we find

$$\Delta S = \Delta S_A - 64 \text{ cal./deg.}$$

where ΔS_A is the bracketed term above. Thus $dE_0/dT = \Delta S/nF = \Delta S_A/nF - 1.39$ mv./degree. The reference cell (S.C.E.) itself has a temperature coefficient of -0.76 mv./degree. Hence, with S.C.E. as the reference electrode

$$\frac{E_{1/2}}{dT} = \frac{\Delta S_A}{nF} - \frac{\Delta S^{\ddagger}}{2nF} - 0.63 \text{ mv.} - 0.66 \text{ mv.}$$

$$= \frac{1}{nF}[\Delta S_A - {}^1/_2\Delta S^{\ddagger}] - 1.29 \text{ mv.} \qquad (15)$$

The observed temperature coefficient of $E_{1/2}$ is -1.03 mv. This leaves for the bracketed term a value of *ca.* 12 e.u., a small value considering the size of the molecules involved, and in keeping with the small difference in entropy between hydrogenated and olefinic hydrocarbons, and the small entropy of activation to be expected for the simple transition (probably involving a hydration) to stable DA.

The coefficient at *p*H 6.67 differs from that at *p*H 3.58 because of the more negative S_{H^+} term and also because the active hydrogen atom in the center of the molecule is dissociated at this *p*H,[2] so that ΔS must also include the entropy of dissociation. The first effect adds an additional -0.62 mv. to equation 15, the second a small positive increment, in view of the small positive entropy of dissociation of most acids. The observed increment in $dE_{1/2}/dT$ is -0.4 mv.

It should be noted that the difference in temperature coefficient at the two acidities cannot be accounted for by a mechanism in which an irreversible oxidation is the rate-determining step. To explain the observed relation between *p*H and $E_{1/2}$ the hydrogen ions must be supposed to take part in a reversible equilibrium preceding the rate determining step. Thus the hydrogen ions are not involved in the formation of the activated complex, and cannot affect the activation energy, which would be the same at both acidities.

CAMBRIDGE, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, IOWA STATE COLLEGE]

On the Mode of Interaction of Surface Active Cations with Ovalbumin and Bovine Plasma Albumin[1]

BY JOSEPH F. FOSTER AND JEN TSI YANG

RECEIVED AUGUST 6, 1953

The combination of a cationic detergent, dodecyldimethylbenzylammonium chloride, with ovalbumin (O) and bovine plasma albumin (A) has been studied by electrophoresis, equilibrium dialysis, viscosity and optical rotation. O reacts in an all-or-none manner accompanied by denaturation of the protein and a consequent increase in viscosity and optical rotation. Determination of the number of ions bound is rendered difficult by the tendency toward gelation and precipitation of a part of the protein. Heat-denatured O, and both A and heat-denatured A, fail to show this all-or-none type of reaction, only a single electrophoretic component being observed. At the *p*H of the experiments (2.5–3) A is denatured, there being a nearly tenfold increase in specific viscosity (at low ionic strength) and a 30% increase in optical rotation even in the absence of the detergent. In view of the absence of streaming birefringence and of the instantaneous character of the changes in viscosity and rotation it is concluded that this denaturation is essentially an isotropic expansion of the molecule.

The combination of proteins with surface active anions has been the object of much study during the past decade. It has been amply demonstrated that in the case of native ovalbumin (O) and horse and bovine serum albumin (A) an all-or-none type of combination occurs, a large number of ions being bound essentially as a unit.[2–4] In the case of heat-denatured proteins, on the other hand, the binding is stepwise[2,4] suggesting that the all-or-none reaction is associated with a configurational change in the protein (denaturation).

Studies with surface active cations are much more limited, in spite of the considerable biochemical interest in such compounds arising out of their use as bactericidal agents. Precipitation of proteins on the alkaline side of their isoelectric points by such compounds has been reported by several workers.[5–8] In a more detailed study of the action of dodecylamine hydrochloride on O, Timasheff and Nord[9] concluded that two stages of binding exist, corresponding to 30 and approximately 100 ions per mole of protein, respectively.

In another study from this Laboratory it has been shown recently that several surface active cations denature O in acid solution.[10] Studies by streaming birefringence and light scattering were in accord with the view that the reaction involves an unfolding of the O molecule to rodlets of length about 600 Å. which then aggregate laterally.

In the present study the nature of the interaction of alkyldimethylbenzylammonium chloride with both O and A is examined in more detail by electrophoresis, equilibrium dialysis, viscosity and optical rotation methods.

(1) Journal Paper Number J-1787 of the Iowa Agricultural Experiment Station, Ames, Iowa, Proj. 1223. Presented in part before the Division of Biological Chemistry of the American Chemical Society, March, 1953. This work was carried out under contract Nonr-803(00) of the Office of Naval Research.

(2) H. P. Lundgren, D. W. Elam and R. A. O'Connell, *J. Biol. Chem.*, **149**, 183 (1943).

(3) F. W. Putnam and H. Neurath, *ibid.*, **159**, 195 (1945).

(4) J. T. Yang and J. F. Foster, THIS JOURNAL, **75**, 5560 (1953).

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(7) K. H. Schmidt, *Z. physiol. Chem.*, **277**, 117 (1943).

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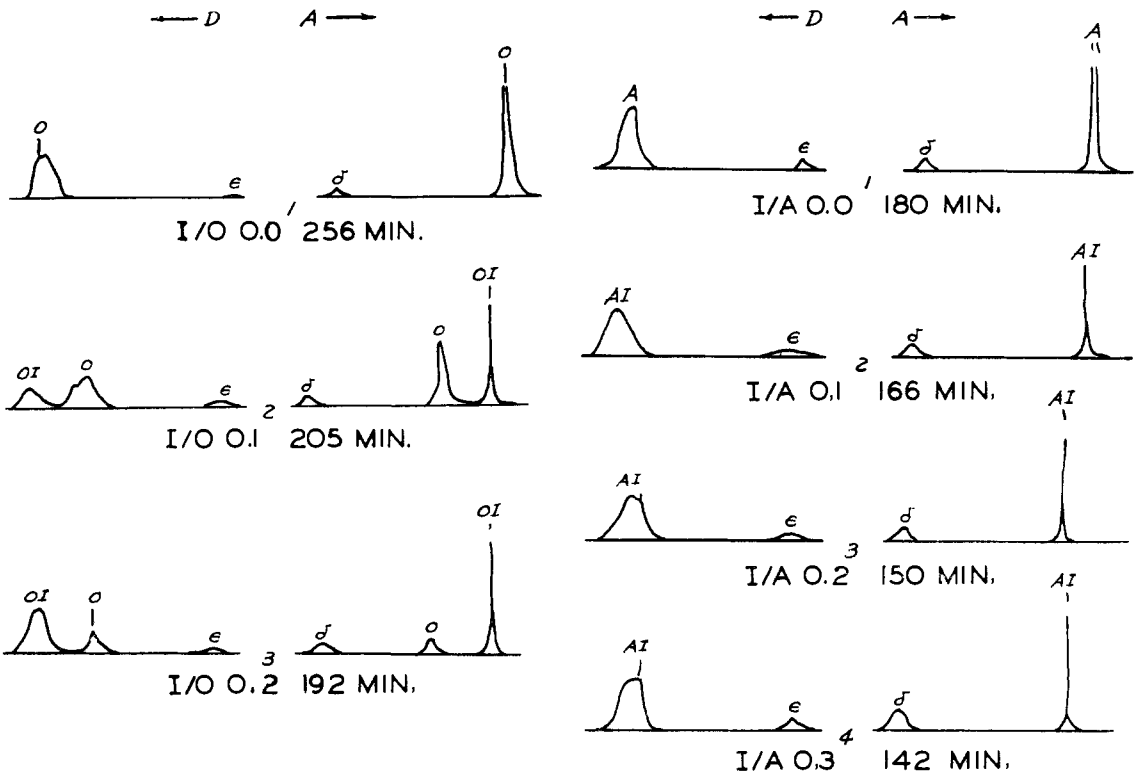

Fig. 1.—Typical electrophoretic patterns of the dodecyldimethylbenzylammonium complexes of ovalbumin and bovine serum albumin. Electrophoresis was at 6.2 ± 0.2 volts/cm.

Materials and Methods

Proteins.—Bovine plasma albumin (A) was obtained through the courtesy of Armour and Company. Ovalbumin (O) was prepared from fresh egg white by ammonium sulfate precipitation at the isoelectric point, recrystallized three times and dialyzed free of salt.

Buffer.—Glycine–NaCl buffer was prepared with reagent-grade chemicals: glycine 0.09 *M*, HCl 0.01 *M* and NaCl 0.09 *M*, *p*H 3.3 and ionic strength 0.10.

Cationic Detergent.—Alkyl (principally, *n*-dodecyl) dimethylbenzylammonium chloride was purchased from Onyx Oil and Chemical Company.

Electrophoresis.—Electrophoretic analyses were carried out at 2.0° in the Tiselius-type cell, with a modified Philpot-Svensson cylindrical lens optical system.

Equilibrium Dialysis.—Twenty-ml. portions of buffered protein-detergent mixtures contained in Visking casings ($^{20}/_{32}$ inches in diameter) were equilibrated against equal volumes of the buffer in glass-stoppered test-tubes, which were shaken gently at 1–3° for two days. The dialyzates were then diluted, if necessary, and analyzed spectrophotometrically. Control experiments with buffer only were frequently carried out to minimize the analytical errors.

Spectrophotometric Analyses.—Both protein and detergent concentrations were determined with the model DU Beckman spectrophotometer. The extinction coefficients of A and O are $E_{1\ cm.}^{1\%}$ 6.70 at 279 mμ and $E_{1\ cm}^{1\%}$ 7.50 at 280 mμ, respectively. The molar extinction coefficient of the detergent was found to be 400 at 263 mμ.[11]

Viscosity.—The viscosity measurements were made in an Ostwald-type viscosimeter at 24.9 ± 0.1°. The flow time for water was 63 seconds.

Optical Rotation.—The optical rotation measurements were made at 25 ± 3° using a sodium lamp as light source.

Results and Discussion

Electrophoretic Behavior.—In Fig. 1 are given examples of typical electrophoretic patterns obtained with the detergent complexes of O and A. The same all-or-none behavior which characterizes the reaction with anionic detergents is clearly seen in the case of O. On the other hand, A exhibits only a single component in all cases.

The all-or-none reaction between O and detergent is unquestionably a consequence of denaturation of the protein molecule. In the case of heat-denatured O only a single electrophoretic component was obtained. Heat-denatured A gave results indistinguishable from native A.

It should be mentioned that certain difficulties were encountered in the electrophoretic study of O-detergent complexes. In buffers of ionic strength 0.1 or over there was a pronounced tendency of the systems to gel after standing for several hours. For this reason it was necessary to either dispense with the conventional dialysis or operate at ionic strengths so low as to render interpretation of the electrophoretic patterns questionable. Consequently it was not possible to determine with any precision the composition of the all-or-none complex as has been done previously in the case of the anionic detergents.[2-4] In experiments without dialysis, analysis of the relative areas indicated the complex to contain 40–60 bound cations per molecule of protein. This value would be reduced by the correction, which would be considerable, for free detergent, but is in rough agreement with the finding of one cation per carboxyl group in the protein.[12]

In the case of A there was no similar problem, the solutions remaining clear and fluid. With both proteins trouble was encountered at high

(11) J. T. Yang and J. F. Foster, *J. Phys. Chem.*, **57**, 628 (1953).

(12) H. Fevold, *Adv. in Protein Chem.*, **6**, 187 (1951), in a summary of the composition of O, gives a value of 45–51 free carboxyls per molecule.

detergent levels due to convective disruption of the boundaries. This precluded experiments at detergent:protein binding ratios above about 0.3. Similarly it was impossible to conduct control experiments on the detergent in the absence of protein.

These limitations in no way invalidate the conclusion that a different mechanism exists in the binding of detergent by the two proteins.

Equilibrium Dialysis Studies.—Results of studies of the binding by means of the equilibrium dialysis technique are summarized in Fig. 2. These verify that the number of ions bound is large in both cases; indeed, no sign of an upper limit to the binding is apparent at the highest detergent levels employed.

TABLE I

COMBINATION OF ALBUMINS WITH DDBAC AT 1–3° IN GLYCINE–NaCl BUFFER (*p*H 3.3, Γ/2, 0.10)

Total concn. of detergent $\times 10^4 M$	Concn. of free detergent $\times 10^4 M$	Concn. of bound detergent $\times 10^4 M$	Av. moles detergent bound per mole protein r	Av. mobilities[a,b] $\mu \times 10^5$ cm.^{2} volt^{-1} sec.$^{-1}$
Native A (concn., 6.0 $\times 10^{-5}$ M)				
0	0	0	0	7.7
5.8	2.3	1.2	2.1	..
14.4	4.4	5.6	9.3	8.0
28.8	7.8	13.2	22	8.8
42.9	11.2	20.5	34	9.4
57.2	14.6	28.0	47	..
85.8	18.3	49.2	82	9.8
Heat-denatured A (concn., 6.0 $\times 10^{-5}$ M)				
5.8	1.9	2.0	3.3	8.0
14.4	4.1	6.2	10	8.1
28.8	7.9	13.0	22	8.8
42.9	11.4	20.1	34	9.7
57.2	13.8	29.6	49	..
85.8	18.7	48.4	81	10.3
Native O (concn., 9.3 $\times 10^{-5}$ M)				
5.8	2.7	0.4	0.4	
14.4	5.3	3.8	4.1	
28.8	8.7	11.4	12	
42.9	11.0	20.9	22	
57.2	13.7	29.8	31	
114	22.8	68.4	72	
Heat-denatured O (concn., 9.3 $\times 10^{-5}$ M)				
5.8	1.5	2.8	3.0	
14.4	4.3	5.8	6.2	
28.8	8.6	11.6	12	
42.9	12.1	18.7	20	
57.2	15.9	25.4	27	
114	23.0	68.0	72	

[a] In all cases, A–detergent mixtures appeared as a single moving boundary. [b] Buffered O–detergent mixtures became too turbid to make electrophoretic analyses.

The curves do not reflect the important difference in binding mechanism demonstrated electrophoretically. It would be expected that the binding curve would be very steep, perhaps nearly vertical, in the case of O in the region where all-or-none binding is taking place. Failure to observe such behavior is probably due to the heterogeneity of the detergent preparations used. Equilibrium dialysis studies on the detergents without

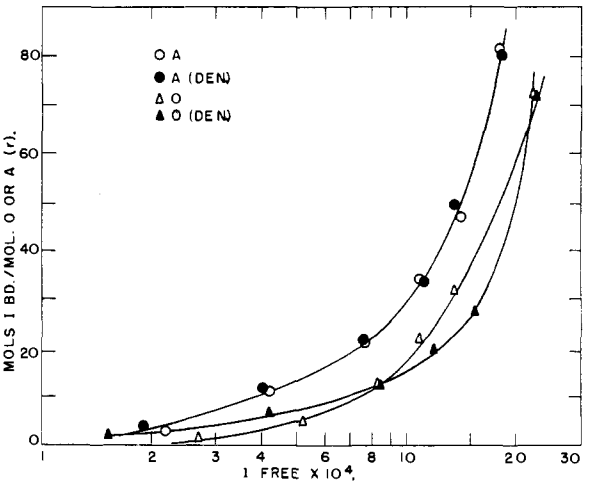

Fig. 2.—Binding curves for combination of native and heat-denatured ovalbumin, and native and heat-denatured bovine serum albumin with dodecyldimethylbenzylammonium chloride.

protein[11] showed a complex behavior suggesting the presence of lower homologs which do not form micelles in the concentration range studied. Doubtless the presence of these homologs masks the binding curves of the true detergent as was postulated in the case of anionic detergents.[4] In that case the curves could be markedly steepened by subfractionation of the detergent. Unfortunately attempts to purify the cationic detergent have not been successful.

The conventional $1/r$ *vs.* $1/I$ plot was non-linear in both cases suggesting that the simple statistical theory is not adequate even in the case of A. In view of the uncertainty as to the character of the heterogeneity of the detergent it did not seem worthwhile to attempt a detailed interpretation of the binding curve.

Also included in Fig. 2 are binding results on heat-denatured O and A. In the case of O there appear to be minor differences, but the curves for denatured and native A are identical within the limits of the experiment.

Viscosity Behavior.—To attempt a further clarification of the differences in behavior between the two proteins, viscosity measurements were carried out on the two proteins with and without detergent. The results are summarized in Fig. 3 in the form of plots of specific viscosity as a function of time. Values for the native proteins, measured near the isoelectric points, are shown by arrows on the ordinate. In all cases the protein concentration was 2.0%.

With O, only a slight and time-independent increase in viscosity takes place upon lowering the *p*H to 2.5 with HCl. The increase is of an order of magnitude similar to that observed by Bull[13] with this protein and his explanation, namely, that it is due to the electroviscous effect, is possibly the correct one. Upon adding detergent, however, either in the presence of acid or at the isoelectric point, a much more pronounced rise takes place. Further, this increase is relatively slow and is undoubtedly

(13) H. B. Bull, *Trans. Faraday Soc.*, **36**, 80 (1940).

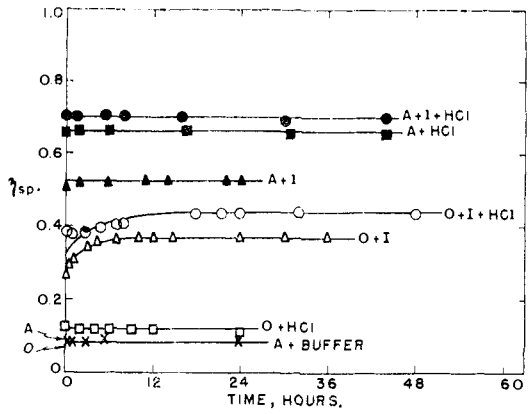

Fig. 3.—Changes with time in the specific viscosity of ovalbumin and bovine serum albumin under various conditions (for conditions see text).

due to the unfolding indicated by the streaming birefringence studies.[10]

With A the situation is very different. Upon adding acid to *p*H 2.5 the specific viscosity increases instantaneously, so far as can be judged, by a factor of almost ten. Addition of detergent at this *p*H results in only a very slight further increase. Addition of detergent at the isoelectric point results in a similar, though not quite so marked, increase. On the other hand, when the *p*H is adjusted to 3.2 and ionic strength 0.05 (with glycine, glycine–HCl and NaCl) no appreciable increase in viscosity takes place.

This last result might at first appear to indicate that the increases resulting under the other conditions with A were due to the electroviscous effect. However, it should be pointed out that they are of another order of magnitude than is to be expected on this basis, at least judging from the results with O. Evidence presented in the next section will clearly rule out this possibility. It is necessary to assume, then, that A undergoes a marked reversible configurational change as the *p*H is lowered from 4 to 2.5, resulting in a pronounced increase in the hydrodynamic volume of the protein. In view of the absence of streaming birefringence under such conditions it seems probable that this change involves essentially a swelling of the molecule, in contrast to the unfolding exhibited by O. Doty and Katz[14] and Scheraga and Mandelkern[15] have previously arrived at the conclusion that the denaturation of A by urea similarly involves essentially an isotropic swelling.

Interpretation of viscosity measurements without extrapolation to infinite dilution is well known to be somewhat dangerous. However, the order of magnitude of the effects observed is such as to render the qualitative conclusions reasonably certain. Moreover, the authors have carried out a rather extensive investigation of the intrinsic viscosities of both of these proteins at low *p*H which verifies fully the above interpretation and these results will be published in the near future.

(14) P. Doty and S. Katz, Abstracts of the 118th Meeting of the Am. Chem. Soc. (1950).

(15) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).

Optical Rotation.—In two recent publications Jirgensons[16] has called attention to the usefulness of optical rotation in the study of protein denaturation. In particular he has shown that the negative specific rotation of A is markedly increased at low *p*H and in solutions of guanidine HCl. Accordingly it was felt worthwhile to make measurements of optical rotation on the solutions prepared for the viscosity studies. These results are summarized in Fig. 4, the values for the native proteins near the isoelectric points again being represented by arrows at the ordinates. The high degree of similarity between the shifts in rotation and in viscosity (Fig. 3) are indeed striking. Again there is only a slight change in O at the low *p*H, a more marked and time dependent shift upon addition of detergent. Again with A acid produces a marked enhancement, actually more than is obtained upon addition of detergent or acid plus detergent. When buffer ions are present at the low *p*H no increase is obtained. The shifts in the case of A appear to be instantaneous in all cases.

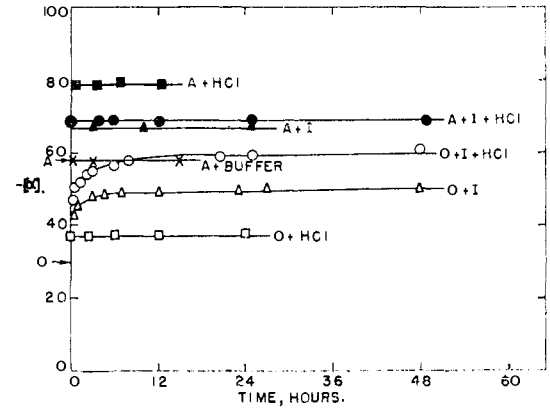

Fig. 4.—Changes with time of the specific rotation of ovalbumin and bovine serum albumin under various conditions (for conditions see text).

These results seem to rule out completely the possibility that the viscosity increases observed are due to the electroviscous effect. The most obvious explanation is that the molecule swells, probably due to coulombic repulsion between the large number of charged groups. The addition of buffer ions presumably reduces this repulsive force in the usual manner, thereby preventing swelling.

The instantaneous character of the changes in both viscosity and rotation of A further suggests that the process is a relatively simple one such as swelling. With O, in view of the fact that the change is evidently from a globular to a rod-like structure,[10] it is not surprising that the process is a slow one.

On the Mechanism of Combination.—The formation of electrophoretically-resolvable components in the protein–detergent interaction clearly implies some step in the combination which is either irreversible or, at most, only very slowly reversible under the conditions prevailing during electrophoresis. The important question arising from

(16) B. Jirgensons, *Arch. Biochem. Biophys.*, **39**, 261 (1952); *ibid.*, **41**, 333 (1952).

these studies, then, is why the two proteins behave so differently in this regard. In the case of O the streaming birefringence behavior[10] indicates that this change is the same typical denaturation produced also by heat and by urea. The absence of such changes in the case of A suggests that either (1) binding in this case does not involve a similar structural change or (2) the change is a readily reversible one. The identical behaviors of native and heat denatured A tend to rule out the first explanation. Furthermore, the pronounced changes in specific viscosity and optical rotation imply a pronounced structural alteration. This change is evidently produced by the low *p*H, even in the absence of detergent. In a more detailed study of this behavior considerable evidence has been presented for the ready reversibility of the changes in both viscosity and optical rotation observed.[17] The conclusion, then, is that the failure to observe an all-or-none type of reaction in the case of serum albumin is due to the reversibility of the structural changes produced in this protein either by acid or by the cationic detergent.

Acknowledgments.—The authors are indebted to Armour and Company, Chicago, for generous donations of crystalline bovine plasma albumin. Mr. Joseph Kucera performed the electrophoretic analyses.

(17) J. T. Yang and J. F. Foster, Abstracts 124th Meeting of the American Chemical Society (1953); THIS JOURNAL, in press.

AMES, IOWA

NOTES

The System: Silver Perchlorate–Dioxane[1]

BY ALAN E. COMYNS AND HOWARD J. LUCAS

RECEIVED OCTOBER 31, 1953

Few complexes in which silver ion is coördinated with an oxygen atom have been isolated.[2] Evidence has been obtained for the formation of a solid complex between silver nitrate and dioxane,[3] and for this complex the authors tentatively assigned the formula $C_4H_8O_2 \cdot 8AgNO_3$, on the basis of silver determinations. Silver perchlorate was found to be insoluble in dioxane by Salomon,[4] who deduced from this that silver perchlorate did not react with dioxane. In the complexes of silver nitrate with pyrone,[5] phenol[6] and cycloöctatetraenyl phenyl ketone,[7] it is not known whether the silver ions are attached to the oxygen atoms or to other parts of the molecules, since similar compounds not containing oxygen also form silver complexes.

We have found that although silver perchlorate is insoluble in dioxane, it reacts slowly with dioxane to produce a white powder of composition $3(C_4H_8O_2) \cdot AgClO_4$, which occupies about six times the volume of the original silver perchlorate. The reaction between the well-dried reactants at 25° is very slow: it is accelerated by traces of water (with 0.5% water, reaction is complete within five days) and by heat. The conversion of silver perchlorate, in the presence of excess moist dioxane, to this complex is quantitative (99%); and dioxane may quantitatively (97%) be removed from the complex by high-vacuum distillation. The same complex may be produced in the form of large crystals by dissolving silver perchlorate in a warm mixture of equal volumes of dioxane and acetone, and cooling to room temperature. When heated the crystalline complex does not melt, but evolves dioxane and is converted to a powdery solid rapidly at 120°. Further heating causes this solid to shrink and finally to melt to a bubbling brown liquid above 400°. On heating in a flame, the complex burns quietly with occasional bright spurting: it does not appear to be explosive.

Experimental

Dioxane and anhydrous silver perchlorate were both of "reagent" grade. Silver analyses were by conventional thiocyanate titration; carbon, hydrogen and chlorine analyses were by Dr. A. Elek.

Preparation of Powdered Complex.—Silver perchlorate (1.81 g.), dioxane (10 ml.) and water (0.05 ml.) were shaken together at 25° for 5 days. Filtration yielded 4.42 g. of white powder. Adhering dioxane was removed by pumping to 5 mm. mercury pressure for 1 min. The product weighed 4.10 g., and evacuation for another minute resulted in the further loss of only 0.01 g.

Anal. Calcd. for $(C_4H_8O_2)_3 \cdot AgClO_4$: C, 30.6; H, 5.10; Cl, 7.52; Ag, 22.9. Found: C, 30.1; H, 5.14; Cl, 8.57; Ag, 23.0, 23.1.

Preparation of Crystalline Complex.—Silver perchlorate (2.2 g.), dioxane (15 ml.) and acetone (15 ml.) were warmed until the solid had dissolved, and then cooled to room temperature. The resulting crystals were filtered off, washed with dioxane, and freed from excess dioxane by a current of dry air; yield 3.4 g.

Anal. Found: C, 29.9; H, 5.13; Cl, 7.60; Ag, 23.0, 23.1.

Recovery of Dioxane from Complex.—A wide, inverted U-tube, connected to a high-vacuum line *via* a stopcock, connected the flask containing the crystalline complex (3.35 g.) with the receiver. High-vacuum distillation from the flask at 70° to the receiver cooled in liquid nitrogen yielded, after 3 hours, 1.85 g. of colorless liquid. A negligible amount collected after a further 45 min. The distillate was treated with sodium until the slight effervescence had ceased, and redistilled in the same apparatus. The distil-

(1) The research herein reported has been made possible by support extended the California Institute of Technology by the ONR, under Contract Nonr-270 (00).

(2) N. V. Sidgwick, "The Chemical Elements and Their Compounds," Oxford University Press, Oxford, Eng., 1950, p. 141.

(3) J. A. Skarulis and J. E. Ricci, THIS JOURNAL, **63**, 3429 (1941).

(4) G. Salomon, *Rec. trav. chim.*, **68**, 905 (1949); "Cationic Polymerisation and Related Complexes," edited by P. H. Plesch, Heffer, Cambridge, Eng., 1953, p. 59.

(5) R. Willstätter and R. Pummerer, *Ber.*, **37**, 3747 (1904).

(6) C. R. Bailey, *J. Chem. Soc.*, 1534 (1930).

(7) A. C. Cope and D. J. Marshall, THIS JOURNAL, **75**, 3208 (1953).