Perhaps the most interesting question of structural significance raised by our results has to do with the variation of K values among the 14 sites which bind alkyl sulfate. Though, obviously no detailed answer is possible now, it does appear that the configuration of the protein in the interacting area around each positive charge is not the same for all the sites. It would be of considerable interest to determine the degree of such heterogeneity for the binding of organic anions of different sizes and shapes.

Acknowledgment.—We are indebted to Professor R. Keith Cannan for the generous hospitality afforded us in his laboratory during the conduct of this investigation. We are also grateful to Dr. I. M. Klotz of Northwestern University for many enlightening discussions.

Summary

The reversible binding of homologous alkyl sulfates by bovine serum albumin has been studied at two temperatures by the method of equilibrium dialysis. This has required the development of a colorimetric method for determining low concentrations (10^{-5} M) of these compounds. Deviations of the binding curves from the simple theory based on the mass action law have been shown to be inexplicable on the basis of electrostatic effects. A new heterogeneity theory based on a particular distribution of the intrinsic binding constants has been proposed and has been found to account quantitatively for our results. The values of the thermodynamic functions $\Delta F°$, $\Delta H°$ and $\Delta S°$, for the binding process have been calculated and the importance of the entropy contribution emphasized. The validity of the determination of binding capacity (n) by linear extrapolation has been subject to question and interpretations of binding data in the literature reconsidered. Finally, some structural implications of our results have been noted.

NEW YORK 16, N. Y. RECEIVED NOVEMBER 15, 1948

[CONTRIBUTION FROM THE LABORATORIES OF PHYSICAL CHEMISTRY AND PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

Biophysical Studies of Blood Plasma Proteins. IX. Separation and Properties of the Immune Globulins of the Sera of Hyperimmunized Cows[1]

BY E. L. HESS[2,3] AND H. F. DEUTSCH

Introduction

The antibody fraction of normal bovine serum has been previously separated in high yield by means of ethanol fractionation.[4] Our attention has now been directed to the application of these procedures for the recovery of the antibody enriched fractions of the sera of cows immunized to *Brucella abortus* organisms and Newcastle virus. The various globulin fractions separated have been subjected to solubility, molecular kinetic and immunological study.

Experimental

The hyperimmune sera were prepared by inoculating two Holstein cows with Newcastle virus in whole egg embryo and with the viable *Brucella abortus* organisms at four-day intervals over a three-month period. In addition, a low titer serum pool of two calves immunized to diphtheria and tetanus toxin was studied briefly. The increase of the serum γ-globulins was followed by electrophoretic analysis and the extent of antibody production was determined by immunological assays.

Brucella abortus antibodies of two types, ag glutinins and bactericidins, were assayed accord ing to the methods published by Huddleson[5] and Irwin and Beach,[6] respectively. Newcastle virus hemagglutination inhibiting and neutralizing antibodies were assayed according to the method of Brandly, Jungherr, Moses and Jones.[7] The titers reported are based upon the assay of fractions reconstituted in phosphate buffer to their original serum concentration.

Except in certain subfractionations as indicated below, the fractionation steps follow the procedure outlined in Fig. 5 of the prior article.[4] The course of the fractionation of the hyperimmune bovine serum is shown by the electrophoretic diagrams in Fig. 1. The designation of the fractions is consistent with that adopted in the earlier report. Precipitates A from various sera were pooled for subsequent subfractionation or rework operations.

Standard electrophoretic and sedimentation procedures were followed in the physical chemical characterizations of the fractions. Solubility studies utilized the customary phase rule solubility procedures as reviewed by Herriott[8] and the successive extraction of the solid phase with

(1) A portion of this material was presented before the Division of Biological Chemistry of the American Chemical Society at the 113th Meeting, Chicago, April 19–22, 1948. This work was supported in part by grants from the Wisconsin Alumni Research Foundation and from the U. S. Public Health Service.

(2) du Pont Fellow in Chemistry in 1947.

(3) Post-doctorate Fellow in Chemistry 1948.

(4) Hess and Deutsch, THIS JOURNAL, **70**, 84 (1948).

(5) Huddleson, "Brucellosis in Man and Animals," Oxford Press, New York, N. Y., 1943.

(6) Irwin and Beach, *J. of Agr. Res.*, **72**, 83 (1946).

(7) Brandly, Jungherr, Moses and Jones, *Am. Jour. Vet. Research*, **7**, 289 (1946).

(8) Herriott, *Chem. Rev.*, **30**, 413 (1942).

TABLE I

FRACTIONATION, YIELD AND IMMUNOLOGICAL ASSAY DATA

	Brucella abortus[a] agg.	Brucella abortus[a] bact.	Newcastle virus[b] h. i. V. dil.[e]	Newcastle virus[b] neutralizing MLD	Total[c] protein g./l.	% γ-(d)-globulins	Wt. γ-globulins g./l.	% Yield γ-globulins g./l.	β[d] globulins
Pre-immunization	0	1:160	0	0	76	28	21		
Hyperimmune serum	1:2560	1:81,920	1:5	10^6	76	41	31		
Precipitate A (pooled)	1:2560	1:81,920	1:5	10^5	34	85	29	93	5
Supernatant (pooled)	1:40	1:20,480	0	10^1	42	7	2		
Precipitate B	1:100	0	0	0	6	17	1		5
Precipitate C	1:2560	1:81,920	1:5	10^5	25.6	95	25.6	83	
Precipitate C-1	1:4096	1:81,920	1:5	10^5	11.4	97	11.4	37	
Precipitate C-2	1:1024	1:7680	0	10^2	10.6	100	10.6	34	

[a] These assays were performed by Dr. D. Berman. [b] These assays were performed by Mr. R. Hanson. [c] N_2 detn. or lyophilized dry weight of precipitates. [d] Electrophoretic analysis. [e] Zero in this column indicates no detectable antibody.

aliquots of buffer. The usual precautions of equilibration and approach of equilibrium from both under and supersaturated solution were observed. Veronal, cacodylate and acetate buffers of $\mu = 0.01$ and 0.1 were used in the solubility and electrophoretic studies.

Results

Analytical and antibody data for the serum and the various fractions are shown in Table I. The assays clearly show that the major portion of the antibodies is precipitated from the diluted serum at *p*H 7.6 and 18% ethanol. This precipitate (A) which amounted to 24 g./liter of serum in the case of normal cows and 34 g./liter of serum with the hyperimmune serum, represents more than 90% of the γ-globulins present in the original serum (electrophoretic analysis). Further fractionation of precipitate C gives products richer in the antibodies. It is apparent from Table I that the major portions of the antibodies separate with the γ_1-globulin fraction (Ppt. C-1).

In an effort further to concentrate the antibodies, certain subfractionations, exploratory in nature, were tried. For example, precipitate C-1 was separated into three fractions at $\mu = 0.003$ by varying *p*H and the ethanol concentrations. Brucella abortus agglutinins and Newcastle virus neutralizing antibody concentrations were doubled (as indicated by serial dilution assays) in the fraction that was soluble in the aqueous phase at $\mu = 0.003$, *p*H = 5.8, protein concentration = 0.5%, but which precipitated upon addition of ethanol to 25% concentration. In another instance, when the *p*H of an aqueous solution of precipitate C (protein = 0.5%, $\mu = 0.013$) was varied over the range 5.5 to 6.3 there occurred a precipitation of from 7.5 to 11.4% of the total protein in solution. The precipitate in each case gave negligible antibody titers.

Both the γ_1- and γ_2-globulins have sedimentation constants of $s_{20w} = 7.4S$ at a protein concentration of approximately 1% in 0.15 *M* NaCl. The sedimentation constants are substantially independent of concentration from 0.25 to 1% protein, and are in exact agreement with those previously reported[4] for the γ_1- and γ_2-globulins from normal bovine serum. The criterion of molecular mass homogeneity previously applied[4] revealed an essentially monodisperse system. Evidence of traces of a heavier component, $s_{20w} = 18S$, was found in the case of the γ_1-globulins, in contrast to its absence in the corresponding precipitate from normal bovine serum.

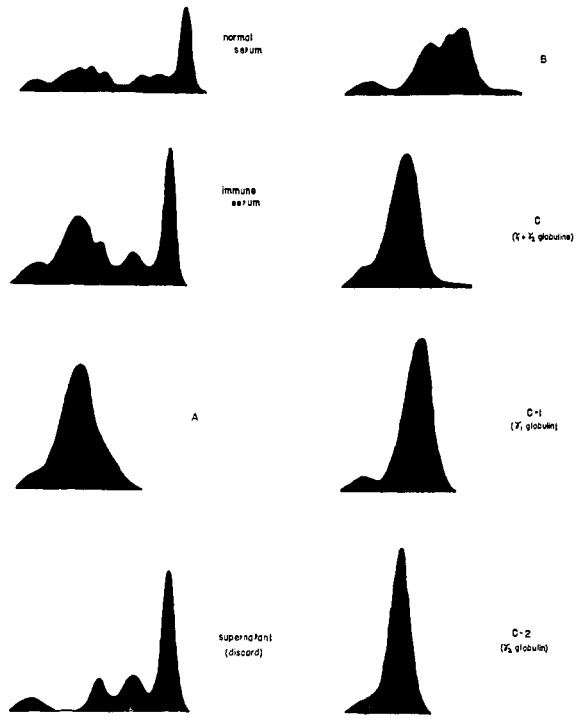


Fig. 1.—Electrophoretic patterns of hyperimmune bovine serum fractions in veronal buffer *p*H 8.6, $\mu = 0.10$ after 7200 seconds at 6.0 volts/cm. Concentration of protein is 3% in all cases; normal serum in barbiturate–citrate buffer at *p*H 8.60, $\mu = 0.088$, $E = 8.5$ volts/cm. Detailed statements of conditions for these separations appear in Fig. 5 of ref. (4).

Isoelectric points (pI_e) determined from the customary plot of mobilities (U) against *p*H are

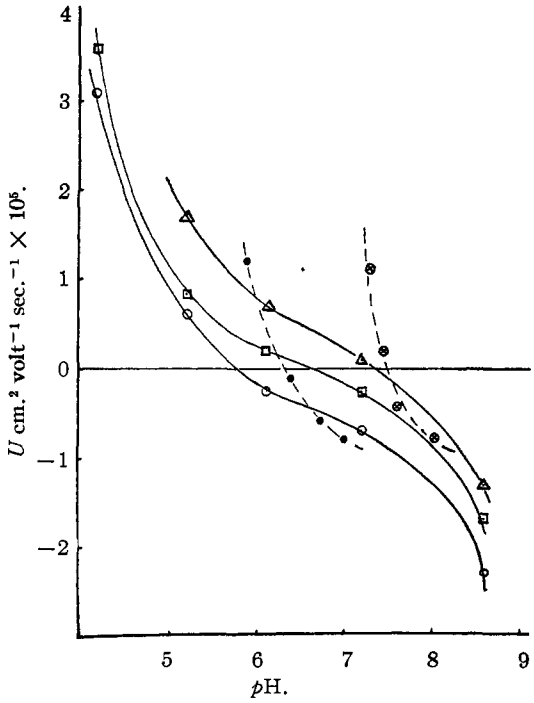


Fig. 2.—*p*H–mobility relationships:

□	γ-globulins	μ = 0.10	pI_e 6.60
○	γ_1-globulins	μ = .10	pI_e 5.85
△	γ_2-globulins	μ = .10	pI_e 7.35
●	γ_1-globulins	μ = .01	pI_e 6.35
⊗	γ_2-globulins	μ = .01	pI_e 7.50

shown in Fig. 2. The pI_e are sensitive to μ and increase with decreasing μ. The isoionic points (pI_i) as determined by extrapolating the pI_e obtained in μ = 0.1 and μ = 0.01 buffers to μ = 0 gives *p*H values of 6.4 for the γ_1-globulins and 7.5 for the γ_2-globulins. In Table II are assembled data obtained from electrophoretic studies at μ = 0.10. The heterogeneity constants (H) are calculated according to the method of Sharp, Taylor, Beard and Beard.[9]

The apparent diffusion constants are calculated from the relation $D^* = (Xi)^2/2t$.[10,11] The time (t) is in seconds, X_i is the half width of the gradient curve of the electrophoretic pattern at the point of inflection (h_i), where h_i is equal to $1/\sqrt{e}$ times the height of the maximum ordinate. The ascending and descending boundaries are virtually enantiographs at μ = 0.10. The values of the heterogeneity and the apparent diffusion constants listed in columns 3 and 4 of Table II are averages for the ascending and descending boundaries.

TABLE II

ASSEMBLED MOBILITY AND HETEROGENEITY DATA

Sample	*p*H	$U \times 10$ cm.² volt⁻¹ sec.⁻¹	$D^a \times 10^7$ cm.² sec.⁻¹	$H \times 10^5$ cm.² volt⁻¹ sec.⁻¹	Buffer[b] μ = 0.1
C1	4.20 ± 0.05	+3.1	67	0.92	Acetate
	5.20	+0.6	80	.98	Acetate
	6.10	−0.3	69	.93	Cacodylate
	7.20	−0.7	65	.90	Cacodylate
	8.60	−2.3	53	.80	Veronal
C2	4.20 ± 0.05	+4.7	63	.87	Acetate
	5.20	+1.6	71	.95	Acetate
	6.10	+0.7	93	1.05	Cacodylate
	7.20	+0.1	73	0.95	Cacodylate
	8.60	−1.3	62	0.87	Veronal

[a] Values are calculated from Schlieren patterns after 120 minutes at E = 5.0 volts cm.$^{-1}$ μ = 0.10 in all cases at ≦ 1° C. [b] All acetate and cacodylate buffers, μ = 0.02 with the sodium salt of the acid and μ = 0.08 with NaCl.

Resolution of the schlieren pattern into more than a single peak was not observed at any of the *p*H values listed in Table II although the γ-globulins showed reversible electrical spreading to a marked degree. At μ = 0.01, the electrophoretic boundaries for both the γ_1- and γ_2-globulins are very asymmetric and actually resolve into several peaks. In Fig. 3 the resolution of these two globulin fractions at μ = 0.01 and 0.1 are compared, while Fig. 4 shows the reversible spreading phenomenon and the resolution at a *p*H close to the "average" pI_e of the γ_1-globulins. The asymmetry and the separation of more or less distinct components precludes the calculation of heterogeneity constants at μ = 0.01 for either γ_1- or γ_2-globulins.

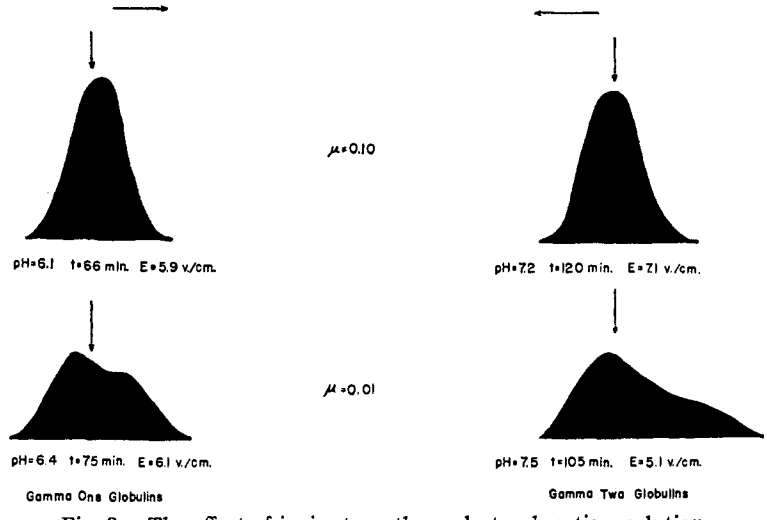


Fig. 3.—The effect of ionic strength on electrophoretic resolution.

The successive extraction of 6.7 g. of γ_1-

(9) Sharp, Taylor, Beard and Beard, *J. Biol. Chem.*, **142**, 193 (1942).

(10) Lamm, Dissertation, Upsala, 1937.

(11) Neurath, *Chem. Rev.*, **30**, 372 (1942).

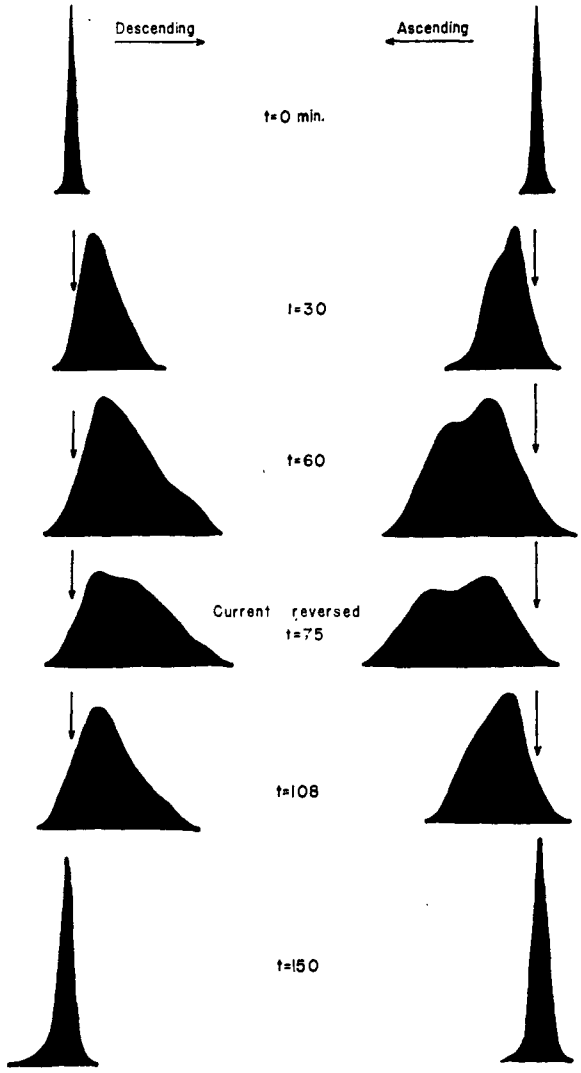


Fig. 4.—Electrophoretic reversible spreading of γ_1-globulins: pH, 5.95, μ = 0.01, E = 6.1 v./cm.

globulins (Ppt. C-1) with 25-ml. portions of sodium cacodylate buffer at pH = 5.95 ± 0.05, μ = 0.01, yielded the data plotted in Fig. 5. It is apparent that the solid phase remaining after ten extractions represents within the limit of error of the nitrogen determination a protein of constant solubility. Electrophoretic patterns of the supernatants from the first, fourth, seventh and tenth extractions are shown in Fig. 6. Progressive increase in symmetry is evident. However, even the residue possessing a relatively constant solubility clearly shows spreading far greater than can be attributed to normal diffusion. Phase rule solubility curves for the γ_1- and γ_2-globulins in μ = 0.01 buffers at their "average isoelectric points" give no evidence of a major component of relatively constant solubility and corroborated the heterogeneous nature of the fractions as revealed by electrophoretic spreading and solu-

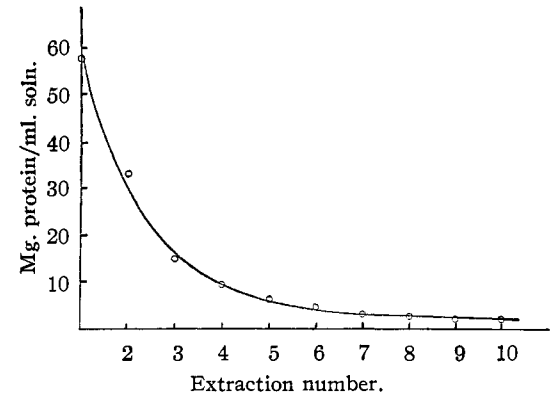


Fig. 5.—Successive extraction of γ_1-globulins at pH 5.95 ± 0.05, μ = 0.01 sodium cacodylate buffer, T = 0°.

bility extraction procedures. Under the above conditions the γ_1-globulin fraction is far less soluble than the γ_2-globulin fraction and even in the most dilute solutions it fails to dissolve completely. The phase rule solubility behavior of the γ_2-globulins is similar to that observed by Englehardt[12] with myosin solutions. In low ionic strength aqueous solutions at higher protein concentrations the γ_2-globulins do not separate into the customary solid and liquid phases but separate into a liquid phase and a gel.

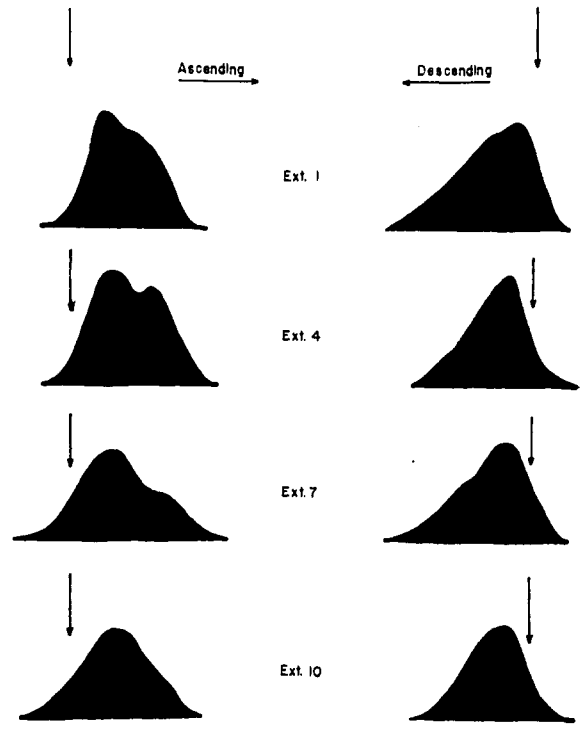


Fig. 6.—Electrophoretic patterns of γ_1-globulin extracts: μ = 0.01; pH 5.95; t = 60 minutes; E = 6.0 volts/cm.

(12) Englehardt, *Adv. in Enzymol.*, **VI**, 147 (1947).

That the procedures used on adult animals apply to calf serum is substantiated both by yield and antibody recovery data. Actual yields are somewhat low, however, due to the lower γ-globulin levels in the younger animals. Percentage yields of each fraction are essentially the same as reported in Table I.

Discussion

The fractionation methods developed for removal of the γ-globulins from normal bovine sera[4] have been found to be applicable to immune bovine sera with the resultant separation of the major portion of the serum antibodies. Larger amounts of antibody fractions are obtained per unit of serum as a consequence of a higher concentration of γ-globulins in the starting serum.

Differences in solubility of the antibodies studied are shown by the greater solubility of the *Brucella abortus* bactericidins as compared with the agglutinins. Thus, a significant portion of the bactericidins remain in the supernatant from the first precipitate (Ppt. A, Fig. 1) although the agglutinating antibodies seem to be almost quantitatively removed. More quantitative antibody assay methods are needed to determine the exact solubility differences.

Subfractionation of the γ-globulins reveals that further separation of individual antibodies by chemical methods is feasible. Thus, while the neutralizing antibodies for Newcastle virus are found in both the γ_1- and γ_2-globulin fractions, the hemagglutination inhibiting antibody appears to reside largely in the γ_1-globulin fraction. However, the concentration of the former antibody as well as those to Brucella organisms are distinctly lower in the γ_2-globulin fraction. The result for the Brucella antibodies may be in contrast to the response of the horse to bacterial antigens where immunization to bacterial cells causes a marked increase in the normal γ-globulins (γ_2) levels[13] as compared to the γ_1-globulin (T-component) area. The actual distribution of the horse antibodies to bacterial antigens is however unknown.

Further aqueous subfractionations at μ = 0.013, however, reveal that both of the Newcastle virus antibodies are concentrated largely in the pseudoglobulin fraction of precipitate C-1. Thus while as much as 15% of this portion may be removed as aqueous insoluble material no significant amounts of the Newcastle antibodies are removed. Such findings indicate that as regards the antibodies studied in this work a larger amount of protein (γ_1-englobulin) can be removed with Ppt. B, Fig. 1 without significant loss of the antibodies if this precipitation is carried out at *p*H 6.1 rather than at *p*H 4.9.

A rework of a γ_1-globulin fraction involving the variables *p*H, ionic strength and ethanol concentration to give three subfractions resulted in the Newcastle virus neutralizing antibody being concentrated entirely in the more soluble (pseudoglobulin) fraction of C-1. No hemagglutination inhibiting antibody was detectable in any of the above three subfractions although recombination of two of the fractions resulted in a low but significant titer. Further studies of this apparent antibody complex are being carried out.[14]

Electrophoretic spreading experiments in μ = 0.1 and 0.01 buffers reveal that the γ-globulin fractions are electrically inhomogeneous. The inhomogeneity (spreading greater than can be attributed to diffusion and which is electrically reversible) is more evident at low ionic strengths (μ = 0.01). Such data indicating the presence of electrokinetically inhomogeneous *molecular* species have been previously noted.[4,10,15,16,17,18,19]

Solubility studies corroborate the heterogeneous nature of antibody fractions that give a single electrophoretic peak at μ = 0.10. Successive extraction procedures utilizing a buffer solution of relatively low ionic strength tend to leave a residue of more uniform electrophoretic composition than the starting fraction, but one that still shows marked reversible electrophoretic spreading. In this respect the reversible spreading type of experiment provides a delicate criterion of homogeneity.

The γ_1- and γ_2-globulins reveal the same degree of mass molecular homogeneity exhibited by the corresponding fractions from normal bovine serum. The sedimentation constants, however, are somewhat higher than one reported by Cohn and his associates[20] but are essentially in agreement with the values obtained by Pedersen[21] for salt fractionated material.

Acknowledgments.—We wish to thank the following individuals who aided us in this work: Dr. David Berman of the Department of Veterinary Science provided us with the *Brucella abortus* antigen, immunized and helped bleed the animals, and performed the agglutination and bactericidal assays. His coöperation, advice and assistance were invaluable. Mr. R. M. Hansen, also of the Department of Veterinary Science, performed the Newcastle virus hemagglutination inhibition and neutralizing assays. Mr. Edwin M. Hanson made the sedimentation velocity runs and designed and constructed much of the multiple stirring apparatus for the solubility studies. We are grateful to Professor J. W. Williams for his continued interest in this research.

(13) Van der Scheer, Wyckoff and Clark, *J. Immunol.*, **39**, 65 (1940).

(14) Hansen, Brandly and Winslow, Abst. Proc. Soc. Am. Bacty., 48th Meeting, May 10-14 (1948), Minneapolis, Minnesota.

(15) Tiselius and Horsfall, *Ark. Kem. Min. Geol.*, **13A**, 18 (1939).

(16) Horsfall, *Ann. N. Y. Acad. Sci.*, **39**, 203 (1939).

(17) Longsworh, Cannan and MacInnes, THIS JOURNAL, **62**, 2580 (1940).

(18) Lauffer and Ross, *ibid.*, **62**, 3296 (1940).

(19) Alberty, Anderson and Williams, *J. Phys. Colloid Chem.*, **52**, 217 (1948).

(20) Cohn, Luetscher, Oncley, Armstrong and Davis, THIS JOURNAL, **62**, 3396 (1940).

(21) Pedersen, Dissertation, Upsala, 1945.

Summary

Two γ-globulin fractions, isoelectric at *p*H 5.85 and 7.35 in $\mu = 0.1$ buffers, were prepared by methods previously described, from sera of cows immunized to Brucella abortus and Newcastle virus. These fractions are inhomogeneous as indicated by electrophoretic spreading experiments and solubility studies, but are essentially monodisperse as regards sedimentation behavior. The antibodies studied are largely concentrated in the fraction of lower isoelectric point (γ_1-globulins). Concentration and separation of certain antibodies were obtained, indicating the feasibility of chemical separation of individual antibodies from polyvalent systems.

RECEIVED APRIL 30, 1948

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT, UNIVERSITY OF MISSOURI]

Hydrodiethylstilbestrol Compounds. IV. The Unsaturated Ketones[1,2]

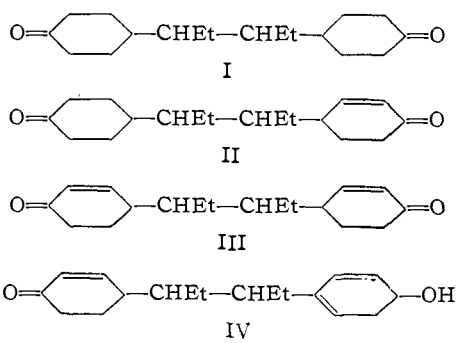
BY HERBERT E. UNGNADE AND PAUL W. TUCKER[3]

Unsaturated ketones derived from perhydrostilbestrol have been described by Schoeller and coworkers.[4] The di-unsaturated diketones are said to be colorless oils, the mono-unsaturated compounds have not been characterized nor is any criterion of purity disclosed in the pertinent examples. Since the compounds are claimed to possess androgenic activity up to one capon unit per mg., it appeared possible that purified preparations might possess considerable biological activity.

The substances have been prepared essentially as described by the previous investigators. The two known perhydrodiketones (I)[4,5] can be brominated rapidly in chloroform[4] or in glacial acetic acid[6] with the introduction of one or more atoms of bromine. In view of the possibility of replacing the tertiary hydrogens of the hexane chain in the diketones (I) the bromination has now been effected by means of N-bromosuccinimide which supposedly does not react with tertiary hydrogens in the absence of peroxides.[7]

Bromination of the diketones (I) with N-bromosuccinimide in carbon tetrachloride proceeds rapidly with the introduction of one atom of bromine. The second and third bromines are introduced more slowly, the second bromine entering preferentially the unbrominated nucleus. On the basis of recovered succinimide (98–100%) the reaction is nearly quantitative. The glassy bromodiketones are best dehydrobrominated by refluxing with collidine. Chromatographic adsorption of the crude glassy substances on alumina from benzene solution yields homogeneous products containing benzene of solvation which can be removed only by vacuum sublimation.

Ultraviolet absorption spectra of the analytically pure sublimates (II) and (III)[8] show relatively low extinction values at 225 mμ and only a weak secondary maximum at 280–285 mμ. Both phenolic ketones (IV) give the expected phenolic band of 280 mμ. Traces of a phenolic by-product (V) have been isolated from the dehydrobromination of the *meso*-tribromodiketone regardless whether it is prepared by direct bromination or with N-bromosuccinimide. This substance has a strong maximum at 300 mμ which may be taken as evidence for the presence of a double bond conjugated with the phenolic ring.[9] A red ketonic by-product is obtained in the same reaction which must possess a multiple conjugated system in view of its color. The formation of these by-products and the small extinction values for the compounds (II) and (III) lend support to the assumption that N-bromosuccinimide may also attack tertiary hydrogens even in the absence of peroxides and that the substances (II), (III) and (IV) probably consist of mixtures of conjugated and non-conjugated unsaturated ketones.



The *meso* compounds (II) and (III) have been tested biologically. Both show little or no androgenic activity in the seminal vesicle test with three daily doses of 1.0 and 0.75 mg. of substance, respectively.[10]

(1) Presented in part before the Division of Organic Chemistry of the American Chemical Society, Chicago, April, 1948.

(2) From the Ph.D. thesis of Paul W. Tucker, 1948.

(3) George Breon Fellow, 1946–1948. Present address: Research Dept., Phillips Petroleum Company, Bartlesville, Okla.

(4) Schoeller, Inhoffen, Steinruck and Höss, U. S. Patent 2,392,-864.

(5) Ungnade and Ludutsky, *J. Org. Chem.*, **10**, 307 (1945).

(6) Ludutsky, Ph.D. thesis, University of Missouri, 1946.

(7) Schmid and Karrer, *Helv. Chim. Acta*, **29**, 573 (1946).

(8) Absorption spectra by Dr. E. E. Pickett, University of Missouri.

(9) Jones, *Chem. Rev.*, **32**, 36 (1943); Kaiser and Koenig, THIS JOURNAL, **68**, 740 (1946).

(10) Androgenic tests by Mr. K. D. Sprague, Control Department, Winthrop-Stearns, Inc., Rensselaer, N. Y.