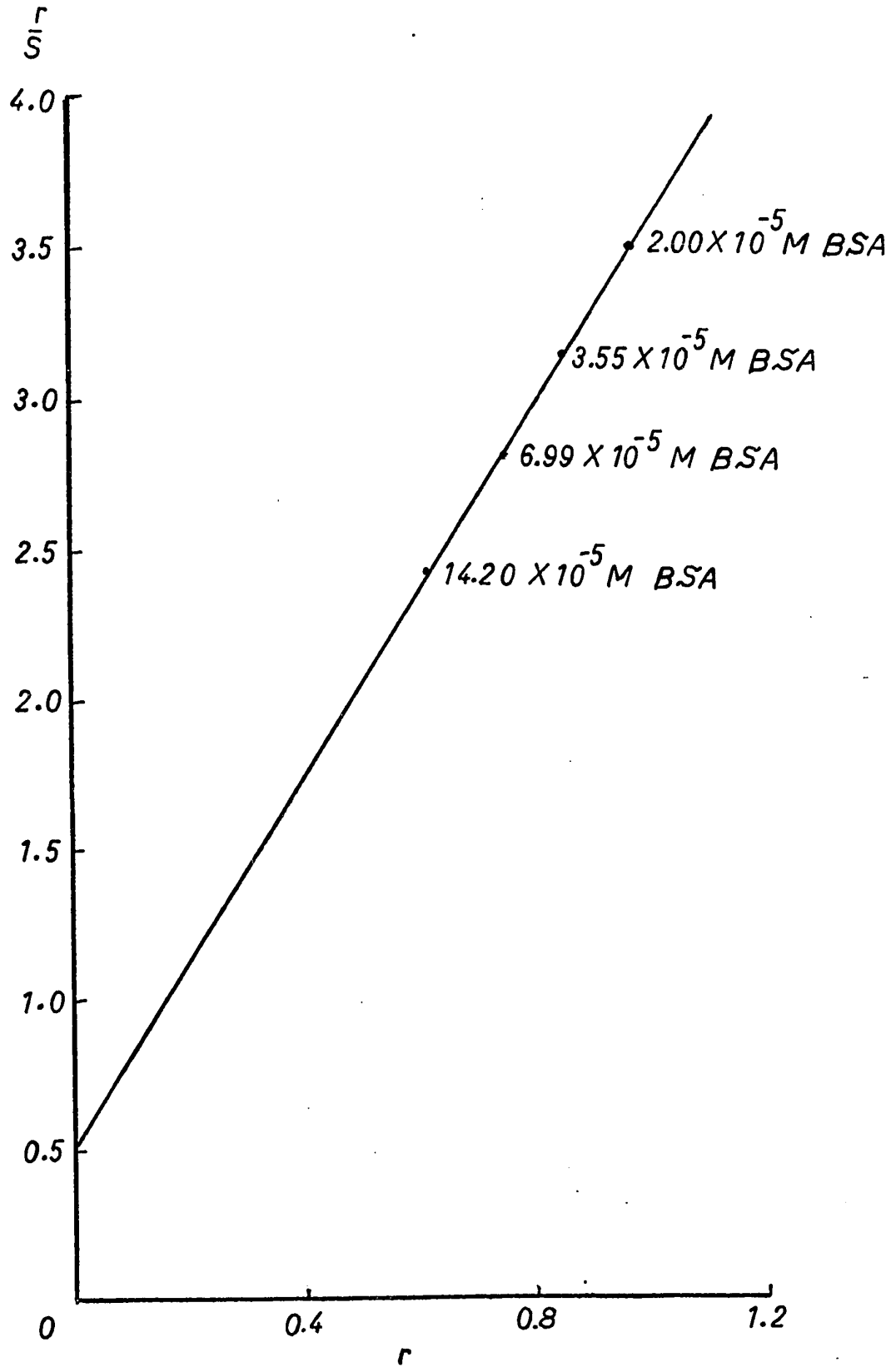


Fig. 24

Reciprocal plot for BSA "Pentex" - testosterone binding by equilibrium dialysis, pH 7.6, 25 °C.

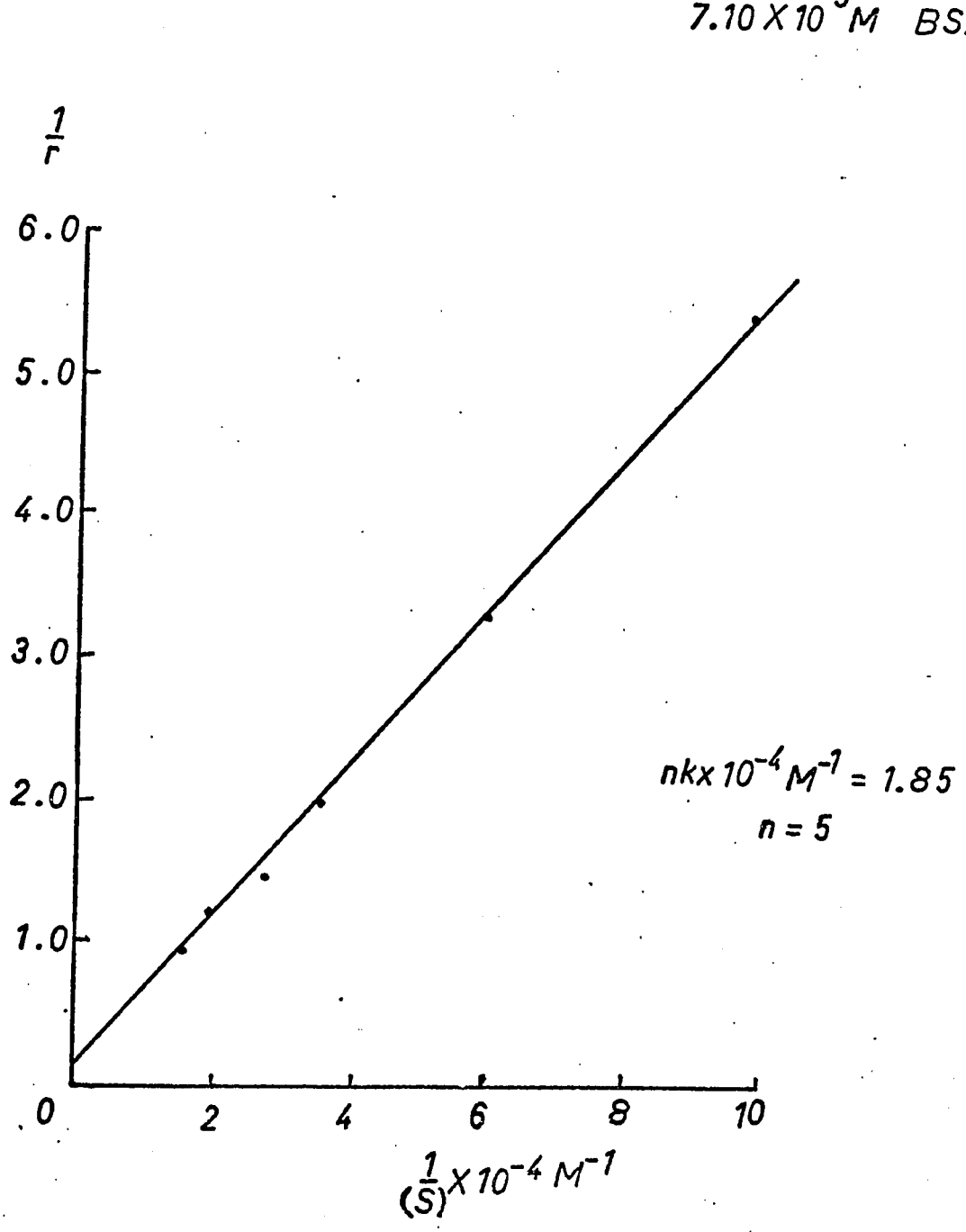
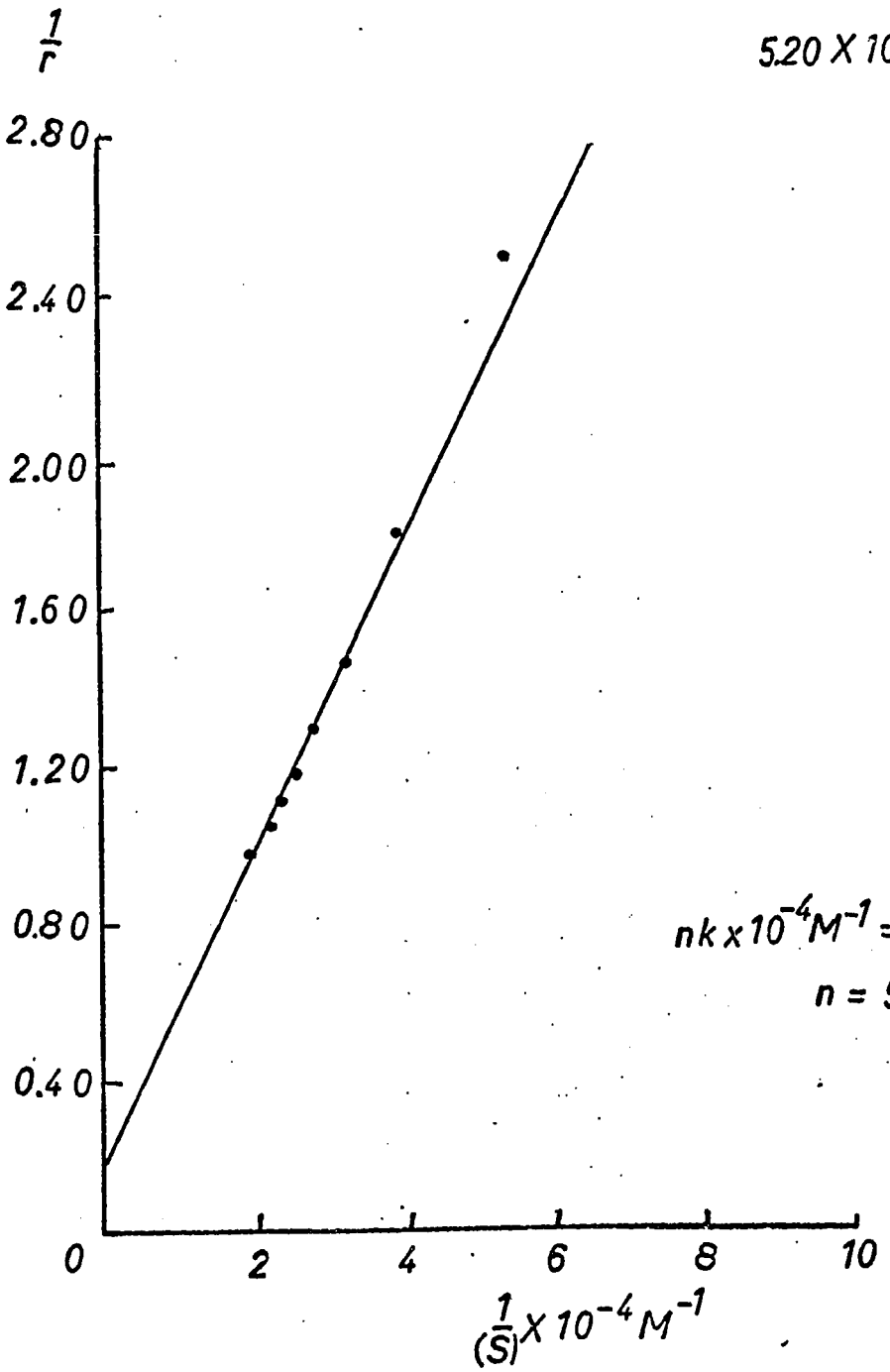
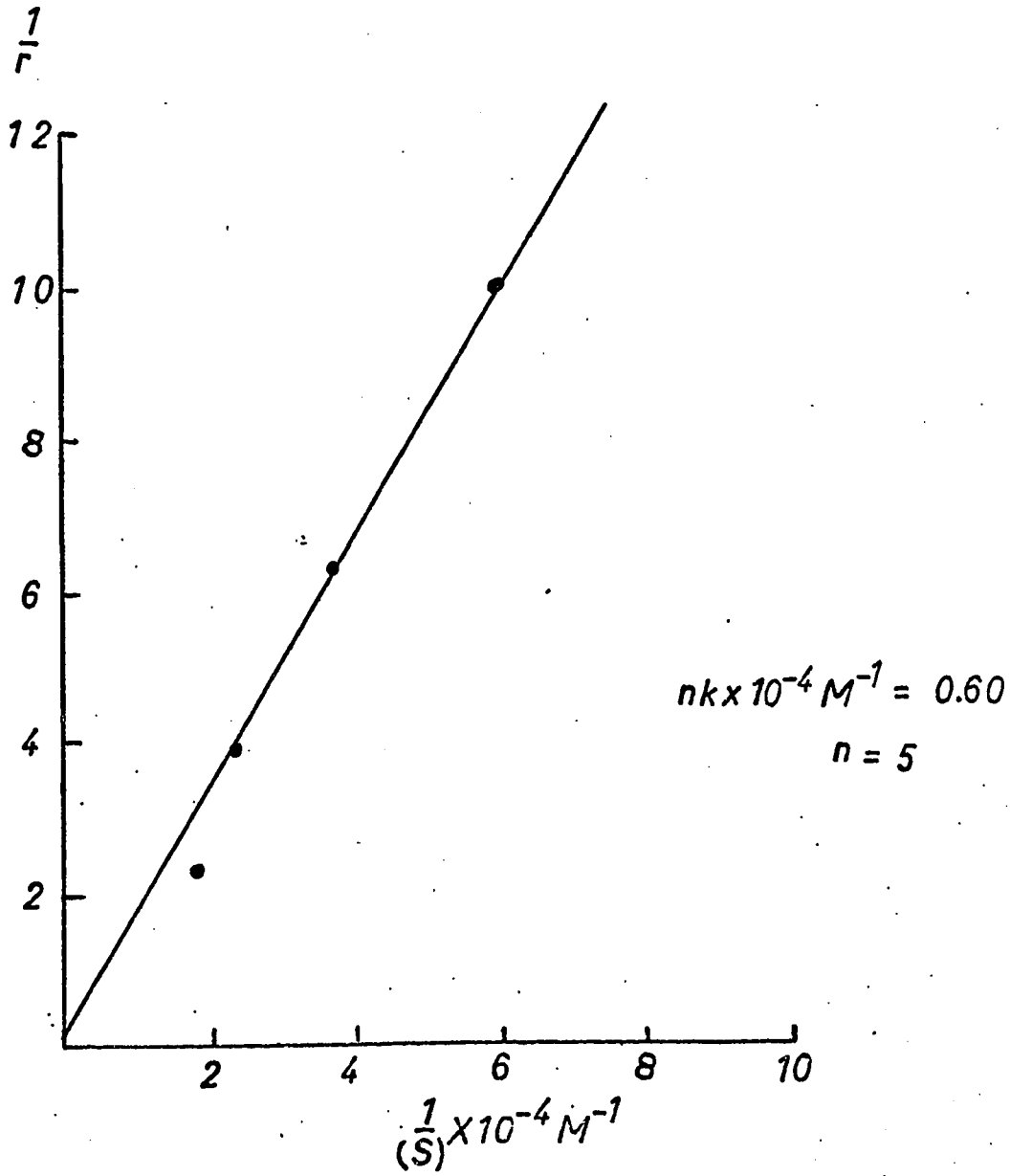


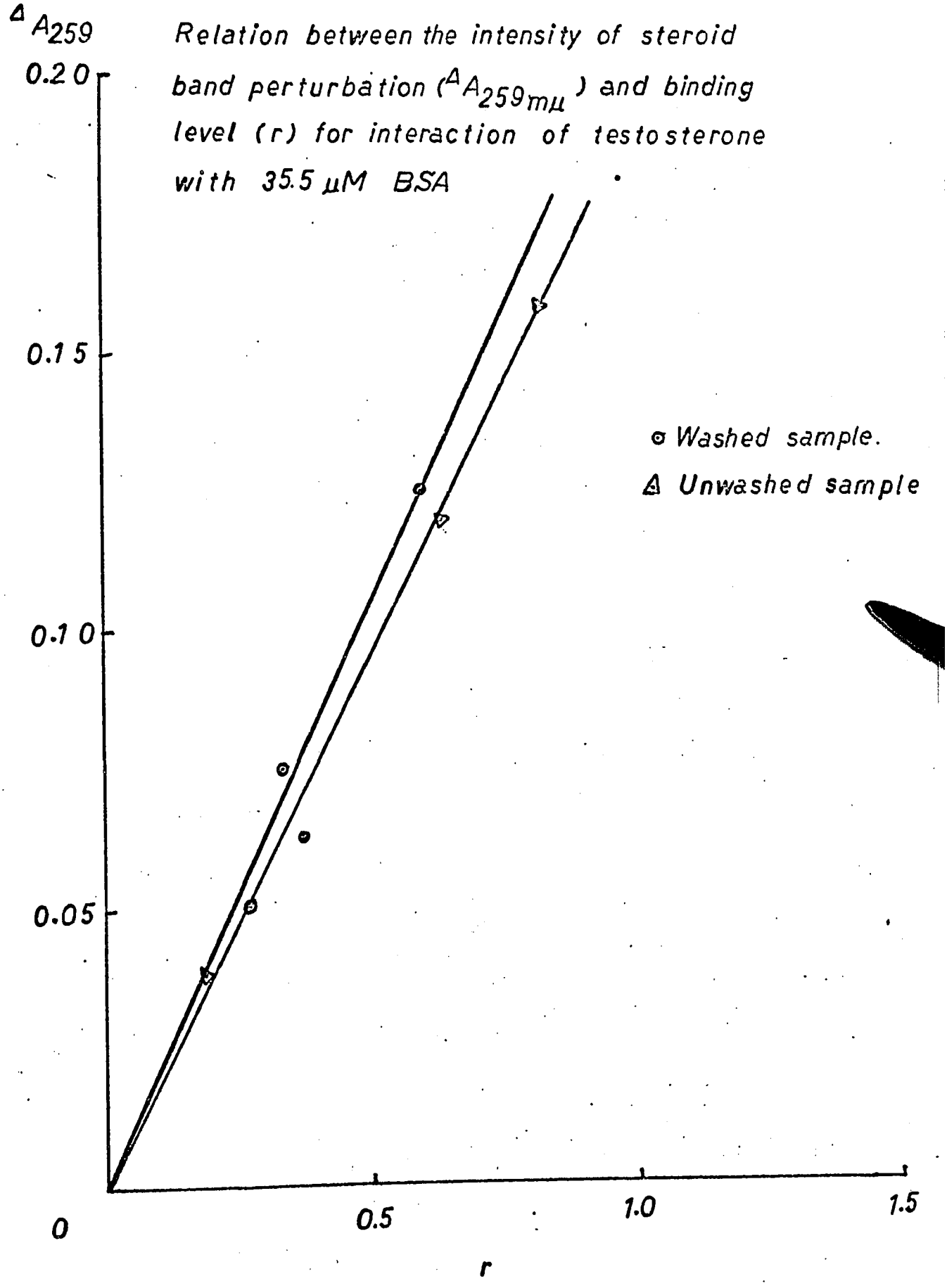
Fig. 25

Reciprocal plot for BSA "Pentex" - testosterone binding by equilibrium ultrafiltration, pH 7.6, 22°C.



Reciprocal plot for BSA-testosterone binding by equilibrium dialysis, pH 7.6, 25°C. The albumin solution was first washed with buffer in the ultrafiltration cell for 12 hours.




Δ A259
Relation between the intensity of steroid band perturbation (Δ A259mμ) and binding level (r) for interaction of testosterone with 35.5 μM BSA
0.20
0.15
0.10
0.05
0
0.5
1.0
1.5
r
⊙ Washed sample.
Δ Unwashed sample

SUMMARY AND CONCLUSIONS

1. Attempts to use an equilibrium dialysis method of Westphal, using visking dialysis membranes, to study the binding of unlabelled Δ^4-3-ketosteroids to albumin proved to be unsatisfactory as a consequence of the failure to eliminate completely ultraviolet absorbing material leached from unwashed membranes when exposed to phosphate buffer. Various procedures for washing the visking membranes have been tried and, while some success in this was achieved, no reliable measurements of binding could be obtained.

2. The classical equilibrium dialysis method using testosterone-4-C^{14} has been used for the determination of binding parameters for bovine albumin-testosterone interaction over a range of protein concentration using two preparations of bovine albumin.

3. A novel equilibrium ultrafiltration technique has been developed for the measurements of the binding parameters of BSA-testosterone complex. The values obtained by this technique were in all cases consistently higher than those obtained by the classical equilibrium dialysis method.

4. A dependency of the binding affinity of testosterone for bovine albumin on the protein concentration has been demonstrated for the first time in these studies, using not only the classical equilibrium dialysis technique but also the equilibrium ultrafiltration procedure developed here.

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