from 177–181° for all determinations. Gas was evolved on melting but no discoloration was observed. (Done and Fowden reported melting with decomposition in a sealed tube between 174–183°.)

As further proof of these relationships, γ-methyleneglutamine from the tulip was hydrolyzed to the γ-methyleneglutamic acid which in turn was hydrogenated and proved to be chromatographically identical with the synthetic γ-methylglutamic acid as furnished by Dr. Fowden.

Through the interest and coöperation of Dr. Alton Meister of the National Cancer Institute, Bethesda, it was possible to investigate the enzymatic oxidation of the isolated γ-methyleneglutamine. The effect of L-amino acid oxidase of rattle snake venom was followed manometrically by the method of Meister[5] and it was found to be oxidized to the extent of 65% as compared with 85% for L-glutamine and 88% for L-asparagine. The isolated material may tentatively be considered to have the L-configuration.

As a result of the evidence documented above, it is now possible to conclude that the substance recognized and described[1] as Unknown No. 12 in the tulip bulb is identical with the substance described as γ-methyleneglutamine by Done and Fowden.[4] Also the substance described as Unknown No. 4 in the tulip bulb is to be regarded as γ-methyleneglutamic acid. Both of these substances exist free in the tulip plant and their metabolic role will be of great interest.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY, SANTA BARBARA COTTAGE HOSPITAL RESEARCH INSTITUTE]

The Dispersion of Testosterone in Aqueous Bovine Serum Albumin Solution

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By means of dialysis through a semipermeable membrane it is shown that the distribution of testosterone between water and aqueous bovine serum albumin solution follows the classic laws of distribution between two phases, based on the ratio of solubilities in these phases, over a sixfold concentration of albumin, a shift of *p*H from 5.2 to 7.4 and a twelve-fold change in testosterone concentration. At *p*H 5.2 and saturation, the albumin adsorbs a maximum of 3 moles testosterone per mole albumin, at *p*H 7.4, 4 moles. According to the Scatchard concept, there is one class of adsorbing points, the number of points being large. Under the conditions of dialysis the testosterone in the water phase is in a metastable state in which the solubility exceeds that previously reported; albumin thus brings about an increase in the fugacity of testosterone in the water phase. The conditions under which the two solubility equilibria are attained are described and considered according to the Gibbs concept. The extinction coefficient for testosterone, not previously reported in aqueous solution, was ascertained.

Introduction

The marked dispersive action of serum albumin solutions upon testosterone and other steroids[1–3] is undoubtedly a governing factor in the transport of steroids in body fluids and has led to the preparation of solutions suitable for intravenous injection.[4–6] Testosterone appeared idealy suited for a study of the nature of the attraction between albumin and an un-ionizable steroid, since its solubility in water is sufficiently high to assure accuracy in analysis. The orientation by adsorbents of organic ions of various sizes, as well as inorganic ions has been the subject of intensive investigation.[7,8]

The original plan, which was successfully consummated, sought to obtain the equilibrium concentrations of testosterone in water and in an albumin solution separated by a semipermeable membrane. The results were such that the solubility of testosterone in water had to be reinvestigated. In the case of progesterone, solubility depended upon two equilibrium phases in aqueous non-protein solution.[1] An analog was therefore sought in the case of testosterone and found.

Experimental

Equilibrium between Water and Albumin.—Armour crystalline bovine serum albumin and Schering testosterone were used in these experiments. The dialyzing chambers were replicas of those described by Jorgensen.[9] The junction between the two chambers and the dialyzing membrane was sealed with paraffin, which is ideally suited for the purpose as it was shown not to adsorb testosterone from aqueous solutions.

The dialysis was carried on in a cabinet at 37.5° with rocking by a mechanical shaker. Preliminary experiments indicated that equilibrium in dialysis was established in 24 hours. This was confirmed by the experiments in which the results for transfer from albumin to water and water to albumin are in substantial agreement.

The membrane used was a vegetable parchment supplied by Central Scientific Company and was selected because the adsorption of testosterone by it was nil. The blank was appreciably reduced by washing with distilled water and air drying. In testing for albumin leakage across the membrane, it was found that the foam test was more sensitive than chemical tests. A concentration of 1 part albumin in 100,000 parts water is detectable by the foam test. In all the experiments reported, the leakage of albumin, if any, into the water phase did not exceed this concentration. It was necessary to reject a considerable number of experiments because of imperfect parchment.

(1) F. Bischoff and H. R. Pilhorn, *J. Biol. Chem.*, **174**, 663 (1948).
(2) F. Bischoff and R. E. Katherman, *Am. J. Physiol.*, **152**, 189 (1948).
(3) F. Bischoff and R. E. Katherman, *Federation Proc.*, **11**, No. 1, 188 (1952).
(4) F. Bischoff, R. E. Katherman and V. Favati, *Am. J. Physiol.*, **165**, 667 (1951).
(5) C. D. West, *Endocrinology*, **49**, 467 (1951).
(6) I. Rothchild, *ibid.*, **50**, 583 (1952).
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(8) I. Langmuir, THIS JOURNAL, **40**, 1361 (1918).
(9) K. S. Jorgensen, *Acta Pharmacol. Toxicol.*, 1, 263 (1945).

TABLE I

DISTRIBUTION OF TESTOSTERONE BETWEEN WATER AND BOVINE SERUM ALBUMIN SOLUTION ACROSS A VEGETABLE MEMBRANE

*p*H	Albumin, g./100 ml.	C_{alb} concn. testosterone in mole/l., alb. side $\times 10^4$	C_{H_2O} concn. testosterone in mole/l., H_2O side $\times 10^4$	Starting side	Recovery, %	$C_{H_2O}S_{alb}/C_{alb}$	$1/k$	$\frac{\nu}{(A)} \times 10^{-4}$
5.4	0.95	4.20	1.47	Alb	100	1.94	5.1	1.4
5.4	.93	3.99	1.36	Alb	101	1.83	4.8	1.5
5.4	.93	3.18	1.09	Alb	99	1.84	4.9	1.5
5.2	1.00	2.88	0.936	Alb	[a]	1.92	4.8	1.5
5.3	0.96	0.62	.21	H_2O	97	1.90	4.9	1.4
5.3	0.95	0.332	.125	H_2O	99	2.08	5.7	1.2
7.3	1.00	3.41	.972	Alb	[a]	1.92	4.0	1.8
7.4	0.92	2.64	.753	Alb	100	1.88	3.7	1.9
7.4	0.93	0.565	.183	H_2O	96	2.01	4.4	1.6
5.2	6.2	5.6	.43	Alb	[a]	1.95	5.1	1.4
						1.95 ± 0.025	*p*H 5.3, 5.0 $\pm$ 0.12	
							*p*H 7.4, 4.0	

[a] Experiments assayed by the Zimmermann reaction for which acknowledgment is given to Mr. Yee Sing Yee.

In order to determine testosterone in water and in aqueous albumin solution, the optical densities were measured at 249 and 239.5 mμ in a Beckman spectrophotometer with silica cells, 1 cm. path. Controls for both the water and albumin phases served as blanks and were subtracted to obtain the testosterone level. The densities of testosterone and albumin in the same aqueous phase were found to be additive. At a concentration of albumin of 0.243 and 0.121 g. per 100 ml., the recoveries of testosterone (6.5 and 3.25 γ per ml.) were complete within the error of experimentation. Neither the extinction coefficient nor the wave length at maximum extinction had previously been reported for testosterone in aqueous solution. The maximum extinction was found to be at 249 mμ with a molecular extinction coefficient of 15,800.

In order to determine the shift of water to the albumin side, a sample of 0.96 g. albumin per 100 ml. was dialyzed against water and against itself for 24 hours at 37.5°. A one-to-eleven dilution was read at 249 mμ. The O.D. of the undialyzed solution read 0.271, the phase dialyzed against water read 0.288 and the phase dialyzed against itself read 0.286. There is no significant dilution of the albumin concentration. The difference between the dialyzed and undialyzed samples is the membrane blank.

Since crystalline bovine serum albumin contains approximately 1 mole fatty acid per mole albumin,[10] it was necessary to establish that this compound or any others that may be adsorbed by the albumin did not dialyze through the membrane and affect the distribution of testosterone in the aqueous phase. A solubility determination of testosterone was made in the aqueous phase which had been dialyzed against an albumin solution for 24 hours. The conditions[1] were those which previously gave a solubility of 1.26×10^{-4} mole/liter in water. At 70 hours equilibration, the results for the aqueous phase of the dialysate were 1.20×10^{-4} at *p*H 5.3 and 1.16×10^{-4} at *p*H 7.4.

The results of the dialysis experiments are given in Table I.

Solubility in Water.—The experiments are recorded in Table II. Experiment 1 was a repetition of the original equilibration conditions,[1] using the original testosterone, m.p. 153.7–154.2°, but assaying by ultraviolet extinction instead of by the Zimmermann reaction. The agreement 1.23×10^{-4} mole/l. *vs.* 1.26×10^{-4} mole/l. is excellent. Experiment 2 shows that the original testosterone has a higher solubility on a short equilibration period with gentle stirring, but the low solubility is approached with time and vigorous shaking. The other experiments are with a new batch of testosterone, m.p. 153.4–154.2°. The results of experiment 3 are much like those of experiment 2. Experiment 4 shows that the high solubility is reached in about 5 hours and that the filtrate remains supersaturated. The insoluble residue does not give the high solubility value. In experiment 5 solubility is approached from supersaturation. The high solubility value is not attained. The m.p. of the solid phase which crystallized out was 154.0–154.2°. On heating this residue to 150° and equilibrating with new solvent, the high value of 1.81 is obtained. The unheated residue gave a value of 1.40. Experiments 6 and 7 demonstrate that with only gentle stirring the equilibrium value is approached with time.

(10) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

TABLE II

THE SOLUBILITY OF TESTOSTERONE IN WATER AT 37.5°

(0) Refers to testosterone previously used[1]; (n) refers to new batch of testosterone; V indicates vigorous agitation with mechanical shaker; G indicates gentle agitation by slow rotation; N indicates no agitation

Expt.	Mg. solute per 100 ml.	Pretreatment of solute and solvent	Equilibration time in hr.	Solubility, mole/l. $\times 10^4$
1	35(0)	None	94V	1.23
2	10(0)	None	7G	1.76
			175G	1.50
			182V	1.38
3	30(n)	None	6G	1.80
			24	1.62
			102	1.47
			270	1.35
			365	1.33
4	20(n)	None	4G	1.93
			5	1.96
		Filtrate of above	237N	2.05
			453	2.04
		New solvent with above residue	22G	1.21
			46	1.29
			77	1.34
5	35(n)	Solution at 80–90°	144G	1.44
			192	1.43
		New solvent with above residue	116	1.40
		Solid phase heated to 150° plus new solvent	194	1.81
		Filtrate of above, seeded with (n)	21	1.82
6	6.0(n)	Solution at 80–90°	28G	1.83
			144	1.50
			456	1.45
			623	1.32
7	7.0(n)	Solution at 80–90°	48G	2.24
			312	1.31

In order to demonstrate that the high solubility values for testosterone obtained under certain conditions were not due to an impurity or to a hydrated or enol form, the following experiment was performed.

Forty-nine mg. of testosterone with 250 ml. of water was equilibrated for 4 hours in a 37.5° bath with gentle stirring.

The optical density of a filtrate was scanned in the range 226 to 274 mμ; the only peak was found at 249 mμ. 240 ml. of the filtrate was evaporated to dryness in a weighed 500 ml. florence flask, by boiling at atmospheric pressure and finally drying over $CaCl_2$ at atmospheric pressure and room temperature. A similar control flask with 240 ml. of water was carried through all operations as a tare. The weight of the testosterone was ascertained. The flasks were then heated to 98° for 20 hours with no change in weight of testosterone. The extinction coefficient by this method was 15,400 and the concentration in the original filtrate 2.02×10^{-4} mole/l. The m.p. of this residue was 152.6–153.2°. There is no evidence from these data that the higher solubility was due to an impurity. The $\Delta^{2,4}$- or $\Delta^{3,5}$-enol forms are definitely ruled out, as the maxima for these forms would be closer to 270 and 230 mμ, respectively.

Although the solubility in the water phase as calculated from the distribution coefficient and solubility in albumin exceeds the solubility in water obtained on prolonged equilibration with solid testosterone, the actual concentration obtained experimentally exceeded this low solubility only in the experiments performed at the higher concentration, *viz.*, with saturated steroid solution. In order to establish that the high solubility was not due to aggregate crystals of testosterone in colloidal solution the following experiment was performed to increase the testosterone concentration in the water phase and test the state of aggregation: A saturated albumin solution containing a suspension of testosterone was equilibrated against water under the usual experimental conditions. After 24 hours equilibration the undiluted water phase and a 1:1 dilution were read in the spectrophotometer at 270 mμ. The optical densities were 0.692 and 0.343, respectively, indicating no aggregation of particles. The concentration of testosterone was 1.67×10^{-4} mole/l. liter in the undiluted water phase or a 35% increase over the low water solubility. Colloidal suspensions of testosterone, prepared by adding an ethanol solution of testosterone to water, did not follow Beer's law on diluting the sample so that solution was complete.

Results

The mathematical treatment of the adsorption from solution of a solute by proteins, either in or out of solution, has involved the testing of the goodness of fit to the Freundlich[11] adsorption isotherm, to derivations of Langmuir,[8] which take into account the lattice surface structure, and to derivations of Scatchard[12] for determining the equilibrium constant for ions bound to points on the protein molecule, the number of such points, and the number of classes of points. Our data are unique in that in addition to the distribution data we have the solubility data for each phase, so that according to the classic distribution coefficient, our distribution between the two phases should be in proportion to the solubilities in each phase, provided the activity coefficient is 1.0. Since we have the distribution data in each phase, and the solubility in the albumin phase, the solubility in the water phase may be calculated according to this concept.

$$S_{H_2O} = \frac{C_{H_2O} \times S_{alb}}{C_{alb}}$$

where S is the solubility and C is the concentration of testosterone in water or albumin solution as indicated by the subscripts.

The solubility of testosterone at 37.5° and at *p*H 5.3–5.5 in 1.00 g. of albumin per 100 ml. of solution was 5.9×10^{-4} mole/l.; at *p*H 7.4, 6.7×10^{-4} mole/l. In 0.92 g. of albumin per 100 ml. it was 5.3×10^{-4} at *p*H 5.3. In the treatment of the data the relation between testosterone solubility and albumin concentration was assumed to be linear over this small range. The solubility of testosterone at 37.5° in 6.2 g. of albumin per 100 ml. is 25×10^{-4} mole/l. at *p*H 5.2.

The calculation of solubility in water, S_{H_2O}, is given in column 7, Table I, and the agreement is remarkably close. The solubility in H_2O for values obtained over a twelve-fold concentration of testosterone, a six-fold concentration of albumin and shift of *p*H from 5.2 to 7.4 varies from 1.83×10^{-4} mole/l. to 2.08×10^{-4} with a mean value of 1.95 ± 0.025. It should be noted that this solubility value is considerably higher than the value obtained on equilibrating water and testosterone until an equilibrium value is obtained.[1]

The agreement of the distribution data is also good when the exponential constant n in the Freundlich equation is equal to 1.0. The value which deviates most is the one for the low concentration of testosterone, in which range the experimental error approaches the deviation. Grollman[7] found the equation to be a good approximation in the study of phenol red adsorption by blood proteins when n was 1. In our experiments the influence of *p*H is noted, k depending upon the *p*H.

$$X/m = kC^{1/n}$$

where X/m is millimoles testosterone adsorbed per g. of albumin and C is concentration testosterone in millimoles per liter in the water phase at equilibrium. $1/k$ for this equation is given in column 8, Table I, with n equal to 1.

When n is 1 in the Freundlich equation the amount adsorbed is proportional to the concentration in the aqueous phase. Langmuir[8] has pointed out that the Freundlich constant n approaches 1 at low concentrations and that the Langmuir equation for simple adsorption approaches the Freundlich equation under these conditions. The general Scatchard equation is[12]

$$\frac{\bar{\nu}_{Aa}\, e^{2w z_P z_A}}{(A)\nu_A} = k^{\circ}_{Aa}\,(n_a - \bar{\nu}_{Aa})$$

The above equation simplifies to

$$\bar{\nu}/(A) = k\,(n - \bar{\nu})$$

under our experimental conditions because (1) the activity coefficient of testosterone[1] is 1.0, (2) z_A is zero because testosterone is not ionized, and (3) there is only one class of points on the albumin molecule, since $\bar{\nu}/(A)$ is a constant over the range of our experimental concentrations (see Table I).

Since $\bar{\nu}/(A)$ is constant giving a curve parallel to the abscissa when plotted against $\bar{\nu}$, n, the number of adsorbing points, must be large in relation to the maximum $\bar{\nu}$ observed (3 moles testosterone/mole albumin, *p*H 5.3, 4 moles testosterone/mole albumin, *p*H 7.4, both at saturation). This result is not unique. We have taken the liberty to plot data obtained by Klotz and Ayers[13] for the distribution of *p*-aminoazobenzene and chrysoidin between crystalline bovine serum albumin solution and aqueous buffers. At *p*H 9.2, 0° in glycinate buffer *p*-aminoazobenzene gives a curve similar to

(11) H. Freundlich, "kapillarchemie," Leipsic, 2nd edition, 1922, p. 739.

(12) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, THIS JOURNAL, **72**, 535 (1950).

(13) I. M. Klotz and J. Ayers, *ibid.*, **74**, 6178 (1952).

the one obtained by us for testosterone. Any adsorption data for proteins, which follow the Freundlich isotherm, when $n = 1$, and the simple Langmuir equation, would give this result with the Scatchard treatment. The conclusion therefore to be reached is that in the case of a neutral molecule like testosterone the adsorption by albumin is quite non-specific.

A summary of our solubility studies indicates that testosterone added to water and equilibrated for a comparatively short period of time produces a supersaturated solution; the maximum amount is dissolved in about 5 hours. After this peak there is a slow fall to the solubility value, 1.26×10^{-4} mole/l., provided some solid phase is present. If no solid phase is present solutions as concentrated as 2.05×10^{-4} mole/l. can be maintained without crystallization for weeks. If the solvent is changed after 5 hours of equilibration, the residue does not again produce a supersaturated solution. Heating the residue to slightly below the melting point restores the form of material required to produce a supersaturated solution.

According to the Gibbs concept of solubility, solubility is dependent on particle size and the energy of the solvent–solute interface; the final equilibrium is attained with a single large crystal.[14] A number of observations in our experiments would support this hypothesis as an explanation of the dual solubility values obtained: (1) The smaller particles would dissolve rapidly giving an initial high solubility; (2) the residue consisting of larger particles would give the lower equilibrium value; (3) heating crystals, which gave the lower solubility, shatters them and produces a product giving the high solubility; and (4) the high solubility in the water phase of the dialysis experiments, in which the solid phase size may be looked upon as the albumin molecule upon which the testosterone is adsorbed. Attractive as these arguments may appear, actual measurement of particle size by microscopic examination showed particles of from 1 to 10 microns in the low solubility solute, as well as in the high. A quantitative estimate of the distribution of particle size was not made, so that the Gibbs theorem cannot be completely ruled out. The only other explanation would be the existence of polymorphous forms of testosterone. In either case, regardless of the interpretation, the albumin increases the fugacity of testosterone in the water phase, a phenomenon which may have biologic implications.

(14) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 51.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY, TOKYO INSTITUTE OF TECHNOLOGY]

Molecular Structures of *trans*-1,2-Dihalocyclohexanes

BY KUNIO KOZIMA, KIYOSHI SAKASHITA AND SHIRO MAEDA

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An investigation of dipole moments and Raman spectra of the addition products of chlorine or bromine to cyclohexene leads to the following conclusions: (1) the compounds are *trans*-1,2-dihalocyclohexanes; (2) their solutions or melts contain the two inverted isomers (1p, 2p)⇄(1e, 2e) in a dynamic equilibrium; (3) the structure (1e, 2e) is more stable in dilute benzene solution than in dilute heptane or carbon tetrachloride solution; and (4) in the solid state the dichloro derivative exists only in the (1e, 2e) form, but the dibromo derivative exists only in the (1p, 2p) form. The potential energy differences of both structures were estimated for various dilute solutions of *trans*-1,2-dichlorocyclohexane and of *trans*-1,2-dibromocyclohexane.

The possible existence of *cis–trans* isomers of 1,2-dihalocyclohexanes which has been assumed for several decades by organic chemists has in recent years also been suggested on the basis of structural considerations of the cyclohexane ring of D_{3d} symmetry.

Only one isomer, however, is obtained by adding chlorine or bromine to cyclohexene. The compound prepared from bromine and cyclohexene is according to Rothstein[1] the *cis* isomer, while according to Mousseron and Granger[2] the addition of chlorine or bromine to cyclohexene leads to the *trans* isomer.

It is highly probable that the *trans*-1,2-dihalo derivatives, like the *trans*-1,4-dihalocyclohexanes,[3] possess two structures in which the valency angles of the carbon atoms of the cyclohexane ring maintain their normal value. These two stable strainless structures, the "inverted isomers"[3] of the *trans*-1,2-dihalocyclohexanes, are represented by the symbols (1p, 2p) and (1e, 2e) and should be in dynamic equilibrium, provided the energy difference between the inverted isomers is not too large. For the *cis*-1,2-dihalocyclohexanes the two stable strainless structures (1e, 2p) and (1p, 2e) are identical and therefore only one isomer can be expected.

This difference between *cis* and *trans* isomers, in connection with measurements of Raman spectra and dipole moments, has enabled us to determine whether the actual compounds are *cis* or *trans* isomers, and to study the equilibrium of the inverted isomers in solution.

Recently, Bastiansen and Hassel[4] concluded from

(1) B. Rothstein, *Ann. chim.*, **14**, 461 (1930).
(2) M. Mousseron and R. Granger, *Compt. rend.*, **205**, 327 (1937).
(3) K. Kozima and T. Yoshino, THIS JOURNAL, **75**, 166 (1953).
(4) O. Bastiansen and O. Hassel, *Tids. Kjemi, Bergvesen Met.*, **8**, 96 (1946).