Anal. Calcd. for $C_8H_{16}O_2$: C, 66.63; H, 11.18. Found: C, 66.83; H, 11.07. The compound did not give an acid when cleaved with constant boiling hydriodic acid, but gave a 2,4-dinitrophenylhydrazone, m.p. 173–174° after recrystallization from methyl alcohol.

Anal. Calcd. for $C_{14}H_{21}O_5N_4$: N, 17.22. Found: N, 16.90.

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[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Preparation and Properties of Serum and Plasma Proteins. XXX. Crystalline Derivatives of Human Serum Albumin and of Certain Other Proteins[1a,b]

BY J. LEWIN[1c]

A systematic study has been made of the crystallization of several proteins under conditions in which a wide variety of ions or neutral molecules may be incorporated into the crystals. The reagents employed included multivalent anions and cations, many containing elements of high atomic number, and also several dyes and drugs. Crystals have been obtained of over 200 derivatives of the mercury dimer of human serum mercaptalbumin, and also of many derivatives of human decanol albumin and bovine serum albumin, together with a few derivatives of the metal-binding β-globulin of plasma. For any given added salt, dye or drug, crystals of the protein derivative could be obtained when the molar ratio of added reagent to protein was varied over a wide range. Such crystals containing ions of heavy metals should provide useful material for X-ray studies. The composition of albumin crystals containing complex ions of platinum has been studied as a function of the composition of the solution from which they are crystallized. For this purpose a new micro method of platinum analysis has been developed, which makes use of the ultraviolet absorption of the chloroplatinate ion.

Introduction

Combinations of proteins with ions of heavy metals have been known for more than a century.[2] It has been widely believed that such ions act primarily as denaturing agents. However, it is now becoming clear that complexes of native proteins with the ions of heavy metals can be formed under carefully controlled conditions, and that the native protein can be regenerated from these complexes. For example, Michael[3] has studied combinations between complex chromium compounds and several proteins; Perlmann[4] has studied metaphosphate binding and has crystallized a metaphosphoric derivative of egg albumin; and Lewin, Baudouin and Hillion[5,6,7] have prepared triiodomercuric (HgI_3^-) derivatives of a number of proteins. The amounts of these anions fixed by the proteins could be varied systematically up to a maximum equivalent to the number of free cationic groups in the protein molecule. As the number of bound groups approached this maximum, the complexes became very insoluble.

In the studies reported here, the range of compounds studied for their ability to form complexes with proteins has been greatly widened. The number of moles of added reagent per mole of protein was generally between one and ten, occasionally more. In this range the complexes of the proteins studied were very soluble in water, but crystallization of the protein salts could readily be achieved at low temperature by addition of methanol or ethanol. The method derives from that developed by Cohn, Hughes, and others for the fractionation of plasma and tissue proteins, and for the crystallization of some of the pure proteins obtained.[8,9,10,11] The added reagents, from which crystalline derivatives were prepared, include simple and complex inorganic compounds, and various organic compounds including a number of dyes and drugs.

Materials

Proteins.—As already stated, four kinds of protein preparations were used during this work. They are in order of increasing frequency of use: (1) crystalline human metal-combining serum β_1-globulin. The sample was prepared by Dr. K. Schmid following the procedure of B. A. Koechlin[10]; (2) crystalline bovine albumin[11] prepared in the Armour Laboratories; (3) human serum albumin crystallized from high ethanol concentration and ionic strengths in the range 0.05–0.3 in the presence of decanol[11]; and (4) the crystalline mercury dimer of mercaptalbumin, obtained from Fraction V of human plasma by the procedure of Hughes.[9,12]

The simple inorganic reagents employed were prepared from commercial Baker or Merck C.P. products. The

(1) (a) This paper is Number 92 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University. (b) This work was supported by the Eugene Higgins Trust, by grants from the Rockefeller Foundation, the National Institutes of Health, by contributions from industry, and by funds of Harvard University. (c) Research Fellow of the French Government Cultural Relations Committee, 1946–1947; present address: Centre National de Transfusion Sanguine, 6 Rue Alexandre-Cabanel, Paris XV, France.

(2) For the early literature see, for instance, G. Mann, "Chemistry of the Proteins," The Macmillan Co., London and New York, 1906; and F. N. Schulz, "Die Grösse des Eiweissmoleküls," Jena, 1908. Other more recent studies are discussed in the "The Chemistry of Amino Acids and Proteins," edited by C. L. A. Schmidt, C. C. Thomas, Springfield, Illinois, 2nd Edition, 1944.

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(11) E. J. Cohn, D. M. Surgenor, M. Hunter, F. W. Kahnt, *et al.*, *Science*, **109**, 443 (1949).

(12) W. L. Hughes, Jr., *Cold Spring Harbor Symposia*, **14**, 79 (1950). This protein, according to Hughes, is a dimer of mercaptalbumin (the fraction of serum albumin containing a single free SH-group per molecule) in which two mercaptalbumin residues are linked through their –SH groups, by a mercury atom. It can be formulated Alb.S–Hg–S–Alb, where Alb.S– represents the mercaptalbumin residue

TABLE I

CONDITIONS FOR PREPARATION OF CRYSTALLINE PROTEIN SALTS

Human Mercoptalbumin Mercury Dimer Derivatives

Ions	Number of moles / mole protein	pH	Methanol, %	Temp., °C.	Γ/2
Derivatives Prepared from Solutions of Salts Containing Multivalent Anions or Anions Containing Heavy Metals					
$WO_4^=(Na^+)_2$	1–4	5.20–5.06	11–21	0	0.030–0.043
$MoO_4^=(Na^+)_2$	1–5	5.20–5.06	8.27	+4	.030– .046
$CrO_4^=(Na^+)_2$	1–5	5.20–5.06	11–21	+4	.030– .046
$SeO_4^=(Na^+)_2$	1–12	5.20–4.93	11–24	0	.030– .055
$VO_4^=(Na^+)_3$	1–2	5.20	17	0	.036– .042
$VO_3^-Na^+$	1–5	5.20	7–17	0	.033– .045
$PO_3^-Na^+$	1–35	5.20–4.90	7–14	0	.030– .060
$Fe(CN)_6^{\equiv}(K^+)_3$	1–4	5.11	11–17	0	.036– .054
$Fe(CN)_6^{=}(K^+)_4$	1–3	5.20–5.11	17[a]	0	.040– .060
$Au(CN)_2^-K^+$	1–3	5.20	11–19	0	.030
$Cu(CN)_4^=(K^+)_2$	1–3	5.12	11–17	0	.036– .048
$Ag(CN)_2^-K^+$	1–12	5.20–4.85	11–21	0	.030– .060
$Nd(NO_3)_5^=(NH_4^+)_2$	1	5.12	6	0	.030
$HgI_3^-K^+$	1–2	5.20	14–17	+4	Approx. 0.050
$CdI_3^-K^+$	1–2	5.20	11	0	Approx. 0.050
$AlCl_4^-Na^+$	1–2	5.20	7	0	0.033–0.036
$AuCl_4^-Na^+$	1–2	5.20	11	0	.033– .036
$SnCl_6^=(K^+)_2$	1–2	5.12	8–14	0	.033– .036
$PtCl_6^=(Na^+)_2$	1–9	5.20–4.76	6–19	+4 to −5	.033– .065
$PtBr_6^=(Na^+)_2$	1–3	5.12	6–13	0	.033– .040
$Fe(CN)_5(NO)^=(Na^+)_2$	1–10	5.20–4.97	11–19	0	.03– .05
Reinecke salt	1–2	5.20	11	0	.03
Diiodo·phenyl sulfonate	1–10	5.12	7–17	0	.03– .05
Flavianic acid	1–5	5.12	7–17	+4 to −3	.03
Cationic Derivatives Containing Multivalent Cations or Cations of Heavy Metals					
Rb^+Cl^-	1–7	5.20	7–11	0	0.030–0.037
$Mg^{++}(Cl^-)_2$	5–10	5.12	7	0	.045– .060
$Ca^{++}(Cl^-)_2$	1–10	5.12–5.35	7–16	0	.033– .060
$Sr^{++}(Cl^-)_2$	1–10	5.12–5.26	7–17	0	.033– .060
$Ba^{++}(Cl^-)_2$	1–10	5.12–5.36	7–17	0	.033– .060
$Ba^{++}(OH^-)_2$	1–15	5.17–7.80	7–17	0	.033– .060
$Zn^{++}(Cl^-)_2$	1–10	5.12–5.20	7–14	0	.033– .060
$Cu^{++}(Cl^-)_2$	1–2	5.20	7–12	0	.033– .060
$Mn^{++}(Cl^-)_2$	1–10	5.12–5.26	7–14	0	.033– .060
$Co^{++}(Cl^-)_2$	1–10	5.12–5.26	7–14	0	.033– .060
$Ni^{++}(Cl^-)_2$	1–5	5.12–5.26	7–14	0	.033– .045
$Ag^+NO_3^-$	1	5.12	11	0	.030
$CdCl_2$	1–3	5.00	7–11	0	.033– .04
$La^{+++}(Cl^-)_3$	1	4.96	7	0	.036
$Co(NH_3)_6^{+++}(Cl^-)_3$	1–5	5.20	4–11	0	.036– .060
trans-$Co(Cl_2en_2)^+Cl$[b]	1–5	5.02	7	−5	.030– .040
cis-$Co(Cl_2en_2)^+Cl^-$	2–15	5.20	7–17	+4	.030– .045
$Co(en)_3^{+++}(Cl^-)_3$	1–10	5.20	4–8	+4	.036– .09
$[Co(en)_2ClH_2O]^{++}(Cl^-)_2$	5–15	5.20	11–14	+4	.045– .075
$Pt(NH_3)_4^{++}(Cl^-)_2$	1–15	5.12–5.68	7–24	−3	.033– .080
Derivatives Containing Dyes					
Orange G	0.5–4	5.00–5.12	7–14	−3	Approx. 0.06
Light Green S.F.	0.5–3	5.00–5.12	11–19	−3	Approx. 0.06
Methyl Green	0.5–4	5.00–5.12	7–14	−3	Approx. 0.06
Evans Blue	0.5–1	5.00	11–14	−3	Approx. 0.06
Derivatives Containing Drugs					
Quinine	1–10	5.00–5.20	4–9	−3	Approx. 0.03–0.05
Strychnine	1–7	5.00–5.20	4–11	−3	Approx. 0.03–0.05
Sulfathiazole	1–7	5.00–5.20	4–7	−3	Approx. 0.03–0.05
Sulfadiazine	1–7	5.00–5.20	4–11	−3	Approx. 0.03–0.05
Barbital	1–7	5.00–4.86	4–7	−3	Approx. 0.03–0.05
Penicillin	1–8	5.00–4.86	7–17	−3	Approx. 0.03–0.05
Miscellaneous					
Aminopterine	1–7	5.00–5.20	4–7	−3	Approx. 0.03–0.05
Clupeine	1	4.96	7	−3	

[a] The solution with 3 moles of $K_4Fe(CN)_6$ per mole of albumin contained 17% ethanol and 10% acetone. [b] The symbol "en" denotes ethylenediamine. In all calculations here, and elsewhere in this paper, the molecular weight of human or bovine serum albumin is taken as 69,000 (G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946); J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947)). The molecule of the mercaptalbumin mercury dimer is of course twice as large, but for the expression of stoichiometric ratios of albumin to other reagents the molecular weight of the albumin monomer has been employed throughout.

same products were used as starting material for preparation of complex inorganic ions, such as the mercury, cobalt, platinum complexes, etc.

The organic reagents were the purest commercially available products, in most cases used directly, though sometimes after redistillation or recrystallization. Methanol, ethanol or acetone of C.P. grade were the principal organic solvents used.

General Methods of Crystallization

The general conditions for crystallization were within the limits

Protein concn. approx.	10 gr. %
*p*H	4.4 to 6.5
Γ/2	0.02 to 0.4
Concn. of org. solvent methanol–ethanol–acetone	5 to 30%
Temperature, °C.	+5 to −5

The protein solution (usually in 10% concentration) was adjusted to the required *p*H by addition of either a *p*H 4.0 acetate buffer or 0.1 *M* NaOH. The final *p*H was checked on an aliquot, diluted to 1% protein, by potentiometric measurement using a glass electrode and the Cambridge *p*H meter. The reagent, usually as a 0.1 *M* solution, was then added, thoroughly mixed with the protein, and the mixture cooled to 0°. Cold 100% methanol or ethanol (chilled to −5°) was then added in small portions with constant stirring until the necessary concentration was reached. Frequently this coincided with the appearance of weak but persistent turbidity.[13] During all the manipulations the temperature was carefully watched and maintained below 0°. The prepared solution was kept in the cold for a long period. Crystals often appeared rapidly, in a matter of hours, especially when the mixture was seeded with crystals previously obtained. In some cases, however, several days or weeks were required. Yields of 50–70% were readily achieved in most of the systems studied. A technique for the preparation of good individual crystals of large dimensions will be described below.

The data in Tables I, II and III give detailed conditions for crystallization of the more important salt derivatives

TABLE II

CONDITIONS FOR PREPARATION OF CRYSTALLINE PROTEIN SALTS

Bovine Serum Albumin Derivatives

Ion	Number of mols / mol protein	*p*H	Methanol, %	Temp., °C.	Approx. Γ/2
$SeO_4^=(Na^+)_2$	1-2	4.96-4.80	11	0	0.03
$CrO_4^=(Na^+)_2$	1-2	4.96	13-19	0	.03
$WO_4^=(Na^+)_2$	1-5	4.96-4.74	11-17	0 to −3	.03-0.04
$MoO_4^=(Na^+)_2$	1-2	4.96-4.80	11	0	.03
$Ag(CN)_2^-(K^+)$	1-4	4.96-4.80	12-17	0 to −3	.03- .04
$Ca^{++}(Cl^-)_2$	1-3	4.96-5.04	11-13	0 to −3	.03- .04
$Ba^{++}(Cl^-)_2$	1-3	4.96-5.04	11-13	0 to −3	.03- .04
$Sr^{++}(Cl^-)_2$	1-3	4.96-5.04	11-13	0 to −3	.03- .04
$Zn^{++}(Cl^-)_2$	1-3	4.96-5.04	7-11	0 to −3	.03- .04
$Ni^{++}(Cl^-)_2$	1-3	4.96-5.04	7-9	0 to −3	.03- .04
$Cu^{++}(Cl^-)_2$	1-2	4.96	9	0	.03
$Mn^{++}(Cl^-)_2$	1-2	4.96	11	0	.03
$Co(NH_3)_6^{+++}(Cl^-)_3$	1-2	4.96	11	0	.03
Quinine	1-2	4.96	11	0	.03

(13) When a mixed solvent was used, ethanol and acetone or methanol and acetone, the alcohol was added first, approximately half the amount of that expected to be necessary for crystallization from the solvent alone, and then the acetone added carefully as before in small fractions. The addition was stopped when the amorphous precipitate locally formed had begun to redissolve with difficulty.

obtained with the three varieties of serum albumin studied. It should be stated that these conditions are not the only ones under which the crystalline derivatives are formed, but they generally correspond to a good yield.

TABLE III

CONDITIONS FOR PREPARATION OF CRYSTALLINE PROTEIN SALTS

Human Serum Decanol Albumin Derivatives

Ion	Number of mols / mol protein	*p*H	Methanol, %	Temp., °C.	Approx. Γ/2
$MoO_4^=(Na^+)_2$	1-3	4.96-4.84	27-23	−3	0.15
$WO_4^=(Na^+)_2$	3-8	4.84-4.69	26-33	−3	.02-0.035
$CrO_4^=(Na^+)_2$	3-8	4.84-4.69	25-31	−3	.02- .035
$Ag(CN)_2^-(K^+)$	3-8	4.84-4.69	21-28	−3	.02- .035
$Mg^{++}(Cl^-)_2$	3-8	5.00-5.18	23-31	−3	.02- .035
$Ca^{++}(Cl^-)_2$	3-8	5.00-5.18	23-31	−3	.02- .035
$Mn^{++}(Cl^-)_2$	3-8	5.00-5.18	18-24	−3	.02- .035
$Zn^{++}(Cl^-)_2$	3-5	5.00-5.12	15-17	−3	.02
$Sr^{++}(Cl^-)_2$	3-8	5.00-5.18	23-31	−3	.02- .035
$Co(NH_3)_6^{+++}(Cl^-)_3$	3-8	5.00-5.18	17-21	−3	.02- .035

Analysis of Solutions and Mother Liquor: Composition of Crystals

The data already given define the conditions of crystallization, but further analysis is required to determine the amount of the added reagent which is present, together with the protein, in the crystalline phase. Such analyses are laborious, and are feasible only if accurate micro-methods are available for the analysis of the added reagent, especially if only a few moles of reagent are present per mole of protein.

Platinum derivatives appeared to be the most convenient for these studies, as it was possible to develop a spectrophotometric method capable of determining amounts as small as 3 to 5 μg. of the metal with ±5% accuracy in a sample containing as much as 100 mg. of protein.[14] Both anionic derivatives containing $PtCl_6^=$, and cationic derivatives containing $Pt(NH_3)_4^{++}$, could be studied by the same analytical method.

Method of Platinum Analysis.—In principle the method was as follows. The solution was dried, and the residue incinerated at 700°. The metallic platinum obtained was dissolved in hot aqua regia and the resulting chloroplatinic solution diluted in a volumetric flask to a known volume. The optical density at 265 mμ was then measured in a Beckman spectrophotometer. The molar absorption coefficient of $PtCl_6^=$ at this wave length is 26,920 and a solution containing 1.5 μg.Pt/ml. has an optical density equal to 0.207 in a 1-cm. cell.

Detailed Procedure

0.5 to 2 ml. of the solution containing 50–200 mg. of protein and the unknown amount of platinum were transferred into a weighed crucible. The product was dried at 105° to constant weight, and the dry residue incinerated in an electric oven at 700° overnight. After cooling, the black residue was treated with 1 ml. of freshly prepared aqua regia and heated on a sand-bath at approximately 200° until the liquid had almost completely evaporated. The platinum was completely dissolved by this first treatment. Another ml. of aqua regia was added and the evaporation repeated once more. The brownish residue was then dissolved in 1 ml. of a solution made up as follows: 2.5 g. of sodium

(14) In the studies reported here, the platinum content of the crystals was determined indirectly, by analysis of albumin solutions before and after crystallization; the method is described in detail below. However, the sensitivity of this method of platinum determination should permit the analysis of single crystals, 1 to 3 mm. in diameter, containing only one mole of platinum per mole of albumin.

chloride and 25 ml. of concd. hydrochloric acid are dissolved in sufficient water to bring the total volume to 100 ml. The mixture was again evaporated on the sand-bath. The subsequent manipulations were carried out with precautions to avoid exposure to bright light, since this brings about a photochemical change in the chloroplatinic ion in aqueous solution.[15] The spectrophotometric reading was carried out promptly after the final solutions were prepared. The yellow residue formed was dissolved in several portions of HCl (approximately 2 *M*) and transferred quantitatively into a volumetric flask which was then made up to volume with the same HCl solution. The dilution was chosen to give a final platinum concentration of 1.5 to 6 μg. Pt/ml. The Beckman ultraviolet spectrophotometer and a 1 cm. quartz cell were used to determine the optical density at 265 mμ. It was in general desirable to make readings at several dilutions; this also provided a check on the applicability of Beer's law.

The simplified method described is directly applicable in cases where no impurities are present which may contribute to absorption at 265 mμ. If such impurities were present, it would be necessary to wash the platinum residue several times with hot nitric acid, followed by filtration on a sintered glass filter. This should eliminate all impurities but the Pt-group metals. It was not determined whether the latter elements would interfere in the analysis, since they were known to be absent from the preparations studied.

The results of control analyses carried out by the method described above on solutions containing known amounts of platinum, both in the absence and in the presence of albumin, are summarized in Table IV. This shows that in the presence of albumin the platinum is quantitatively recovered.

TABLE IV

PLATINUM DETERMINATION IN PLATINUM-PROTEIN SOLUTIONS CONTAINING 100 MG. PROTEIN/ML.

Mg. of platinum in the sample	
Added	Found
5.1	4.8
10.0	10.3
14.6	15.1

In the presence of protein the absorption spectrum of the $PtCl_6^=$ ion is unmodified, as is shown in Fig. 1. The effect of exposure to light was also found to be unmodified by the presence of the albumin.

Method of Protein Analysis.—Protein was determined by dry weight, the solution being dried at 103–105° to practically constant weight (approximately 18 hours). Ash determinations were made after incineration. The correction for ash was generally of the order of 2% of the weight of the protein.

Composition of Crystals.—Certain other protein crystals such as β-lactoglobulin[17] and horse methemoglobin[18] have been studied by direct analysis of the crystal after it had been removed from the mother liquor and any remaining adhering mother liquor wiped off. In the present studies, a different procedure had to be adopted. Because of the relatively high solubility of the crystals in the solutions with which they were washed in order to remove the mother liquor, a direct analysis of the crystalline mass appeared to be excluded. Therefore, analysis of platinum and of albumin was carried out on an aliquot of the albumin solution before crystallization, and again on an aliquot of the mother liquor after extensive crystallization had taken place. From the changes in composition which had occurred in the liquid, it was possible to calculate approximately the ratio of platinum to albumin in the crystalline phase. This calculation is not completely unambiguous, since the result obtained depends on a factor, which we shall denote as f, representing the ratio of the total volume of the crystals to the volume of the albumin in the crystals and expressing the degree of hydration of the crystals. However, calculation shows that uncertainties in the value of this factor produce relatively little alteration in the calculated ratio of platinum to albumin in the crystals (see columns 8 and 9, Table V). The values of f considered ($f = 1$ and $f = 2.5$) correspond, respectively, to 0 and 53% of hydration by volume.[19]

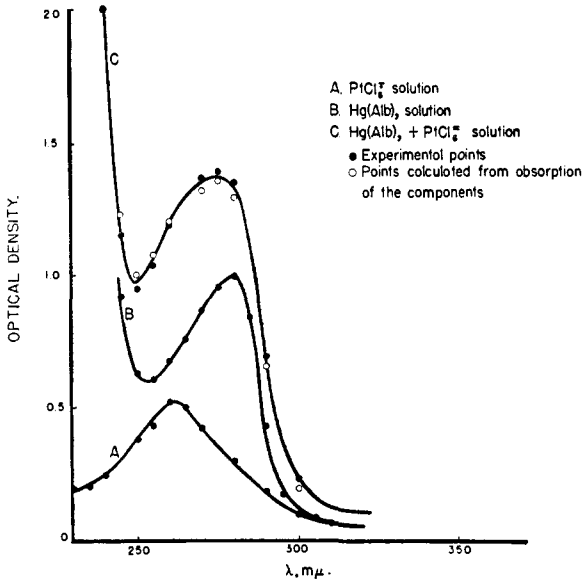


Fig. 1.—Ultraviolet absorption spectra of (A) chloroplatinate solution, (B) mercaptalbumin mercury dimer, and (C) the two solutions combined. Approximate concentration of both albumin and chloroplatinate was 2×10^{-5} molar.

The detailed procedure of calculation is as follows: in the solution before crystallization let a_0 denote the mg. of albumin per ml. solution and Pt_0 the corresponding mg. of platinum/ml. The crystals formed from 1 ml. of solution contain A mg. of albumin, Pt mg. of platinum; these are the quantities which are to be determined by calculation from the experimental measurements. The volume of anhydrous albumin in the crystals may be taken as $\bar{V}A$, where $\bar{V}$, the partial specific volume of the anhydrous albumin, is taken as 0.733 ml./g. or 0.733×10^{-3} ml./mg.—the same value found in aqueous solutions.[20] The total volume of crystals from 1 ml. of solution is $f\bar{V}A$, where f is the volume ratio factor defined in the preceding paragraph. Unpublished studies in this Laboratory[19] indicate that f may be of the order of 2.5. For purposes of calculation, we shall assume a lower limit for f of 1 and an upper limit of 2.5.

If it is assumed that there is no change of volume on crystallization,[21] then the volume of supernatant liquid after crystallization, from 1 ml. of original solution, is $1 - f\bar{V}A$. Let the concentrations (mg./ml.) of albumin and platinum in this supernatant be a_s and Pt_s, respectively. Then, since the total albumin in the supernatant is $a_0 - A$ and the total platinum is $Pt_0 - Pt$, a_s and Pt_s are given by the relations

$$a_s = \frac{a_0 - A}{1 - f\bar{V}A} \qquad (1)$$

(15) A more detailed discussion of the method of platinum analysis will be given later in a separate paper. Boll[16] showed that the change in the absorption spectrum of a fresh $PtCl_6H_2$ solution after exposure to bright daylight is due to the hydrolysis of the chloroplatinic acid with formation of various hydroxychloroplatinic derivatives, and liberation of HCl.

$$(PtCl_6)^= + H_2O \rightleftarrows (PtCl_5OH)^- + HCl$$

$$(PtCl_5OH)^- + H_2O \rightleftarrows (PtCl_4(OH)_2)^= + HCl$$

The hydrolysis is avoided by keeping the platinic complex solutions in the dark and by adding a large excess of HCl in order to shift the equilibrium to the left.

(16) M. Boll, *Compt. rend. acad. sci. Paris*, **156**, 138 (1913); **156**, 691 (1913).

(17) T. L. McMeekin and R. C. Warner, THIS JOURNAL, **64**, 2393 (1942).

(18) J. Boyes-Watson, E. Davidson and M. F. Perutz, *Proc. Roy. Soc. (London)*, **A191**, 83 (1947).

(19) The composition and density of the crystals are now being studied by Mr. F. M. Richards and Dr. B. W. Low in this Laboratory.

(20) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(21) Recent measurements of F. M. Richards indicate that this assumption is true to better than 1 part in 10^3.

TABLE V

CALCULATION OF ALBUMIN, PLATINUM AND PLATINUM/ALBUMIN RATIO IN CRYSTALS OF HUMAN SERUM ALBUMIN CONTAINING $PtCl_6^{=}$ OR $Pt(NH_3)_4^{++}$ IONS

Expt. No.	a_0	a_s	Pt_0	Pt_s	Mole Pt/mole Alb = R: R_0	R_s	$R_{cryst.}$ (f = 1)	$R_{cryst.}$ (f = 2.5)	A/a_0
Crystallization from $PtCl_6^{=}$ Solutions									
322	103.6	54.4	0.221	0.123	0.75	0.80	0.71	0.71	0.53
323	102.0	57.9	.418	.232	1.45	1.42	1.46	1.46	.48
324	92.4	56.8	.609	.386	2.33	2.40	2.21	2.24	.43
1189	83.0	46.0	.772	.452	3.28	3.47	3.06	3.10	.49
1190	80.0	28.2	.903	.349	4.00	4.37	3.75	3.76	.68
1191	82.0	49.3	1.078	.697	4.65	5.00	4.15	4.19	.44
Crystallization from $Pt(NH_3)_4^{++}$ Solutions									
1688	76.8	32.7	0.772	0.597	3.56	6.5	1.53	1.70	.61
1689	73.3	30.5	1.530	1.167	7.38	13.5	3.26	3.56	.62
1690	67.4	35.9	2.040	1.675	10.72	16.6	4.38	4.82	.50

a_0 = albumin content of liquid before crystallization (mg./ml.); a_s = albumin content of supernatant liquid after crystallization. Pt_0 and Pt_s denote the corresponding values of platinum content (mg. Pt/ml.) before and after crystallization. $R_0 = 353.3\ Pt_0/a_0$; $R_s = 353.3\ Pt_s/a_s$.

Hence

$$Pt_s = \frac{Pt_0 - Pt}{1 - f\bar{V}A} \quad (2)$$

$$A = \frac{a_0 - a_s}{1 - f\bar{V}a_s} \quad (3)$$

$$Pt = Pt_0 - Pt_s(1 - f\bar{V}A) = \frac{Pt_0(1 - f\bar{V}a_s) - Pt_s(1 - f\bar{V}a_0)}{1 - f\bar{V}a_s} \quad (4)$$

$$\frac{Pt}{A} = \frac{Pt_0(1 - f\bar{V}a_s) - Pt_s(1 - f\bar{V}a_0)}{a_0 - a_s} \quad (5)$$

The data from six experiments on solutions containing $PtCl_6^{=}$ ions and from three experiments on solutions containing $Pt(NH_3)_4^{++}$ ions are given in Table V. The calculated weight ratio, Pt/A, is given by equation 5. The mole ratio, R, of platinum to albumin in the crystals, as presented in Table V, is obtained from the weight ratio by multiplying by the factor 353.3. In the chloroplatinate solutions, R is very nearly the same in the original solution and in the supernatant liquid after crystallization. Correspondingly the value of R calculated for the crystals is nearly the same as in the solution and, in this case, is practically independent of the assumed value of f. On the other hand, the value of R in the solutions containing $Pt(NH_3)_4^{++}$ ions is much higher in the supernatant after crystallization than in the original solutions. Correspondingly R is much lower in the crystals than in the solution from which they were crystallized, whatever value of f may be assumed. However, the assumption of an f value of 2.5 gives a calculated value of R about 10% greater than that obtained on the assumption that $f = 1$. The higher values are probably the more reliable. Table V also contains, in the last column, estimates of the fraction of the total albumin in the system found in the crystals after crystallization. These values were calculated from equation 1, assuming $f = 2.5$. It appears that in all experiments the fraction of albumin crystallized lies above 0.4 and below 0.7, generally not very far from 0.5.

Methods for Preparation of Large Crystals.—It was observed that many protein salts, among the large number studied in this work, crystallized more readily than the isoionic protein. Also, in work with certain albumin derivatives, crystals of considerable size and with particularly well developed faces were obtained. From human decanol albumin, the $Co(NH_3)_6^{+++}$ derivatives gave crystals 2–3 mm. long and of considerable thickness. Mg^{++} and $MoO_4^{=}$ derivatives were also large, although somewhat more difficult to grow.

Cu^{++} salts were the only derivatives of bovine serum albumin to give relatively large and well-developed crystals; These were six-sided prisms. On the other hand, numerous derivatives of the mercury mercaptalbumin dimer gave large, well formed crystals—notably those containing PO_3^{-}, Cd^{++}, Sr^{++}, $Co(NH_3)_6^{+++}$ and $Pt(NH_3)_4^{++}$ ions. The largest crystals obtained were as long as 7 mm. Photographs of some of the crystalline samples are shown in Figs. 2 and 3.

In preparing large crystals, many details of technique were important. Preliminary exploration of the optimum conditions for maximum yield of small crystals as a function of *p*H, salt concentration, protein concentration and temperature was always necessary. The conditions for growing large crystals were generally found to diverge slightly from the optimum conditions for maximum total yield of crystals. Several virtually identical samples were always prepared. The solutions were adjusted rapidly to the chosen conditions, with effort to avoid shaking, bubble formation or mechanical disturbance. After preparing the cold solution, it was often found advantageous to warm the solution a little, in order to dissolve the majority of the sub-microscopic crystals which had formed rapidly, and conserve in the medium only a few of them as centers for further growth. The solution was then cooled slowly to the desired final temperature. Protection from vibration or jarring was found important. To permit slow growth of large crystals, it was desirable to reduce the area of air–liquid interfaces, or to eliminate them entirely. Small bottles completely filled with solution and stoppered, were employed. The capacity of these bottles was 3–5 ml., with neck of inner diameter 10 mm. Before the rubber stopper was pushed in, it was pierced by an injection needle (1 mm. bore) which permitted the escape of all air from the bottle. The liquid was then allowed to rise in the needle, under the pressure applied through the stopper; the needle could then be withdrawn.

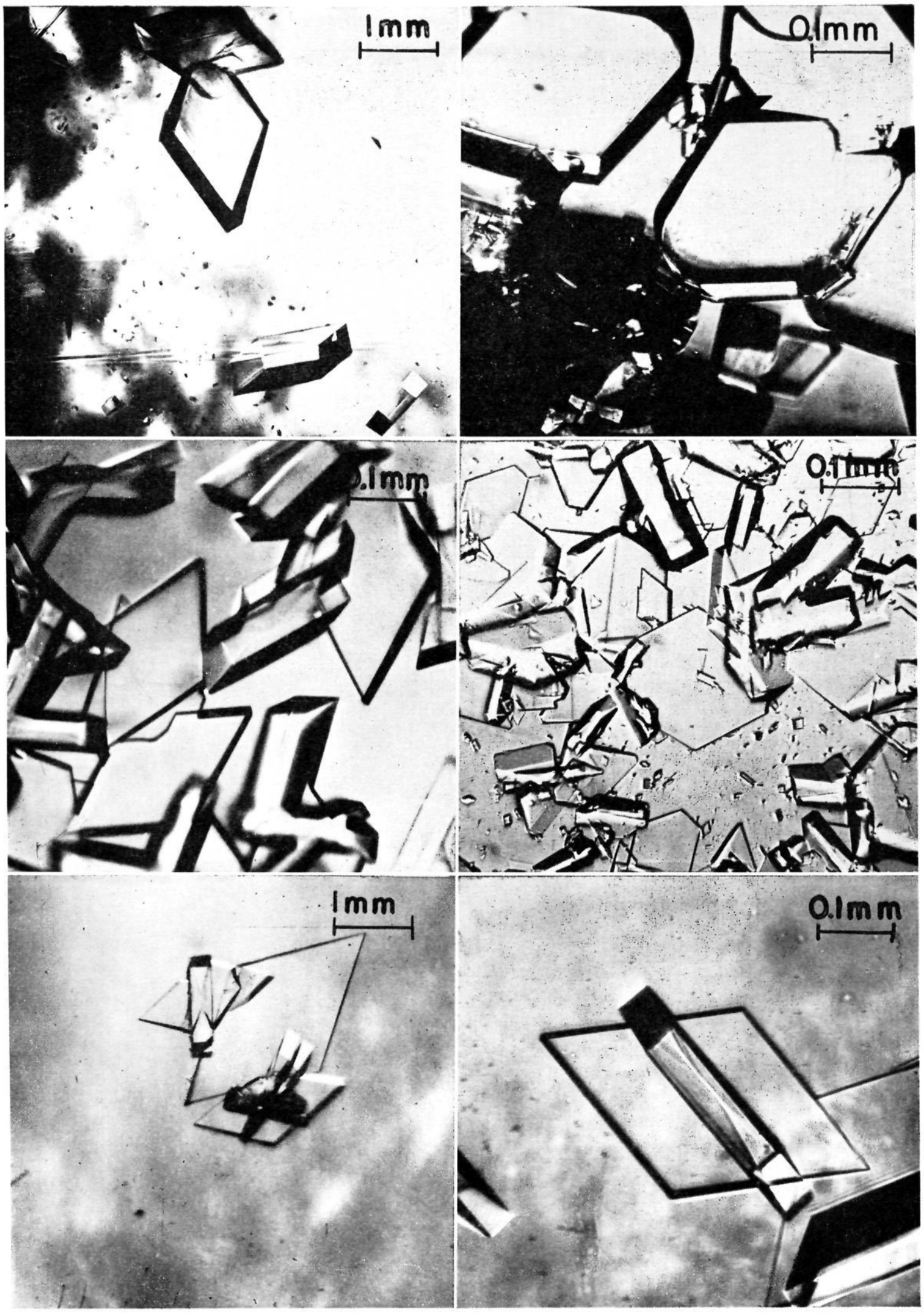
Use of Mixed Solvents.—In some cases, when crystallization appeared difficult from alcohol or acetone solutions, a mixture of these solvents was found to induce crystallization. Optimum proportions were generally about 40% alcohol and 60% acetone by volume. In this way crystallization of derivatives in the presence of high concentrations of reagents such as nitroprusside, which did not crystallize from a single solvent, was achieved.

Discussion

We believe that this investigation has contributed toward showing the extraordinary ability of the protein molecule to form salt derivatives and the usefulness of these derivatives for the crystallization of proteins.

The general nature of the reaction has been demonstrated by the large number of derivatives prepared from four different proteins. The degree of binding, of course, varies with the protein and the reagent used. Insulin has been combined with a series of similar reagents, to form crystalline derivatives, by Oncley and Ellenbogen[22] in this

(22) E. Ellenbogen, Doctoral dissertation, Harvard University, 1949.



Upper left: Cadmium, Cd^{++}, Derivative of Mercaptalbumin Mercury Dimer.

Center left; Barium, Ba^{++}, Derivative of Mercaptalbumin Mercury Dimer.

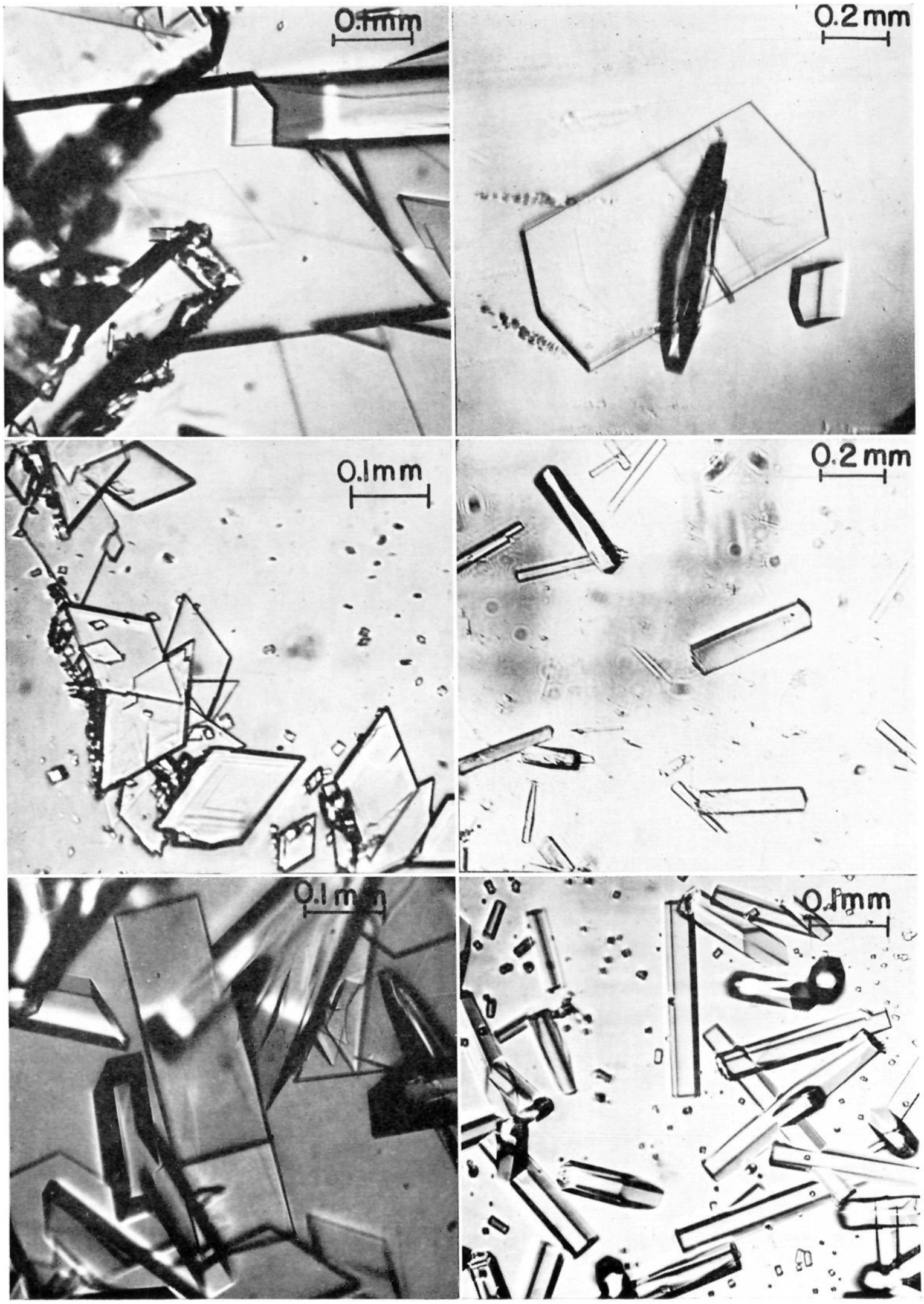
Lower left: Strontium, Sr^{++}, Derivative of Mercaptalbumin Mercury Dimer.

Upper right: Metaphosphate, PO_3^-, Derivative of Mercaptalbumin Mercury Dimer.

Center right: Mercury Iodide, HgI_3^-, Derivative of Mercaptalbumin Mercury Dimer.

Lower right: Platinum Amine, $Pt(NH_3)_4^{++}$, Derivative of Mercaptalbumin Mercury Dimer.

Fig. 2.



Upper left: Strychnine Derivative of Mercaptalbumin Mercury Dimer.

Upper right: Magnesium, Mg^{++}, Derivative of Human Serum Decanol Albumin.

Center left: Orange G. Derivative of Mercaptalbumin Mercury Dimer.

Center right: Cobaltammine, $Co(NH_3)_6^{++}$, Derivative of Human Serum Decanol Albumin.

Lower left: Barbital Derivative of Mercaptalbumin Mercury Dimer.

Lower right: Copper, Cu^{++}, Derivative of Bovine Serum Albumin.

Fig. 3.

department. Perlmann[4] previously succeeded in preparing a crystalline salt derivative of egg albumin and metaphosphoric acid. McMeekin, *et al.*,[23] have prepared crystalline derivatives of β-lactoglobulin with dodecyl sulfate, and Alderton and Fevold[24] some crystalline salts of lysozyme.

Thus already eight different proteins have been shown to give crystalline salts, five of them—namely, insulin and the four studied in this paper—with a large variety of reagents.

The derivatives described here are distinguished from certain others by the fact: (1) that they may be ascribed to very common functional groups (principally the basic and acidic) of the protein molecule and for these reasons can be prepared from a large variety of proteins, and (2) they generally represent very slight modification of the protein molecule, being obtained under very mild conditions involving little or no danger of denaturation.

The study of these derivatives in this Laboratory is continuing.

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BOSTON, MASS. RECEIVED NOVEMBER 29, 1950

(23) T. L. McMeekin, B. D. Polis, E. S. Della Monica and J. H. Custer, THIS JOURNAL, **71**, 3606 (1949).

(24) G. Alderton and H. L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Preparation and Properties of Serum and Plasma Proteins. XXXI. An Optical and Morphological Study of Some Crystalline Human Serum Albumin Preparations and of Their Derivatives[1a,1b]

BY BARBARA W. LOW AND E. J. WEICHEL

A study has been made of the optical and morphological properties of two series of crystalline human serum albumin preparations. Crystals of human serum decanol albumin, mercaptalbumin mercury dimer, and a wide range of the crystalline derivatives, described by J. Lewin in the preceding paper (*q.v.*), of both these protein preparations have been examined. Measurements have been made both on wet, and on air-dried crystals, and observations of changes during drying have been recorded. The morphological and optical constants of the crystals have been measured. Close relationships between the optical and morphological properties of the crystals of the parent proteins and crystals of their derivatives have been established.

Introduction

Measurements of the optical and morphological properties of crystalline preparations are made both for purposes of identification,[2] and also for the valuable light they may throw upon the general features of a crystal structure.[3]

Morphological features and optical constants may provide definite information concerning the shape, orientation and packing of molecules in a crystal structure.

In their X-ray study of methaemoglobin, Boyes-Watson, Davidson and Perutz[4] have deduced the orientation of the heme groups with respect to each other and their general packing direction in the cell, from measurements of the optical properties of the methaemoglobin crystals.

The difficulties involved in measuring the optical constants of protein crystals have been discussed by F. Jones[5] who has studied lysozyme chloride crystals and reported the most detailed measurements of the optical constants of a single protein species so far recorded.[6]

The observations recorded here were made on (1) human serum decanol albumin crystallized at high ethanol concentrations (23–40%) and ionic strengths in the range $\Gamma/2 = 0.05$–0.30[7] and some of its derivatives,[8] and (2) mercaptalbumin mercury dimer, crystallized from ethanol–water mixtures,[9] and some of its derivatives.[8]

The purpose of this study has been to measure the morphological and optical constants of these two series and to investigate the relationships between the crystals of the parent proteins and of their derivatives.

Materials.—With the exception of the mercaptalbumin mercury dimer crystals prepared for us by Dr. W. L. Hughes, Jr., all the crystals used in this study were prepared by Lewin.[8]

(1) (a) This work was supported by the Eugene Higgins Trust, by grants from the Rockefeller Foundation, the National Institutes of Health, by contributions from industry, and by funds of Harvard University. (b) This paper is Number 93 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University.

(2) E. M. Chamot and C. W. Mason, "Handbook of Chemical Microscopy," Vol. I, 2nd Edition, John Wiley and Sons, Inc., New York, N. Y., pp. 319–324.

(3) W. H. Hartshorne and A. Stuart, "Crystals and the Polarizing Microscope," 2nd Edition, Edward Arnold and Co., London, 1950, pp. 130–159.

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(6) For other studies consult the references given by Jones (ref. 5).

(7) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947). This crystal structure is one of two crystallographically quite different crystalline modifications of human serum decanol albumin described by these authors. The range of conditions for these two preparations are wholly different and mutually exclusive. (The second modification is crystallized from water saturated with decanol and at ionic strengths $\Gamma/2 < 0.001$.)

(8) J. Lewin, *ibid.*, **73**, 3906 (1951).

(9) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).