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Influence of Steroid Binding on the Tryptic Hydrolysis of Serum Albumin†

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ABSTRACT: The interactions of steroids, ethanol, and Ca^{2+} with serum albumin have been found to influence the rate of tryptic hydrolysis. This effect has been attributed to conformational change induced in the protein by the ligand. Testosterone, 3β-hydroxy-5-androsten-17-one, progesterone, and cortisol inhibit hydrolysis of *human* albumin in a manner suggestive of an involvement of a maximum of four sites in the case of the two androgens. However, inhibition only occurs above a minimum binding level of 0.4 mol of steroid/mol of protein. All neutral and charged derivatives of estrane and androstane inhibit hydrolysis of *bovine* albumin to a degree which varies with structure of the steroid, while all pregnane and cholestane derivatives stimulate hydrolysis. Unlike results published for dyes, steroid inhibition in the case of human albumin leads to only slight change in the relative amounts of the tryptic fragments observed by gel filtration on G-100 Sephadex, whereas Ca^{2+} produces a larger effect. The inhibitory effect of testosterone increases markedly during the course of the hydrolysis. Measurements of bound steroid have been made for the interaction of testosterone and 3β-hydroxy-5-androsten-17-one with human serum albumin over the entire binding range ($\rightarrow \bar{\nu} = 5.25$ for testosterone and 6.6 for 3β-hydroxy-5-androsten-17-one). Results of these indicate simple binding up to $\bar{\nu} = 2$–3 and departure from this in the higher binding range in a manner suggestive of cooperativity rather than heterogeneity.

The elucidation of the structure of hemoglobin and the recognition of the precise and sophisticated molecular engineering involved at every level of structure and function in the interaction of this molecule with small ligand molecules has focused attention on the possible general significance of this phenomenon in biochemistry. This is particularly true in the steroid field where several groups have discovered the existence of specific binding proteins, not only in plasma (Westphal, 1971a) but also in the target organs (Williams-Ashman and Reddi, 1971), thus providing hope for the elucidation of the mechanism of action in these tissues. The interaction of ligands and proteins has an intrinsic physicochemical interest, even where the physiological relevance may seem remote, and has been studied for many years (Steinhardt and Reynolds, 1969a). While the high-affinity (formation constants of 10^{8}–10^{14}) specific functional proteins of tissues and plasma must have properties not shared by other proteins which bind the same ligand, it seems likely that a full understanding of low-affinity interactions (10^{3}–10^{5}) of steroids and proteins such as serum albumins will prove relevant to ultimate understanding in the case of the high-affinity proteins. In at least some instances the steroid binding sites of albumin (Slaunwhite *et al.*, 1963; Plager, 1965) are heterogeneous in nature. We have been attempting to apply different approaches (Ryan, 1968; Ryan and Gibbs, 1970a,b) to the study of this problem in seeking to clarify its nature further. It is well known that interactions of some ligands with proteins tend to produce conformational changes which "stabilize" the protein (Markus *et al.*, 1967a; Steinhardt and Reynolds, 1969c). One of the most sensitive indicators of such conformational change is the altered susceptibility to enzyme-catalyzed hydrolysis. Thus, Markus and coworkers have been able to demonstrate such an effect in the combination of aspartyl transcarbamylase and ribonuclease with their substrates as well as of Methyl Orange and other dyes with human serum albumin. Such binding-induced conformational changes [or "ligand-stabilized" conformations (Markus *et al.*, 1967a,b)] have also been demonstrated by other means, as in the evidence of "cooperativity," or unmasking, in the binding of some ligands to serum albumins (Steinhardt and Reynolds, 1969d). In the work presented here we have attempted to apply this experimental approach to the steroid–albumin problem and to correlate our findings on the effects of steroids on the tryptic hydrolysis of bovine and human serum albumina with steroid structure and level of bound steroid. One report (Bellamy and Leonard, 1966) has appeared in the literature in which a less developed form of this approach has been used in studying the effect of steroids on bovine albumin. We have also had reason to study the effects of non-steroid ligands, Ca^{2+} and ethanol, on tryptic proteolysis. The former has been shown many years ago (Gorini and Audrain, 1952) to alter the susceptibility of albumin to hydrolysis and we have demonstrated its influence on steroid binding to human albumin (Ryan and Gibbs, 1970b). Its effect on trypsin activity is also well known (Sipos and Merkel, 1971). The low solubility of steroids in water has required that they be added to the protein solutions in small volumes of concentrated (10^{-1}–10^{-3} M) ethanol solutions, thus yielding much higher binding levels than is usually the case. It has been shown that alcohols become hydrogen bonded to the backbone of synthetic peptides (Strassmair *et al.*, 1969), so an effect of ethanol on tryptic hydrolysis of albumin might be anticipated. It has also been shown (Milar

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et al., 1962) to protect albumin against denaturation. Some control studies have also been made on the effect of these various substances on the activity of trypsin towards the synthetic substrate L-Tos-Arg-OMe.[1]

Materials and Methods

Albumin. Crystalline bovine and human serum albumins were obtained from Pentex (Miles Laboratories). Before use they were dissolved to a concentration of about 5% in deionized distilled water and passed through a column of mixed-bed ion-exchange resin (Bio-Rad, AG-501-X8) layered over a 1-in. column of AG50W-X4 cationic resin in hydrogen form. Albumin treated in this manner has been used extensively in binding studies and is known to retain up to 1 mol of bound fatty acid/mol of protein (Polet and Steinhardt, 1968). Differences both in spectral behavior (Steinhardt *et al.*, 1972) as well as in the pH dependence of various parameters (Sogami and Foster, 1968) of albumin subjected to different deionizing and defatting procedures have been recorded. Little is known of the role of bound lipid in steroid binding, although data of Westphal (1971d) indicate inhibition of progesterone binding to human albumin by lauric acid. Since varying values (Sober, 1970) have been reported for $E^{1\%}_{279}$, albumin concentrations were determined on a direct weight basis, using values obtained by drying an aliquot of the deionized solution in a weighing bottle at 110°.

Steroids. Unlabeled steroids were obtained from Schwarz–Mann. When necessary, these were recrystallized from the appropriate solvents. ^{14}C- and ^{3}H-labeled steroids were obtained from New England Nuclear Corp. and Amersham Searle and were used shortly thereafter. Occasional checks revealed that the radiochemical purity, measured as the percentage of radioactivity applied in a thin-layer or paper chromatographic system which travels with the main peak and in parallel with unlabeled reference compound, was better than 97%. Trypsin, twice crystallized, was obtained from Worthington Biochemicals. Tos-Arg-OMe was a product of Schwarz-Mann. Ethanol was redistilled before use.

pH-Stat Assembly. The assay of trypsin activity and the monitoring of proteolysis were achieved by means of the following pH-Stat assembly: (i) Radiometer TTT1a titrator–pH meter, (ii) Radiometer magnetic relay, (iii) Radiometer semimicro jacketed titration assembly with water thermostatted at 25° circulating from a water bath, (iv) a Beckman combination probe electrode mounted on a flexible electrode arm, (v) a Sargent multirange recorder, and (vi) a specially made syringe drive with associated simple bridge circuit (Neilands and Cannon, 1955) for potentiometric recording on the recorder. The signal from the magnetic relay is fed to a reversible 10-rpm Bodine synchronous motor which powers, through a gear train, a reversible screw actuating the plunger of a syringe buret (MicroMetric Instruments). The luertip of the syringe is attached to a two-way stopcock which connects to an alkali reservoir (a 50-ml hypodermic syringe) and 0.016-in. polyethylene spaghetti tubing acting as a delivery tube to the reaction vessel. The outer element of the screw drive rotates without translational motion (thus transmitting translational motion to the inner screw) and is fitted with a brass collar inserted in a sprocket wheel. The latter is connected by a system of non-slip chains and sprocket gears to the shaft of a 20-turn precision potentiometer which forms part of the bridge circuit. This circuit is energized by a 1.5-V Mallory M-900 battery and the output is fed to the 25 mV range of the Sargent multirange recorder. The powered end of the screw mounting is connected by means of a slot in its shaft to the shaft of a revolution counter. By means of this, the linear travel of the plunger of the interchangeable constant bore syringe is converted to an electrical signal which is measured on the recorder. The revolution counter and potentiometers are used to establish the chart calibration. With a 1-ml/1 in. syringe, the full-scale pen travel (250 mm) can be conveniently adjusted to 0.25–1 ml (1–4 μl/division) with the facility of resetting the chart zero so as to use the total 2.5-ml capacity of the syringe before refilling. A selection of gear-reduction ratios between the motor shaft and screw assembly permits adjustment of the maximum rate of titrant delivery to that necessary to fulfill pH-Stat requirements (Jacobsen *et al.*, 1957). As ordinarily used the system was found reproducible and accurate to 0.5 μl.

Trypsin assays were run in the pH-Stat at pH 8.5, using 4–8 μg of enzyme in 50 μl of 10^{-3} N HCl added from a microliter syringe to 3.95 ml of 10^{-4} M Tos-Arg-OMe in 0.1 M NaCl. The reaction rate was calculated from the line traced on the recorder. In studying the effects of solvents and steroid solutions, appropriate volumes were substituted for an equivalent volume of 0.1 M NaCl in the reaction mixture.

pH-Stat Monitoring of Proteolysis. This was carried out on a total volume of 4 ml containing 25 mg of deionized albumin under a stream of water-saturated nitrogen. The appropriate volume of the albumin stock solution was added to 3.2 ml of NaCl solution in the titration vessel. The pH was adjusted to 8.5 with 2.5×10^{-2} N NaOH from the syringe and the volume required was recorded. The agent whose effect was being studied was then added and the total volume was adjusted to 3.6 ml by addition of NaCl solution. On addition of 1.6 mg of trypsin in 0.4 ml of 10^{-3} N HCl, the pH-Stat neutralizes the added acid within 6–12 sec and faithful recording of proteolysis begins within 12–24 sec from the initiation of the reaction. The amount of alkali consumed by neutralization of the trypsin is obtained by extrapolation of the curve back to zero time over the short 24-sec time interval. In many instances, when the reaction had slowed down considerably, the reaction mixture was titrated rapidly to pH 9.0 so as to obtain a factor (1.19) whereby equivalents of alkali consumed could be converted to bonds split (Markus *et al.*, 1967b).

Gel filtration was performed in 2.5×100 cm columns on G-100 Sephadex (Pharmacia) using flow adapters. The gel was soaked in water for 3 days and then poured as a thin slurry. After washing, the water was replaced by 0.05 M borate buffer (pH 8.5) in 0.1 M NaCl. Twenty to forty milligrams of either albumin or its hydrolysis products was diluted to 5 ml with buffer and placed on the column. The column was fed by downward flow from a reservoir at the rate of 12–15 ml/hr using an LKB peristaltic pump. The column was monitored at 280 mμ using a 1-ml flow-cell in a Bausch and Lomb precision spectrophotometer. Fractions were collected in an LKB fraction collector fitted with an event-marker pen. Eluate volume and absorbance were recorded on a Sargent SRL recorder. In some experiments, where the effect of bound steroid on the apparent molecular weight of human albumin was studied, the

[1] The following nonconventional trivial names and abbreviations have been used: Tos-Arg-OMe, tosyl-l-arginine methyl ester; dehydroisoandrosterone, 3β-hydroxy-5-androsten-17-one; androsterone sulfate, 17-oxo-5α-androstan-3β-yl sulfate; androsterone hemisuccinate, 17-oxo-5α-androstan-3β-yl hemisuccinate; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; 1-dehydrostestosterone, 17β-hydroxy-1,4-androstadien-3-one; 6,17α-dimethyl-6-dehydroprogesterone, 6,17α-dimethyl-4,6-pregnadiene-3,20-dione; 16-dehydroprogesterone, 4,16-pregnadiene-3,20-dione.

column was preequilibrated with a saturated solution of testosterone in 0.05 M Tris-chloride buffer (pH 8.5) in 0.1 M NaCl. Albumin (20 mg) was dissolved in 2 ml of this buffer and 0.04 ml of 10^{-1} M testosterone in ethanol was added with stirring. The excess was removed by centrifugation and the supernatant was placed on the column. When elution of the peaks with the testosterone-saturated buffer was complete, the latter buffer was displaced by steroid-free buffer. The elution pattern was then obtained using the steroid-free Tris-chloride buffer. There was only a slight difference of 3 ml in the elution volume for the two experiments and hence no significant change in the apparent molecular weight.

Binding measurements were performed using a modification of the method of Pearlman and Crépy (1967). Experiments were designed to duplicate the conditions of the pH-Stat investigations. For this purpose, an appropriate volume of an ethanol solution of [^{14}C]steroid of known specific activity was added slowly with stirring to a solution of albumin in 0.1 M NaCl which had been preadjusted to pH 8.5. The volume and concentrations of the ethanol solutions were adjusted so that the final volume was 12.5 ml, the total ethanol 1.25%, and the concentration of steroid after addition ranged from 10^{-6} to 10^{-3} M. As in the pH-Stat experiments, steroid precipitated at high steroid concentration and this was removed by centrifugation at room temperature. The concentration of steroid remaining in solution was obtained by counting an appropriate aliquot of the supernatant. Aliquots (2 ml) of the supernatant were introduced into screw-cap vials containing 400 mg of Sephadex and one glass bead and which had been preequilibrated overnight with 2 ml of 0.1 M NaCl containing 1.25% ethanol. In the case of the higher steroid concentrations, the preequilibration solution was saturated with steroid of the same specific activity as that used in preparing the protein solutions. The volume of the external phase was 3 ml and the concentration of albumin 9.1×10^{-5} M. Each steroid concentration was run in triplicate. After equilibration for 2 hr at 25°, duplicate 100-μl aliquots were transferred from each tube into vials containing 4 ml of scintillator fluid (250 ml of toluene, 120 ml of ethanol, 2.22 g of 2,5-diphenyloxazole, 0.037 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene) and were counted, together with the appropriate number of blanks and standards, to 2% standard error in the appropriate window of a Beckman LS250 counter. The values for K' were obtained either by substituting an equivalent volume of 0.1 M NaCl for the protein solution, or by adding 2 ml of a dilution of a saturated solution of [^{14}C]steroid in 0.1 M NaCl–1.25% ethanol to tubes after preequilibration. Unlike the results reported by Pearlmann and Crépy (1967) for buffered aqueous solution, we have found the value for K' to be invariant over the complete concentration range in a given experiment. However, the range for all experiments (1.1–1.46 for testosterone, 1.1–1.5 for dehydroisoandrosterone) is wider than that for previous experiments (Ryan and Gibbs, 1970b) using buffered aqueous solutions. We have not found any significant effect of ethanol at these concentrations on the value of K'.

Results

As may be seen from Table I, none of the following had any significant effect on the activity of trypsin in the Tos-Arg-OMe assay: testosterone, progesterone, cortisol, dehydroisoandrosterone. The ratio of steroid to enzyme in these assays is much higher than in the proteolysis experiments. The low concentrations of ethanol used in most of our experiments (0.5–1.25%) had little effect on the enzyme, but higher concentrations had a marked stimulatory effect. This was also true for ethylene glycol and dioxane. The effect of alcohol has been reported previously (Schwert and Eisenberg, 1949). While Ca^{2+} has a marked effect on the stability and activity of trypsin at higher temperature and pH (Sipos and Merkel, 1971), we have observed only a 6.8% stimulation by 2×10^{-2} M Ca^{2+} at 25° and pH 8.5. This is lower than the approximately 18% which may be calculated from the data of Sipos and Merkel (1971) for pH 8.0 and 25°. We have no explanation for this discrepancy.

TABLE I: Esterase[a] Activity of Trypsin toward Tos-Arg-OMe in the Presence of Various Substances.

Substance	Concn	Effect[b]
Ethanol	0.5%	0
	1.25%	+1
	2.5%	+5
	10%	+24
Ethylene glycol	5%	+15
	10%	+21
Ca^{2+}	2×10^{-2} M	+6.8
	4×10^{-2} M	+8.7
Progesterone	5×10^{-4} M	+2
Dehydroisoandrosterone	5×10^{-4} M	−4
Testosterone	5×10^{-4} M	+3
Cortisol	5×10^{-4} M	−3

[a] Assayed in 0.1 M NaCl (pH 8.5), 25°, in the pH-Stat under nitrogen. [b] Expressed as percentage increase (+) or decrease (−) of the rate with enzyme and substrate alone.

Tryptic Hydrolysis of Albumin. An excess of enzyme relative to the protein was used (1.6 mg of trypsin and 25 mg of albumin in 4 ml of 0.1 M NaCl) in order to have a measurable rate (Bellamy and Leonard, 1966; Markus *et al.*, 1967a,b) and to minimize influences on the specific activity of the enzyme (Gorini and Audrain, 1952). The instantaneous rate of the reaction (obtained from tangents drawn using the mirror method) decreases smoothly during the course of the reaction to a value which is only a small fraction of the initial rate. Markus *et al.* (1967a) used a higher substrate:enzyme ratio (33:1) and observed some linear segments in the time curve.

When undigested human albumin was chromatographed on G-100 Sephadex, it was found to consist of 88% monomer and 12% dimer. As noted earlier, when such chromatograms are run in the presence of saturating levels of testosterone no influence on the apparent molecular weight is detected (see Methods). Gel filtration patterns on G-100 Sephadex are seen in Figure 1 for products of tryptic hydrolysis after consumption of 10 (~12 bonds) mol of alkali/mol of protein. Calibration of the column indicated that, in addition to the dimer and monomer, fragments of the following molecular size appear in the tryptic digest: 49,000, 27,500, 21,400, ~11,000, and much smaller fragments beyond the calibration range of this column. These are presumed to correspond to fragment "L," "pre-A," "A," "B," and "C" of Markus *et al.* (1967a). A further fraction of mol wt 38,400 is presumed to be the complex of trypsin and inhibitor.

Effect of Ligands on Tryptic Hydrolysis of Albumin. As noted earlier, the high concentration of enzyme relative to protein provides conditions in which the rate of hydrolysis is substrate-limited and ligand-induced variations in enzyme

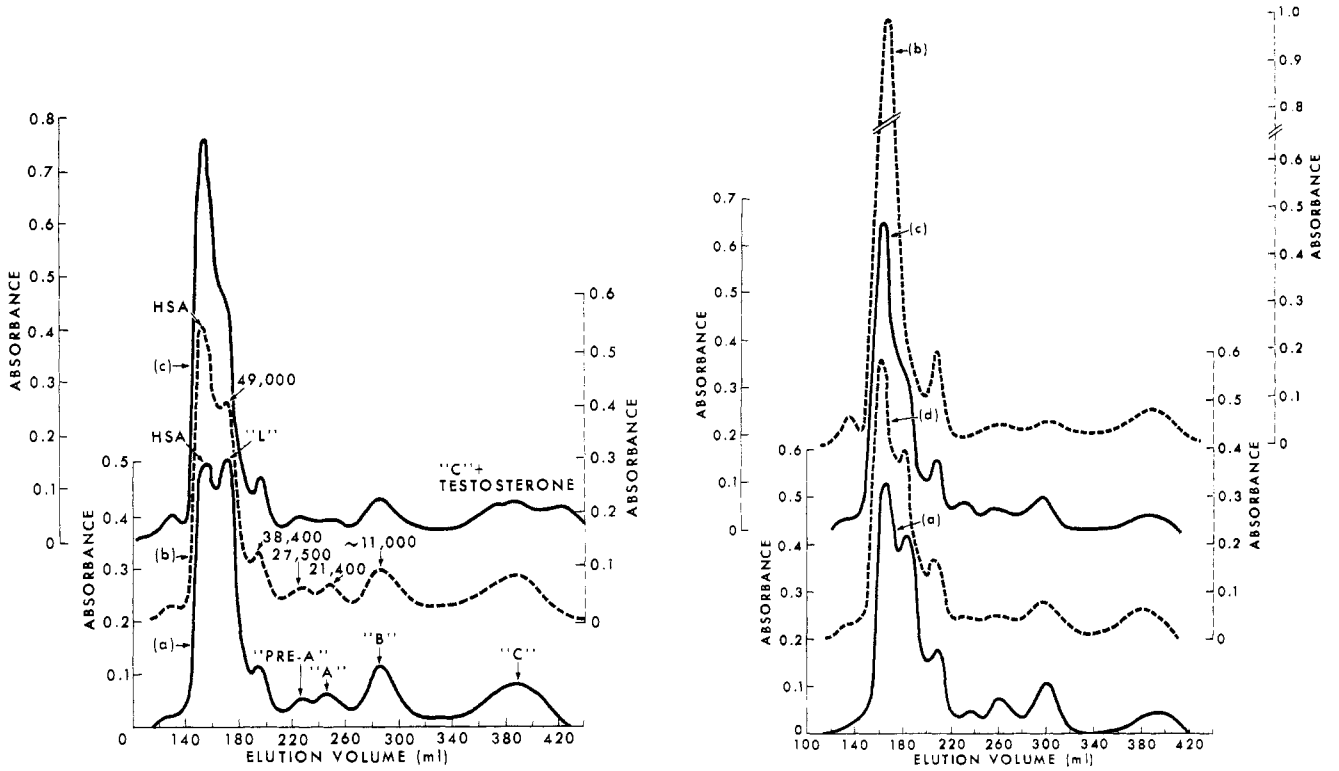

FIGURE 1: (A, left) Gel filtration patterns obtained on chromatography of 40 mg of human serum albumin (HSA) which had been digested by trypsin until 10 mol of alkali/mol (~12 bonds) of protein had been consumed, on G-100 Sephadex in 0.05 M borate buffer (pH 8.5): (a) no additives, (b) digested in presence of 2% ethanol, (c) digested in presence of 2% ethanol and excess testosterone. Enzymatic reaction stopped by addition of soybean trypsin inhibitor. Column monitored at 280 mμ using a flow cell and Bausch and Lomb precision spectrophotometer. Numbers above the peaks are molecular weights derived by calibration of the column. (B, right) Legend as in part A: (a) protein digested in presence of 2% ethanol, (b) digested in presence of 0.02 M Ca^{2+}, (c) digested in presence of 2% ethanol and excess dehydroisoandrosterone, and (d) digested in presence of 2% ethanol and excess progesterone.

specific activity are damped. Thus for example doubling the enzyme concentration (to 3.2 mg/4 ml) results in an approximately 35% increase in rate of hydrolysis of albumin. The effects of ligands in the Tos-Arg-OMe assay were zero or stimulatory and would only be quantitatively significant in the case of high concentrations of ethanol. However corrections for such stimulation have not been applied in the case of inhibitory effects in albumin hydrolysis described below.

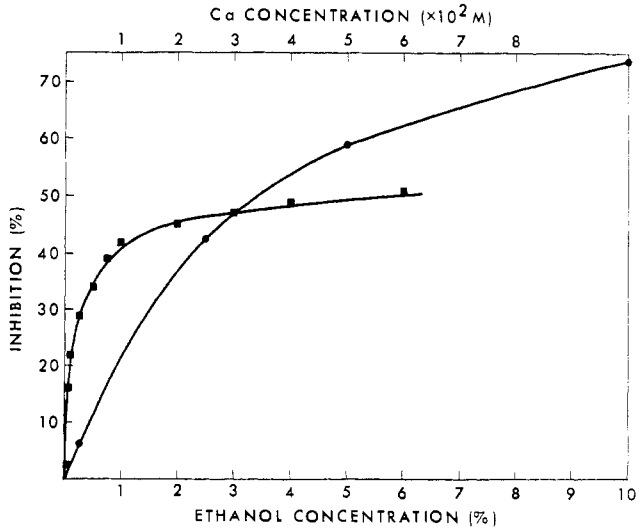

FIGURE 2: Inhibition of tryptic hydrolysis of HSA by increasing concentration of ethanol and Ca^{2+}; measurements derived from 16-min reaction in the pH Stat using 25 mg of HSA and 1.6 mg of trypsin: (●) ethanol; (■) Ca^{2+}.

Non-Steroid Ligands. ETHANOL. In contrast to its marked stimulatory effect on the esterase activity of trypsin at high concentration (Table I), ethanol has an inhibitory effect on trypsin-catalyzed hydrolysis of bovine and human albumins. The inhibition increases with increasing ethanol concentration (Figure 2). It may be noted that the inhibitory effect of ethanol is detectable at concentrations far below the 20–30% at which hydrodynamic changes are not detectable (Herskovits and Laskowski, 1962; Zakrewski and Goch, 1968), emphasizing the sensitivity of the pH-Stat technique. In the range of 0.5–1.25% ethanol used in the steroid studies, the inhibition ranges between 10 and 18%. When the product obtained by digesting 40.5 mg of albumin in the presence of 2% ethanol, until 10.4 equiv of alkali/mol is consumed, is chromatographed on G-100 Sephadex, the results of Figure 1A are obtained. The only influence exhibited by the ethanol is a slight reduction of the "L" peak and a corresponding increase in the monomer peak. Integration of the curve with a planimeter indicated that the combined monomer plus "L" fraction constituted 57.5% of the total material eluted from the column in comparison with 59% in the case of digestion in the absence of ethanol.

CALCIUM. The presence of Ca^{2+} also lowers the susceptibility to tryptic hydrolysis of serum albumin (Figure 2). As noted earlier we have observed only a small stimulatory effect of 2×10^{-2} M Ca in the Tos-Arg-OMe assay. The trypsin concentration in proteolysis is approximately 200 times that

TABLE II: Effect of Steroids on the Trypsin-Catalyzed Hydrolysis of Bovine and Human Serum Albumins.[a]

Steroid	Concn ($\times 10^4$)	%[b]
a. Bovine Serum Albumin		
C_{18} Steroids		
17β-Estradiol	5	−38
C_{19} Steroids		
Testosterone[d]	5	−4
5α-Dihydrotestosterone[d]	5	−8
Androsterone[e]	5	−23
Androsterone sulfate[e]	2.4	−37
Androsterone sulfate[e]	3.83	−55.5
Androsterone sulfate[e]	7.83	−63.5
Androsterone hemisuccinate[e]	5	−49
1-Dehydrotestosterone[d]	5	−8
Dehydroisoandrosterone[e]	5	−19
5-Androstene-3β,17β-diol	5	−17
5α-Androstane-3β,17β-diol	5	−11
C_{21} Steroids		
Progesterone	5	+21.5
6,17α-Dimethyl-6-dehydroprogesterone[c]	5	+21.5
16-Dehydroprogesterone	6	+16
3β-Hydroxy-5α-pregn-16-ene-11,20-dione	5	+12
5-Pregnene-3β,20β-diol	5	+11
5α-Pregnane-3β,20α-diol	5	+12.5
Cortisol	5	+6
C_{27} Steroids		
4-Cholesten-3-one	3	+4
b. Human Serum Albumin		
Testosterone	5	−38
Progesterone	5	−24.5
Dehydroisoandrosterone	5	−38
Cortisol	7.5	−3

[a] Based on 16-min reaction runs in the pH-Stat, using 1.6 mg of trypsin and 25 mg of albumin in a volume of 4 ml of 0.1 M NaCl containing 0.5% ethanol. [b] Expressed as percentage change in alkali consumed when compared with the blank. [c] Gift from Dr. S. Solomon, McGill University. [d] 3-Keto steroid. [e] 17-Keto steroid.

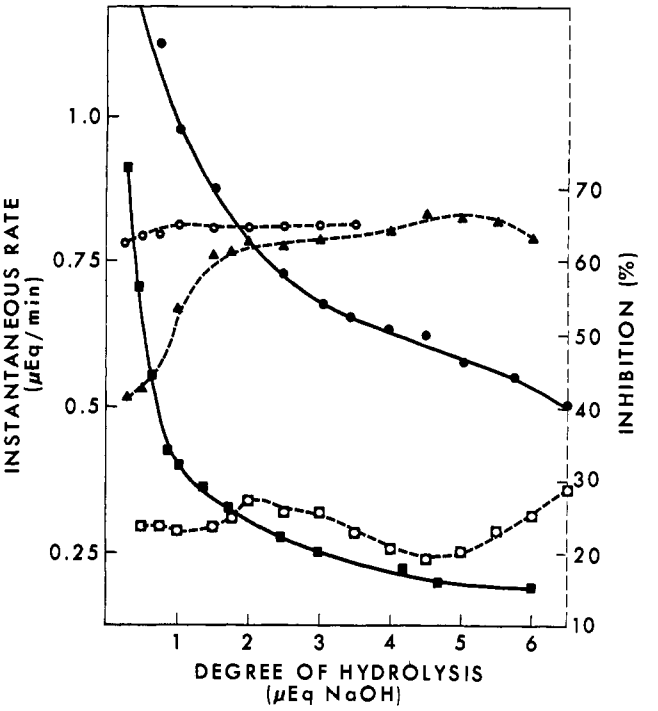

FIGURE 3: Dependence of Ca^{2+} inhibition of the instantaneous rate of tryptic hydrolysis of HSA on the degree of hydrolysis. Based on measurements in the pH-Stat using 25 mg of HSA and 1.6 mg of trypsin at pH 8.5, 25°. Values for instantaneous rates are from tangents drawn to the curve traced by the recorder: (●—●—●) instantaneous rate for albumin alone; (■—■—■) instantaneous rate in presence of 10^{-2} M Ca^{2+}; (▲—▲—▲) inhibition of the instantaneous rate for 10^{-2} M Ca^{2+}, (□□□) inhibition of the instantaneous rate for 7.5×10^{-4} M Ca^{2+}; (○- - -○- - -○) inhibition of the instantaneous rate for 6×10^{-2} M Ca^{2+}.

of the Tos-Arg-OMe assay and the rate is substrate rather than enzyme limited. Under these conditions stimulation of trypsin specific activity is restricted to only about 35% of its potential value. If one uses the value of about 18% stimulation inherent in Figure 1 of Sipos and Merkel (1971) rather than the 6.8% observed by us, the stimulation of trypsin specific activity by 2×10^{-2} M Ca would be approximately 6% under conditions where a 45% inhibition of proteolysis was observed. No attempt has been made to correct for such stimulation of the enzyme in the reporting of the results. Such corrections would of course increase the inhibition values recorded. The Ca^{2+} effect also increases with increasing concentration and the results in Figure 2 are in the form of a typical saturation curve with a minimum K_{ass} of $1-2 \times 10^3$ for this effect. This is similar to the value inherent in the results in Figure 6 of Gorini and Audrain (1952) and very much higher than either the minimum value reported by us for an inhibitory effect of Ca^{2+} on steroid binding (Ryan and Gibbs, 1970b) or that due to overall binding of Ca^{2+} to serum albumin (Pedersen, 1972). It is of interest that if the results for Ca^{2+} are plotted (Figure 3) as inhibition of the instantaneous rate *vs.* the degree of digestion, so as to control the latter as a variable, the inhibition fluctuates during the course of digestion at the low Ca^{2+} concentrations whereas a constant high level of inhibition is observed at the high levels of Ca^{2+}. This must be treated with caution until further investigation is possible, but it might reflect something of changes occurring during the digestion. In any event, the results differ somewhat from those described below for testosterone.

The pattern of products observed on G-100 Sephadex for 40.6 mg of albumin digested to a consumption of 10.4 equiv of alkali/mol in the presence of Ca^{2+} is seen in Figure 1B. Comparison with those for the blank and ethanol shows that it is characterized by an almost total elimination of fragment L, with pre-A, A, and B being considerably reduced. No new peaks appear. Integration of the curve indicates that the combined human albumin and "L" fractions now constitute 69% of the total digest. Thus, either the aromatic amino acid composition of the fragments is altered when digestion is performed in the presence of Ca^{2+}, or the splitting of ~12 bonds under these conditions is less efficient in producing fragment L, pre-A, A, and B than it is in its absence. This is similar to what has been found for Methyl Orange (Markus *et al.*, 1967b).

Effect of Steroid Ligands. STRUCTURE OF LIGAND. The effect of steroids has been studied using both bovine and human albumins. A wide range of steroids was examined in a qual-

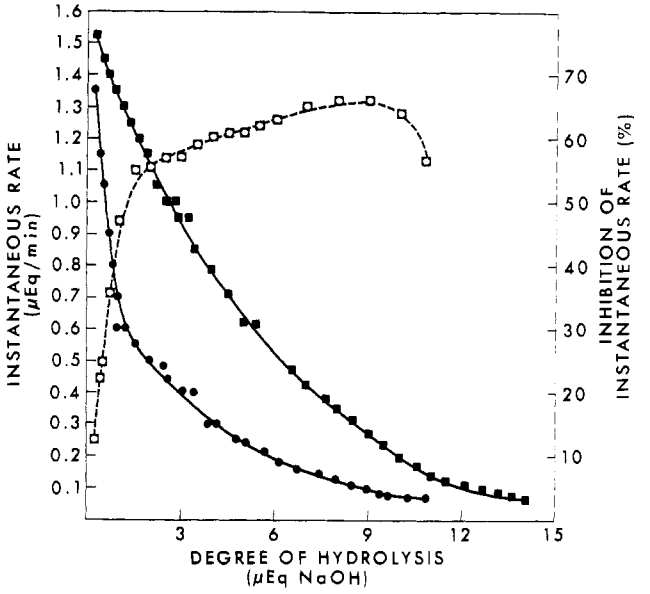

FIGURE 4: Influence of 5×10^{-4} M testosterone in the long-term hydrolysis of HSA. Instantaneous rate of hydrolysis for albumin alone (■) and in the presence of the steroid. Inhibition of the instantaneous rate (□). Measurements based on hydrolysis of 25 mg of HSA catalyzed by 3.2 mg of trypsin at pH 8.5, 25°. Instantaneous rates obtained from tangents drawn to curve traced by the recorder.

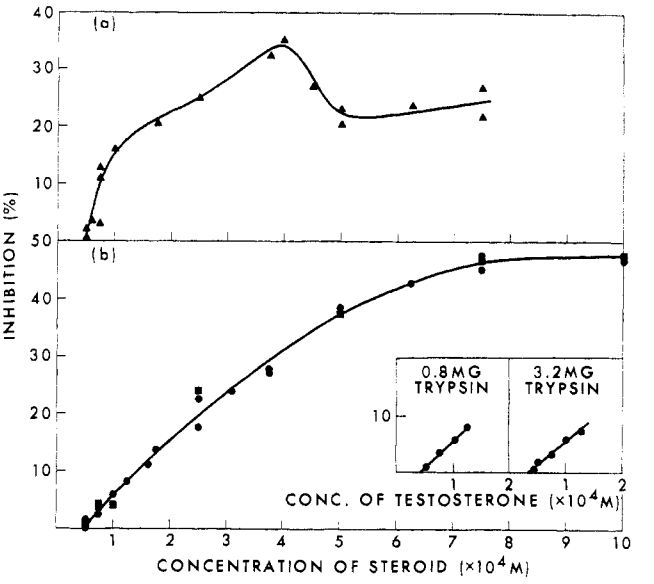

FIGURE 5: Dependence of inhibition of tryptic hydrolysis of HSA on steroid concentration; based on 10-min reaction runs in the pH-Stat at pH 8.5, 25°, using 25 mg of albumin and 1.6 mg of trypsin. Inset: 0.8 mg of trypsin and 3.2 mg of trypsin (▲), progesterone, (●), testosterone, (■) dehydroisoandrosterone.

itative manner in 16-min reactions in the case of bovine albumin, while the interactions of a few steroids with human albumin was investigated more completely using this and other techniques. Steroids (except for androsterone sulfate) were introduced into the protein solution in 10^{-3}–10^{-1} M ethanol solutions to yield a final ethanol concentration of either 0.5 or 1.25%. The significant inhibitory effect of these ethanol concentrations is eliminated from our calculations by use of the appropriate blanks. Bovine albumin (lots 16 and 18) and human albumin (lots 30 and 31) were employed. Generally similar results have been obtained with different lots and these are summarized in Table II.

Almost all of the steroids studied influence the rate of tryptic hydrolysis of the albumin, the results differing for a given steroid in the case of bovine and human albumins and also in the effects of different steroids on a given protein. In most instances, steroids were added to yield the same total concentration although in several instances (progesterone, 4-cholesten-3-one) precipitation occurred.

In the case of bovine albumin the effects fall into four categories. (i) The charged steroids, androsterone sulfate, androsterone hemisuccinate, and estradiol, have marked inhibitory effects. (ii) The C_{19} free steroids have a more moderate inhibitory effect which differs from one steroid to another, the values for the 3-keto compounds (testosterone, 5α-dihydrotesterone, 1-dehydrotestosterone) being significantly lower than the 17-keto compounds (androsterone and dehydroisoandrosterone), while the 5-androstene-3β,17β-diol and 5α-androstane-3β,17β-diol have intermediate values. (iii) The C_{21} compounds, on the other hand, all produce a stimulation of hydrolysis of bovine albumin in a manner which is in part related to the oxygen content of the molecule (*e.g.*, progesterone as compared to cortisol) although it is evident that there are other structural influences involved (contrast progesterone, 16-dehydroprogesterone, and 5α-pregnane-3β,20α-diol. (iv) The C_{27} steroid, 4-cholesten-3-one, has only a small stimulatory effect, emphasizing the difference between the two-carbon and eight-carbon side chain. The interactions of the four ligands with human albumin (Table IIb) produced only inhibitory effects. These results will be referred to further below, but we may note the marked quantitative difference in the effects of testosterone and dehydroisoandrosterone with respect to the two proteins.

EFFECT OF TEMPERATURE. In view of the fact that the specific interactions with proteins (Westphal, 1971b; Steinhardt and Reynolds, 1969e) tend to show a marked temperature dependence of the binding affinity, a study was made of the role of temperature in the steroid inhibition of tryptic hydrolysis of bovine and human albumin. In the case of human albumin, the temperature sensitivity of the inhibitory effect is almost identical for progesterone, testosterone, and dehydroisoandrosterone, the inhibition decreasing from 45 to 30–35% in the temperature range of 10–45°. The differences between bovine and human albumin noted above for measurements made at 25° extend over a wide range of temperatures. In the case of bovine albumin there is only a slight drop in the effect of the C_{19} steroids between 10 and 35° and at 45° the inhibition is slightly higher than at 35°. The stimulatory effect of progesterone on bovine albumin hydrolysis shows the greatest temperature dependence, the value at 35° being only 20% of that at 10°.

GEL FILTRATION PATTERNS WITH STEROID LIGANDS. As can be seen from Figure 1, the pattern obtained on chromatography of the products formed after tryptic hydrolysis of human albumin in the presence of saturating levels of steroid ligands does not differ greatly from that obtained in the absence of steroid. There is a definite lowering of the amount of the 49,000 fragment and an increase in the monomer fragment in the case of testosterone and dehydroisoandrosterone but only a slight effect in the case of progesterone. The effect of the first two steroids is very much less than that produced by 2×10^{-2} M Ca, although these concentrations give about the same level of inhibition of hydrolysis. Approximate quantitation by integration shows that Ca also leads to a greater

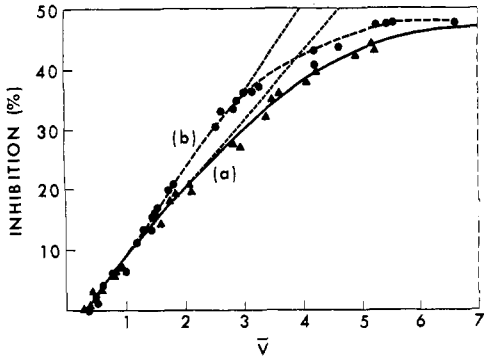

FIGURE 6: Relation between inhibition of tryptic hydrolysis of HSA and level of bound steroid ($\bar{\nu}$) for testosterone (▲) and dehydroisoandrosterone (●).

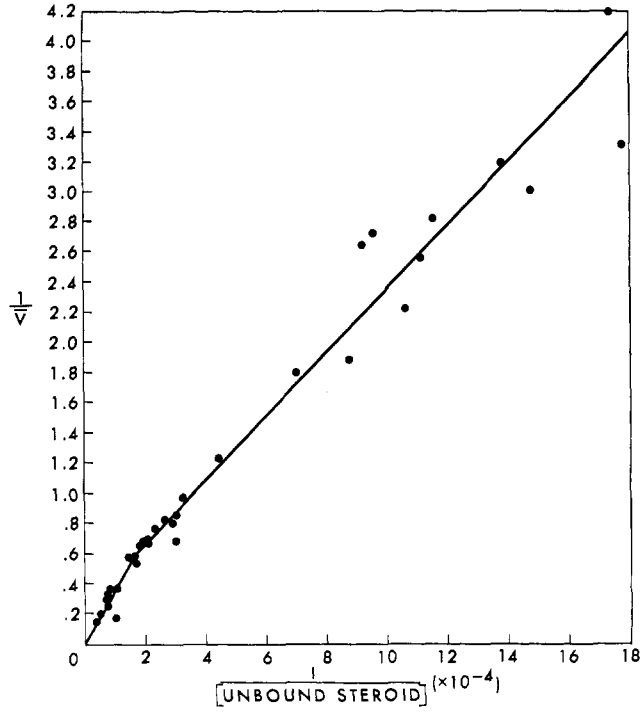

FIGURE 7: Reciprocal plot of binding data for the interaction of dehydroisoandrosterone with 9.1×10^{-5} M HSA in 0.1 M NaCl–1.25% ethanol at pH 8.5, 25°.

diminution of pre-A, A, and B; in fact pre-A could not be detected when Ca is present.

DEPENDENCE OF INHIBITION ON DEGREE OF HYDROLYSIS. The pathway of tryptic hydrolysis of human albumin is, like the sequence structure of the molecule, unknown but undoubtedly complex. Since albumin has 86 trypsin-sensitive bonds, of apparently different degrees of susceptibility, it was of interest to follow the steroid inhibitory effect over a longer period of time, until approximately 30% of the trypsin-sensitive bonds had been split. From the data so obtained instantaneous rates of hydrolysis were obtained, and were plotted against degree of hydrolysis as rate profiles. Comparison of the shape of the curve for testosterone in Figure 4 with that of the blank suggests that the pathway of tryptic hydrolysis of human albumin must be more influenced by the presence of steroid than is suggested by the gel filtration data. The plot of "instantaneous" inhibition against degree of hydrolysis obtained from these rate profiles shows that the inhibitory effect of the testosterone starts out at a low value of 15% and increases with degree of hydrolysis to about 65% when 25 mol of alkali/mol of protein have been consumed. This differs somewhat from what is observed in the case of Ca^{2+}.

RELATION BETWEEN INHIBITION AND STEROID CONCENTRATION. Because of the multiplicity of binding sites in human albumin for a given steroid, we examined the dependence of inhibition on concentration in the case of testosterone, dehydroisoandrosterone and progesterone (Figure 5) and also, in the case of the first two steroids, on the level of bound steroid (Figure 6). The curves for testosterone and dehydroisoandrosterone are rather similar, the inhibition increasing in the form of a saturation curve with increasing steroid concentration until a maximum of about 47.5% is obtained at 10^{-3} M total added steroid. Progesterone differs in that the maximum inhibition is about 35% at a concentration of 4×10^{-4} M. At higher concentration the inhibition drops abruptly to about 25% suggesting a possible transition of the protein to a more digestible form. In spite of this difference however, the three curves are similar at lower concentration and all are characterized by the striking fact that they do not pass through the origin but intercept the concentration axis at about 5×10^{-5} M, corresponding to $\bar{\nu} = 0.4$ in the case of the C_{19} compounds (Figure 6). The later value is independent of the concentration of trypsin (Figure 5 inset).

Results of Figure 6 show also that, in both cases, not all bound steroid is directly linked to inhibition; extrapolation of the linear portion of the curve suggests that a *maximum* of four sites are implicated—less than the measured saturation values (see below). The number could be even less than this.

Representation of Binding Data. While the results of binding measurements performed under the conditions of the pH-Stat experiments have been adequate to establish the relationship between inhibition and binding, their precision has been less than desirable for attempts to derive values of the binding parameters. It was considered that the use of ethanol solutions of steroid would facilitate measurement of binding over the entire binding range. A reciprocal plot for dehydroisoandrosterone is presented in Figure 7. The scatter of the points may be due in part to an accentuation of an inherent drawback of the Sephadex method under the conditions of our measurements or to inherent instability of the steroid–protein equilibrium in the presence of ethanol and absence of buffer. Ethanol clearly affects the digestibility of the protein (Figure 2) but the concentrations used by us are far below what is required to affect the hydrodynamic properties of the protein. Medium chain alcohols are known to bind to bovine albumin with an affinity similar to that of steroids (Ray *et al.*, 1966). While these also exhibit very marked effects on the rate of denaturation of hemoglobin (Cassat and Steinhardt, 1971) they are bound with an affinity too low to be measured. The estimate of the binding affinity (*nk*) which may be obtained (Klotz and Hunston, 1971; Schellman *et al.*, 1954) from the lower binding range of a reciprocal plot (not shown) of the data for testosterone yields a value of 3.74×10^4. This is in keeping with values published by us and others (Ryan and Gibbs, 1970b; Westphal, 1971c) and does not suggest any significant influence of the concentrations of ethanol used. The corresponding value for dehydroisoandrosterone, derived from the data of Figure 7, is 4.6×10^4. The data in the higher binding range of these plots (*e.g.*, Figure 7) departs from linearity and the number of sites is not the value of 5 suggested by extrapolation of the curve in the lower binding

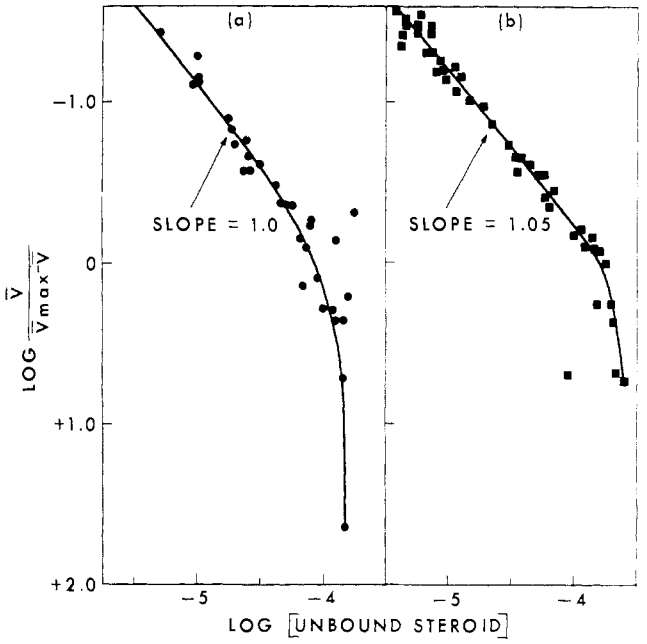

FIGURE 8: Hill plot of data (a) for testosterone (b) and dehydroisoandrosterone. $\bar{v}_{max}$ is the saturation molar binding ratio.

region. We have obtained saturation molar binding ratios of 5.25 and 6.6 for testosterone and dehydroisoandrosterone, respectively. If the binding data for the two steroids is cast in the form of titration curves (not shown) the binding range corresponding to 10–90% of these saturation values covers 1.1–1.2 log units of free ligand concentration, rather than the value of 1.9 expected for simple binding or >2 for heterogeneous binding (Daniel and Weber, 1966). This suggests that the departure from linearity in the high binding region is to be attributed rather to a "cooperative" phenomenon and is more convincingly shown by Hill plots (Figure 8) of log $\bar{v}/\bar{v}_{max} - \bar{v}$ against log [unbound steroid], where $\bar{v}_{max}$ is the saturation value referred to above. These plots exhibit a straight line with a slope of 1 up to binding levels of 2 and 3 for testosterone and dehydroisoandrosterone and a marked increase in slope at higher binding levels. A plot of $\bar{v}$ against [unbound steroid] (not shown) shows a clear inflection between $\bar{v} = 2$ and 3 in the case of dehydroisoandrosterone, but not in the case of testosterone.

Discussion

It is known that steroids influence the catalytic sites of some enzymes (Douville and Warren, 1968) but this does not seem to be the case for trypsin. Analogy with the results of a study made with dyes (Markus *et al.*, 1967b) suggests that the effects observed in this study of the tryptic hydrolysis of serum albumins are due to ligand induced conformational changes which alter either the choice of susceptible bonds or their accessibility to the enzyme, leading to a reduction of the rate of hydrolysis. That inhibitory effects are produced by such different ligands as dyes, steroids, Ca^{2+} and ethanol bears out the above interpretation. The Ca^{2+} effect was originally observed by Gorini and Audrain (1952) who attributed it to blocking of the susceptible bonds, both directly by Ca^{2+} and indirectly by displacement of a mixture of two forms of the protein as a consequence of complexation. According to Steinhardt and Reynolds (1969g) stabilization of proteins by ligands is a very general phenomenon. It occurs—at low levels—even in the case of such unfolding ligands as dodecyl and tetradecyl sulfates interacting with serum albumin (Steinhardt and Reynolds, 1969h). Destabilization effects are also known, of which the data reported here for pregnane and cholestane derivatives interacting with bovine albumin might be taken as an example. However, the effects detected by enzymatic approaches such as this might involve no more than a change in the motility of the peptide segment containing the susceptible bond (Linderstrøm-Lang and Schellman, 1959). There is some optical rotation evidence (Alfsén, 1963) that testosterone induces conformational change in albumin, but no general study of such conformational effects has been made. The results of this paper, however, tend to suggest that the mechanism by which inhibition is effected differs from one ligand to another, *i.e.*, site occupancy by different ligands stabilizes different conformations. Thus the study of the gel filtration patterns of hydrolysis products, obtained after a given degree of hydrolysis, indicates marked deviation from the uninhibited pattern in the case of Ca^{2+} and of dyes but little alteration in the case of ethanol or steroid. The dependence of inhibition on the degree of hydrolysis, as seen in the rate profiles (Figures 3 and 4), also differs. In the case of testosterone, the data could be interpreted either as a diversion of the initial pathway of hydrolysis so as to lead to fragments which are more trypsin resistant or to a progressive increase in the affinity for testosterone as hydrolysis proceeds. In this connection, it is known (King and Spencer, 1970) that the 40,000 fragment liberated by trypsin from the carboxyl terminal of bovine albumin has a diminished affinity for ligands. The gel filtration data however, while underlining the differences between ligands, tend to suggest that it is Ca^{2+} rather than steroids which influences the pathway of initial fragmentation.

The lack of specificity (Westphal, 1971c) as well as the multiplicity of binding sites characteristic of serum albumins might seem to preclude any rigid structural requirements in interactions with steroids. Study of the inhibitory effect indicates that there are significant qualitative and quantitative differences, not only in the interactions of different steroids with either bovine or human albumin, but in the interactions of a given steroid with both proteins. It is evident that, since the data listed in Table I could involve either an effect of structure variation on binding affinity or of site occupancy on conformational change, clear inferences can be made only when all of the appropriate binding measurements have been made. However, the marked qualitative differences between C_{21} and C_{19} steroids in the interactions with bovine albumin, as well as the quantitative differences between, for example, progesterone and 4-cholesten-3-one, C_{19} 3-keto steroids and C_{19} 17-keto steroids, and the differences in temperature sensitivity between progesterone and testosterone, clearly involve more subtle structural influences in the relation between site occupancy and conformation stabilization than in the case of Methyl Orange and Crystal Violet interacting with human albumin (Markus *et al.*, 1967b). Furthermore while the reported binding affinity (*nk*) for the interaction of estradiol with bovine albumin is of the same order ($9.1–72 \times 10^4$; Westphal, 1971c) as for the steroid sulfates, the inhibitory effect of the latter is much greater. The marked effect of charge on the sulfate ligand is evident in a comparison of the data for androsterone and its sulfate (Table II). This is undoubtedly related to the existence of single higher affinity site in bovine albumin for this compound ($k_{ass} = 3.6 \times 10^5$; Plager, 1965).

It should be noted here that our results differ from those of Bellamy and Leonard (1966) who, in a study of the effects of ligands on the liberation of trichloroacetic acid soluble peptides by tryptic hydrolysis of bovine albumin, found only stimulation of hydrolysis by testosterone, corticosterone, 17β-estradiol and other ligands.

Marked differences in behavior are encountered when human albumin is contrasted with bovine albumin. The four steroids studied (Table Ib), including the C_{21} compounds, produced only inhibitory effects and, unlike the interactions with bovine albumin, testosterone and dehydroisoandrosterone produced very similar values for inhibition (see also Figure 6). This is in reasonable agreement with our binding data. The very low value for cortisol (Table II) is almost certainly due to the fact that its binding affinity for human albumin is less than 10% of that of testosterone (Ryan and Gibbs, 1970b) but a comparison at the same binding level would be of interest in view of the apparently small contribution of hydrophobic forces in the binding of this steroid (Ryan and Gibbs, 1970a). The binding affinity of progesterone for human albumin is higher than that of testosterone (Westphal, 1971c), yet its inhibitory effect is significantly lower. Its effect on the gel filtration pattern (Figure 1) is also less than that of testosterone. These differences between progesterone and the other steroids are accentuated further at slightly higher concentrations (Figure 5). It is possible here that the inhibitory effect in the case of progesterone is a composite of a stimulatory effect (as seen with bovine albumin) and an inhibitory effect.

The most interesting feature of the data reported in this paper, which further distinguishes steroid from non-steroid ligands, is that the inhibitory effect in the case of human albumin appears only above a threshold level of $\bar{\nu} = 0.4$ (Figure 6). The binding data, when plotted as $\bar{\nu}$ *vs.* [unbound steroid] (not shown), do not show any indication of high-affinity contaminants in the albumin and any possible explanation involving binding to trypsin has been eliminated by the demonstration that the threshold value is independent of the concentration of trypsin (Figure 5, inset). The possibility of a steroid induced dimerization, or further aggregation, of albumin was discounted by results of gel filtration studies (see Methods). That this threshold effect is not an artefact is attested to by the fact that the plot of inhibition against steroid concentration is markedly influenced by the presence of Ca^{2+}. The latter produces a significant increase in the value of the intercept on the concentration axis (M. T. Ryan, unpublished). It is conceivable that the inhibition is mediated by a dimerization of the albumin in the transition-state complex and in this context it is of interest that trypsin apparently has two neighboring active sites in the molecule (Keil, 1971). It is hoped that further investigation of the Ca^{2+} effect will lead to some insight into the phenomenon.

The results of binding measurements, performed in these studies over a wider concentration range than is generally the case for steroids, indicate that the binding behaviour may be more complex than might have been anticipated. Published data suggest that the value for the number of androgen binding sites is between 2 and 10 (Westphal, 1971c; Ryan and Gibbs, 1970b). Such data have generally involved measurements over the lower binding range and is subject to criticism, at least with respect to values for n (Klotz and Hunston, 1971; Schellman *et al.*, 1954). We have obtained experimental saturation values of 5.25 and 6.6 for testosterone and dehydroisoandrosterone and plots of the data (Figure 8) suggest that the simple binding occurs only to two to three sites. It does not seem that the difference between these values and the saturation values is due to the presence of a preexisting second set of weaker sites, as is true in the case of corticosterone and steroid sulfates (Slaunwhite *et al.*, 1963; Plager, 1965). The deviation from linearity of the Hill plot is more in keeping (Daniel and Weber, 1966) with a binding induced conformational change which exposes, in a cooperative manner, new weaker sites. This is the kind of behavior which has been established for the interaction of dodecyl sulfate and bovine albumin, where the binding data can be fitted to a two-state binding isotherm (Steinhardt and Reyholds, 1969g). Both the polarimetric data of Alfsen (1963) and the inhibitory effect favor the notion of a conformational change. While the binding data indicate a maximum of four sites linked to inhibition, this number could be smaller, such as the value 2 obtained for a similar effect by Methyl Orange (Markus *et al.*, 1967b). This dye is known to exhibit a weak inhibitory effect on testosterone binding (Schellman *et al.*, 1954). It is thus tempting to suggest that those sites whose occupancy induces the inhibition phenomenon are the same as those to which simple binding occurs and that the conformational change suggested as being responsible for inhibition is that which provides for the appearance of new sites. It may finally be noted that the location of the midpoint of the simple binding range suggests a value for k_{ass} for testosterone of $\sim 3 \times 10^4$, close to the "average" value derivable from published data (Westphal, 1971e; Ryan and Gibbs, 1970b), if one assumes a value of $n = 2$, but lower than that quoted above for the single site for steroid sulfates. The study of relationships in the case of bovine albumin remains for future work, particularly that between binding and both stabilization and destabilization with different classes of steroid.

It is hoped that further quantitative investigation, correlated with direct studies of protein conformation by techniques such as ORD, viscosity, etc., in the case of these and other steroids, may lead to an understanding of the structural and other subleties in the above data, as well as to a better definition of the nature of the binding site and the conformational changes which its occupancy induces.

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