[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Countercurrent Distribution of Serum Albumin

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In the search for systems which might permit fractionation of proteins by countercurrent distribution, a study of the behavior of serum albumin has been made. It was found possible to distribute this protein with no detectable denaturation in a system containing a small amount of trichloroacetic acid. While these systems did not provide resolution of the closely related forms of serum albumin, resolution from a number of other protein types was easily accomplished. More interesting from the standpoint of countercurrent distribution was the fact that a band far too narrow was obtained. An explanation of the phenomenon was found. Of further interest is the fact that a molecule as large as 68,000 can be successfully distributed. The use of acetyltryptophan was found helpful in recovery of the protein.

With the present day prospects of a much more detailed inquiry into the more subtle aspects of protein structure it has become increasingly important to be critical of the problem of purity with these substances. Countercurrent distribution (C.C.D) is a technique known to be one of those most effective for this purpose with polypeptides,[1] but thus far it has had only limited application to proteins. However, it has been very enlightening for those cases where it has been applied. These include insulin,[2] ribonuclease,[3] lysozyme,[4] the adrenocorticotropins,[5] lactogenic hormone,[5] growth hormone[5] and casein.[6] Even the serum proteins have been separated by a few stages in two phase systems[7] but here the separation time of the phases has been excessively long.

The first protein to be precisely studied by the method was beef insulin.[2] In this study the use of complexing agents such as trichloroacetic acid and dichloroacetic acid in appropriate concentrations was tried for the first time with a protein to provide a usable partition ratio in a two phase liquid–liquid system. The relatively high selectivity of the method was demonstrated by the separation of a des-amido insulin. Later a somewhat different system[8] permitted an artificial mixture of beef and sheep insulin to be resolved. These separations have not thus far been accomplished by any other method.

Since the experience with insulin, systems employing the chloro acids have been widely used in fractionating the peptide and protein hormones of the pituitary[5] and again have demonstrated their selectivity by being able to distinguish and separate two hormones of molecular weight in the 5000 class in which the only detectable difference is the replacement of a single amino acid with a different amino acid. On the other hand, there were also cases of failure to separate closely related hormones as might be expected.

In general, the systems must be of such a composition as to be very near the critical point, an obvious requirement which has been pointed out by other workers.[6] Because of this it might be expected that the selectivity of the systems would be poor. Quite the contrary appears to be the case. A system which gives a partition ratio in an acceptable range for a given protein will usually not be satisfactory for other proteins. The problem of selectivity accordingly does not present the greatest difficulty. Rather it is the problem of the prevention of transformation, reversible or irreversible, during the operation. Since the selectivity of the systems is usually good even minor alterations in the molecular structure during the run or isolation procedure are quickly recognized by the type of pattern obtained or by a shift in partition ratio.

It has long been known that proteins are likely to undergo denaturation under the conditions required for C.C.D. Here instability may not stem entirely from the mere contact with a second liquid phase which must be largely composed of an organic solvent. The shaking required for rapid interchange of the solute between the two phases could also play a role since it is known that certain proteins denature at an interface. Such considerations as these are sufficient to emphasize the view that a successful approach to the fractionation of proteins by C.C.D. must include a study of certain phases of the phenomenon of denaturation and the search for conditions which will prevent it.

Distributions can be performed under the restricted conditions optimal for protein stability such as low temperature, favorable *p*H range, etc., even though these may be technically bothersome. Somewhat more interesting could be the possibility of adding some complexing or binding agent which would give greater stability to the molecule. A number of instances have been cited in the literature where such binding agents have given protection against heat and urea denaturation.[9] As an extreme case[10] it has been reported that a 1% aqueous solution of bovine serum albumin (BSA) can be heated to 175° for one hour without denaturation of the protein, provided it is stabilized by a small amount of a complexing agent such as perfluorooctanoic acid. These claims were later refuted by Ellenbogen and Maurer[11] who found, however, that if the heating was eliminated no physical-chemical or immunochemical evidence of denaturation could be found. Sodium acetyltryptophanate and so-

(1) L. C. Craig, "Third International Congress of Biochemistry," Brussels, 1955, p. 416.

(2) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

(3) T. P. King and L. C. Craig, Abstracts of 132nd Meeting of the American Chemical Society, New York, N. Y.

(4) E. Craenhals and J. Leonis, *Bull. soc. chim. Belges*, **64**, 58 (1955).

(5) C. H. Li, "Advances in Protein Chemistry," Vol. XI, Academic Press, New York, N. Y., 1956, p. 101.

(6) P. von Tavel and R. Signer, "Advances in Protein Chemistry," Vol. XI, Academic Press, New York, N. Y., 1956, p. 237.

(7) P. von Tavel, *Helv. Chim. Acta*, **38**, 520 (1955).

(8) L. C. Craig, in "Internal Secretions of the Pancreas," Ciba Foundation Symposium, Vol. IV, p. 106.

(9) E. L. Duggen and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1947).

(10) H. B. Klevens, *Nature*, **176**, 878 (1955).

(11) E. Ellenbogen and P. H. Maurer, *Science*, **124**, 266 (1956).

dium caprylate are routinely added to human serum albumin (HSA) to stabilize it. The sodium caprylate permits sterilization of this protein by heating at 60° for several hours.

A number of recent very careful studies[12,13] have shown that when either human or bovine serum albumin was precipitated at room temperature with excess trichloroacetic acid (TCA) the precipitate was soluble in alcohol. Upon removal of the alcohol by dialysis against water and recovery of the albumin no sign of transformation could be detected. It would therefore appear interesting to make a countercurrent distribution study of the complexes of serum albumin. This paper will report some of the experiments made thus far.

Experimental

All the samples of human serum albumin studied were obtained through the courtesy of Drs. H. Kunkel and R. Trautman of this Institute.

One was a Red Cross sample of a Cohn V fraction in 25% aqueous solution. The preparation also contained sodium acetyltryptophanate and sodium caprylate, each present in 0.02 *M* concentration to serve as stabilizers. Since this sample, when used directly, caused emulsions in most systems, possibly due to the caprylic acid, this component was removed by a short dialysis against distilled water at 4°. Upon exhaustive dialysis the protein became less soluble and again caused emulsions. Exhaustive dialysis against 0.02 *M* sodium acetyltryptophanate, however, gave a preparation entirely satisfactory for the distribution study.

A second preparation was a crude albumin sample obtained by fractionation of human serum in the ultracentrifuge. It contained no stabilizers.

A third preparation was the serum albumin fraction obtained from human serum by zone electrophoresis, but with the cut taken so as to include the α_1-globulin fraction.

Two crystalline preparations of bovine serum albumin which had been obtained from the Armour Company were used. One was a recent preparation while the other had been purchased several years earlier, but had been stored in the cold room.

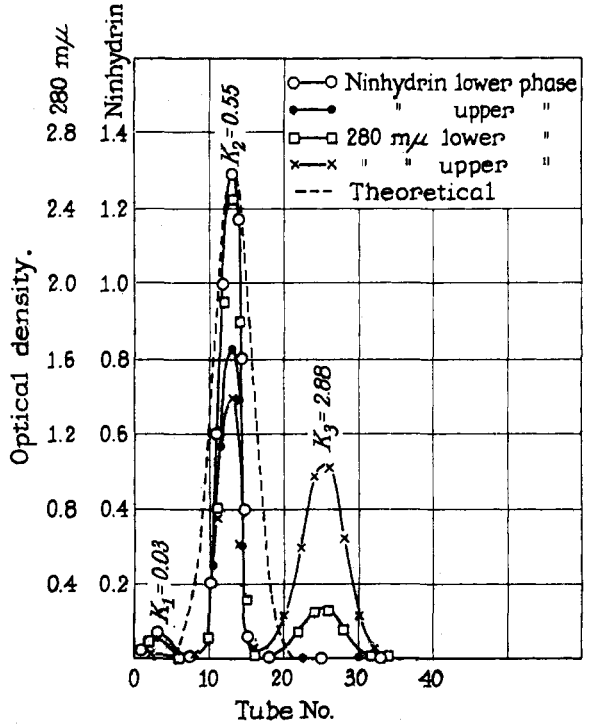

Fig. 1.—Distribution curves of undialyzed HSA Cohn V fraction in System 1 at 31 transfers.

Systems.—The following two systems were used for the distributions reported in this paper. (1) 2-Butanol,[14] 0.1% TCA in 0.01 *M* aqueous acetic acid and ethanol in the volume proportions of 25, 25, 1, respectively. The *p*H of the lower phase was 2.54. (2) 2-Butanol, 0.1% TCA in 0.02 *M* sodium acetate–acetic acid buffer (made from 0.2 *M* buffer at *p*H 4.14) and ethanol in the volume proportions of 15, 15, 1. The *p*H of the lower phase of this system was 3.37.

Distributions. Run 1.—A volume of solution containing 220 mg. of HSA-Cohn V was dissolved in 30 ml. of the lower phase of System 1. Thirty ml. of upper phase was added and the mixture was equilibrated. The initial protein concentration was 3.68 mg. per ml. system, giving rise to an initial *K* of about 0.4. The sample was put into tubes 1, 2 and 3 of a 100 tube hand-operated glass C.C.D. apparatus. The settling time was 12 minutes at first, but gradually increased to over 30 minutes due to emulsification. The pattern obtained after 31 transfers is given in Fig. 1. Three bands were present with travelling *K*'s: 0.03, 0.55 and 2.88, respectively.

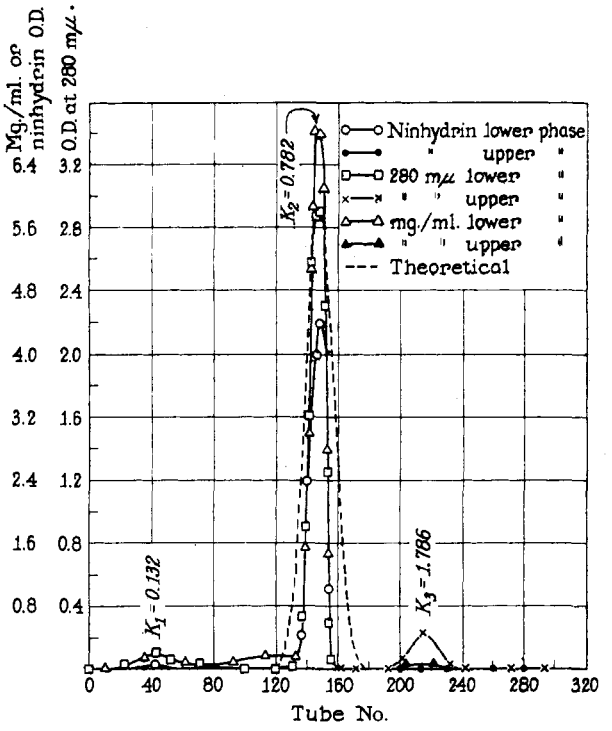

Fig. 2.—Distribution curves of partially dialyzed HSA Cohn V fraction in System 1 at 326 transfers.

Run 2.—Five ml. of HSA-Cohn V solution containing 1.25 g. of protein was dialyzed against running distilled water overnight, lyophilized and dissolved in 100 ml. lower phase of System 1. One hundred ml. of upper phase was added and the sample was put into tubes 1–10 of a 420 tube automatic distribution apparatus. The initial protein concentration was 6.3 mg. per ml. system. The settling time remained at 12 minutes throughout the run with no emulsification. The pattern obtained after 326 transfers is given in Fig. 2. Three bands were present with partition ratios 0.13, 0.78 and 1.8. The slowest moving band was not investigated further. The main band contained HSA. The third band was acetyltryptophan. A cut of tubes 136–153 was concentrated to approximately 100 ml. in a rotary evaporator[15] at 25°. This protein solution was then dialyzed in 23/32 Visking cellophane against 20 l. of slowly running distilled

(12) A. Korner and J. R. Debro, *Nature*, **178**, 1067 (1956).
(13) G. W. Schwert, THIS JOURNAL, **79**, 139 (1957).
(14) Obtained peroxide free from Eastman Organic Chemicals.
(15) L. C. Craig, J. D. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

water at 5°. Upon lyophilization 1.41 g. of HSA was obtained which contained 6.3% chlorine.

This product was redistributed in exactly the same manner as in the previous run except for the protein concentration which was initially 2.5 mg. per ml. system. After 344 transfers the pattern in Fig. 3 was obtained.

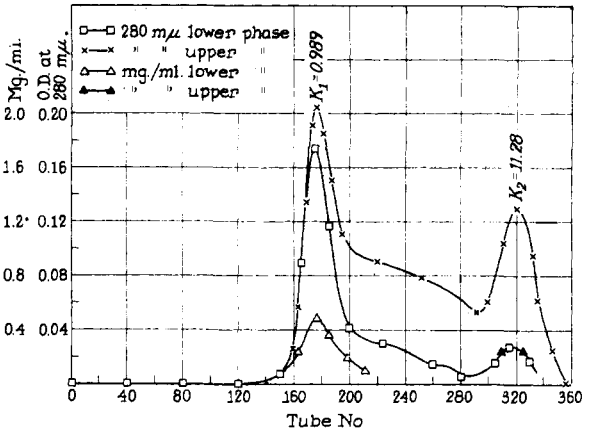

Fig. 3.—Redistribution of protein recovered from the run shown in Fig. 2; 344 transfers.

Run 3.—A volume of solution containing 1 g. of HSA from a fraction obtained by ultracentrifugation of serum was dialyzed, lyophilized and distributed as in run 2 at an initial protein concentration of 2.5 mg. per ml. system. The result after 401 transfers is given in Fig. 4. Essentially four bands and a shoulder of a fifth were present. Bands 1, 2 and 4, travelling with K's of 0.11, 0.40 and 12, respectively, were not investigated further. The main peak 3 moved with a K = 0.86 and contained the albumin. It was isolated as before except that dialysis was permitted to proceed three times as long. The residue then contained 2.75% chlorine.

Run 4.—A volume of the HSA-Cohn V solution containing 1 g. of protein was dissolved in 140 ml. lower phase of System 1. One hundred and forty ml. upper phase was added and the system was equilibrated. The whole mixture was then titrated with 1 N NaOH to pH 7 using phenol red as indicator. The partition ratio of HSA was then found to be very low, hence the upper phase was discarded. The lower phase was dialyzed against 20 l. of slowly running distilled water at 4° which contained 1.6 g. of Na-acetyl-tryptophanate. The protein solution was then lyophilized at 25°. The resulting product contained only traces of chlorine.

Two hundred and twenty mg. of this recovered HSA-Cohn V was distributed in System 1 at an initial concentration of 3.68 mg. per ml. system. Distribution to 31 transfers gave a pattern practically identical with that in Fig. 1. The albumin band was again isolated by neutralization and the use of acetyltryptophanate as a stabilizer.

One hundred and thirty-two mg. of this recovered albumin was redistributed at an initial concentration of 3.31 mg. per ml. System 1 and again essentially the pattern of Fig. 1 was obtained after 31 transfers. The HSA band was two tubes wider. The small amount of low K material was entirely absent. Upon isolation as described earlier the albumin had an optical rotation of $[\alpha]^{25}D$ −65° (c 0.372 in the lower phase of the system).

One ml. of lower phase from tubes 11, 12, 16 and 17 was made alkaline with sodium carbonate, evaporated to dryness and the residue analyzed for chlorine in a Parr bomb. The TCA content was calculated from the result; 1.24, 1.4, 0.71 and 0.57 mg. of TCA per ml., respectively, were found.

In order to determine the influence of albumin concentration on the distribution behavior an identical run was made using 22 mg. of HSA-Cohn V fraction (initially 0.367 mg. per ml. system). The pattern in Fig. 5 represents the results.

Run 5.—Twenty ml. of an HSA-solution obtained by zone electrophoresis and known to contain α_1-globulin was dialyzed against 25 l. of slowly running distilled water containing 1 g. of Na acetyltryptophanate and subsequently lyophilized. One hundred and sixty-two mg. of protein so obtained was added to 20 ml. lower phase of System 1 and 20 ml. of upper was added. After thorough mixing and centrifugation the insoluble part was discarded. The

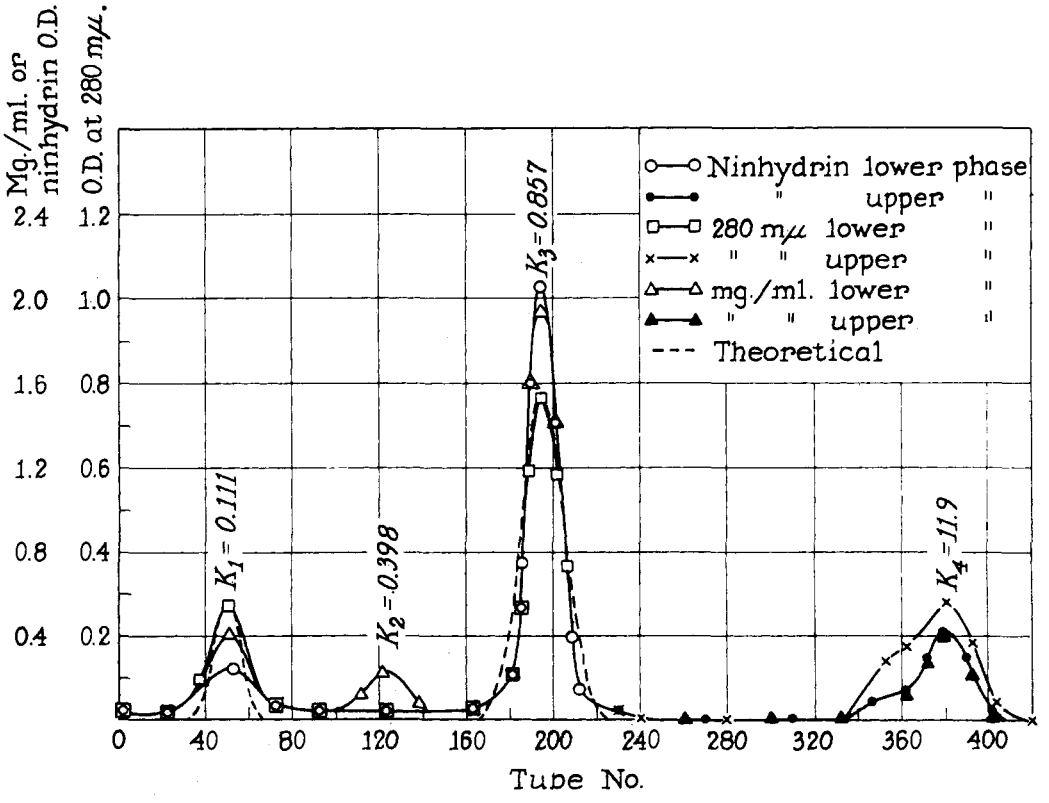

Fig. 4.—Distribution curves of an HSA fraction obtained by ultracentrifugation at 401 transfers.

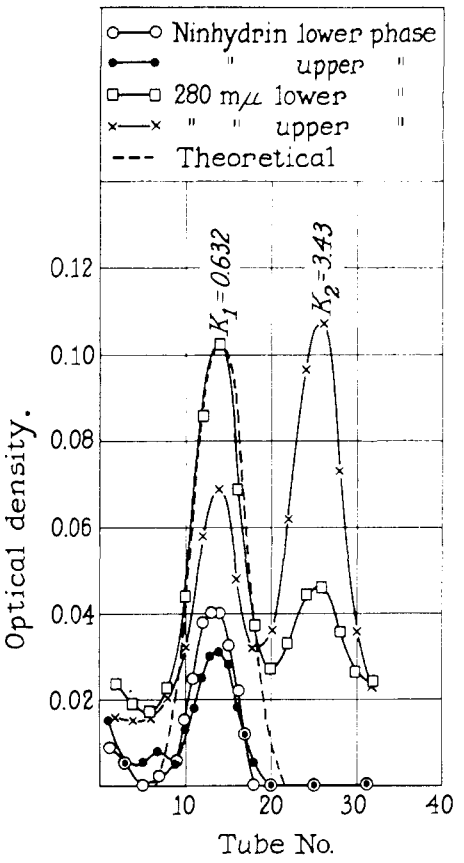

Fig. 5.—Distribution curves of HSA Cohn V fraction in System 1 with approximately one tenth the load of that in Fig. 1; 31 transfers.

settling time was then 30 minutes. The distribution pattern after 31 transfers is given in Fig. 6.

Run 6.—For comparison a similar run was carried out with a crystalline sample of Bovine Serum Albumin (BSA) obtained from the Armour Laboratories. Two hundred mg. of BSA was put into tubes 1, 2 and 3 and taken to 31 transfers. The settling time was 14 minutes. Analysis at this point gave the pattern in Fig. 7.

Run 7.—Two hundred and twenty mg. (0.88 ml.) of HSA-Cohn V solution was dissolved in 60 ml. of System 2. This gave a settling time of 10 minutes. The sample was put in tubes 1, 2 and 3. The distribution curve after 31 transfers is given in Fig. 8.

Discussion

In the earlier stages of the work many different systems involving 2-butanol, 1-butanol, *t*-amyl alcohol, ethanol and the ethylene glycol ethers as the organic layer were studied. Combinations of these with the different complexing agents at different *p*H values gave an almost limitless number of possible systems. Some way of screening out the most promising ones appeared desirable but an entirely satisfactory approach to this problem was not forthcoming.

The factors which were kept in mind during the screening phase of the study included solubility, time of separation of the phases, *p*H, and a usable range of partition ratio as determined by optical density at 280 mμ. If these appeared acceptable, a three stage distribution was made to see if the K was strongly concentration dependent. The K was redetermined after the protein had stood in the system for 24 hours at the temperature to be used for the distribution and from time to time during the course of a week. A shift in partition ratio was a good indication that a slow denaturation was taking place. Often this was confirmed by the separation of insoluble material at the interface. Since in every system tried the partition ratio was more or less concentration dependent, an arbitrary concentration level of about 1.0 mg. per ml. of lower phase was chosen for the initial exploration. Dissolution of the protein in the lower phase first was usually to be preferred.

Nearly 100 different systems were screened in this way, many of which showed considerable partitioning of the protein in the alcohol phase. All of these contained a complexing agent which rendered the protein more soluble in organic solvents. The following compounds were helpful in this respect: trichloroacetic acid, dichloroacetic acid, monochloroacetic acid, trifluoroacetic acid, perfluorobutyric acid, chloral hydrate, azo dyes and triphenylmethane dyes.

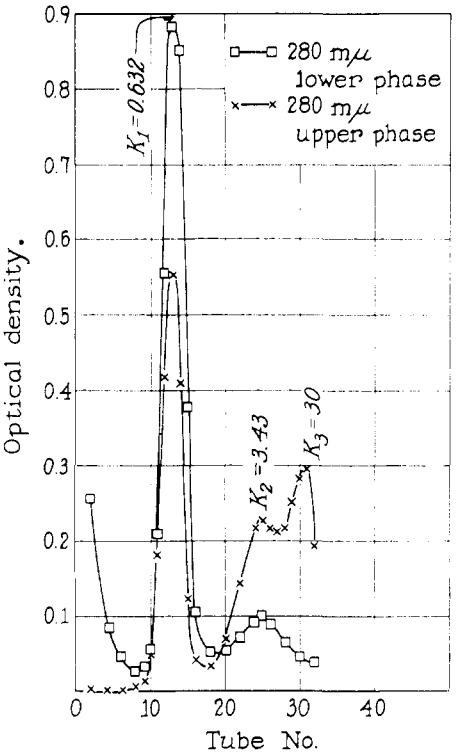

Fig. 6.—Distribution curves of an HSA fraction obtained by zone electrophoresis.

Human serum albumin is generally thought to be stable in the *p*H range of 4 to 9. At first systems falling within this range were chosen. Later, as discussed below, it was found that this restriction was not necessary.

From this screening it seemed that TCA offered the most promise as a complexing agent. TCA seemed to bind HSA only below *p*H 4 and if no other strong acid was present in the system. In the special case of acetic acid both acids competed for the complexing sites. In general, the K increased sharply with decreasing *p*H.

When TCA was used with 2-butanol at a concen-

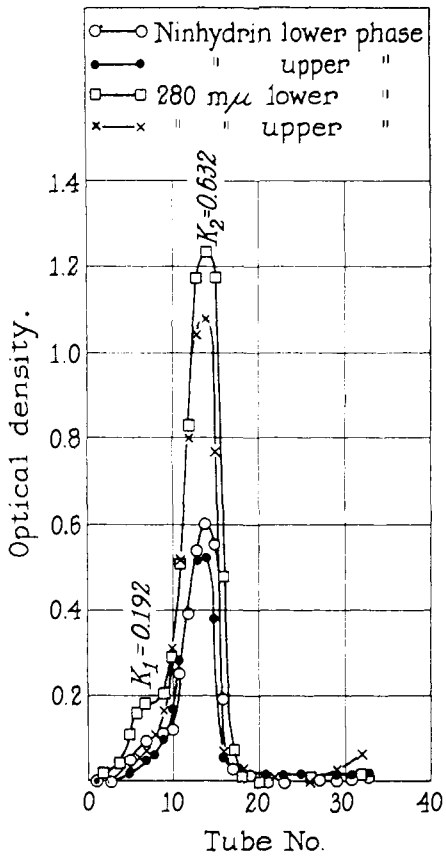

Fig. 7.—Distribution curves of a crystalline BSA preparation in System 1 at 31 transfers.

tration of 0.1% in the aqueous phase an acceptable value for the partition ratio was obtained but the phases separated too slowly. Addition of a little acetic acid improved the settling time and increased the K, but more acetic acid lowered the K value. Addition of a little ethanol further shortened the settling time. The system 2-butanol, 0.1% TCA in 0.01 M acetic acid and ethanol in the volume proportions of 25, 25, 1 was selected for a more careful study by countercurrent distribution.

The long settling time of approximately 30 minutes in run 1 probably was due to caprylic acid. Since the sample originally contained Na acetyltryptophanate and Na caprylate, these two compounds were present as the free acids in System 1. As shown later acetyltryptophan did not cause slow settling. Rapid interchange of the solute between the phases always appeared to occur. After 31 transfers the pattern shown in Fig. 1 was obtained.

Three bands were revealed by analysis by optical density at 280 mμ but the one on the right was not revealed by ninhydrin.[16] Therefore it was thought to arise from the acetyltryptophan, a conclusion confirmed by comparison of the ultraviolet spectrum and the K with those obtained for this substance. The central band was the protein of interest but at first it was thought that some mistake had been made because it appeared far too narrow. Calculation of a theoretical curve confirmed that it was indeed too narrow as the chart shows. However, it was quite symmetrical, with the curves determined by ninhydrin and optical density in agreement and with the curves for one phase superimposing the ones for the other in an acceptable way. The cause of the narrow band will be treated later. A small band of ninhydrin positive material occurred near the origin.

(16) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954).

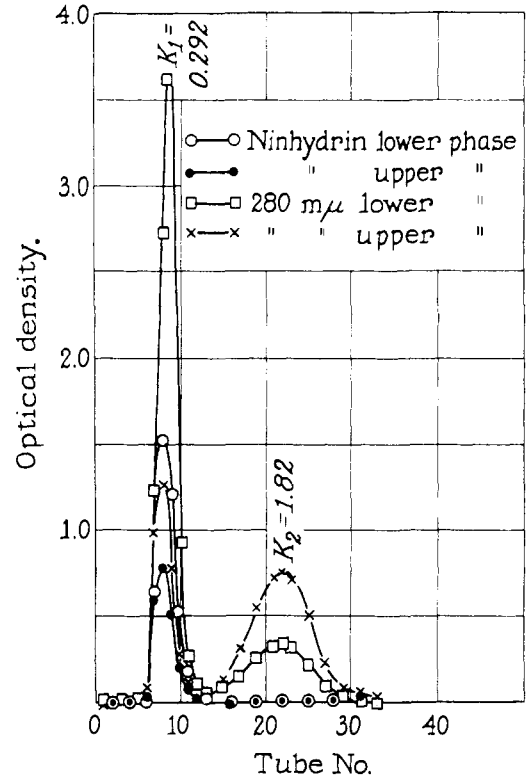

Fig. 8.—Distribution curves of HSA Cohn V fraction in System 2 at 31 transfers.

A second distribution was made with a larger sample in the same system but in a 200 tube automatic distribution apparatus. It was thought that the caprylic acid might be causing the slow separation of the phases. Accordingly, before use the sample was dialyzed against running water at 25° overnight and lyophilized. The settling time was found to be 12–13 minutes. Upon analysis after 326 transfers the pattern of Fig. 2 was obtained. This was a good confirmation of the first run, although the position of the main band was shifted due to the unique TCA and concentration effect as discussed later. In this run analysis was also made by weight. Again the main band was much more narrow than the calculated one.

At this stage of the study two points of interest appeared uppermost. One concerned whether or not the distribution had altered the protein and the other concerned the cause of the narrow band. An answer to the first was given primary attention in the thought that if a slow transformation during the run was occurring, it might produce the narrow band. Accordingly, an attempt to recover the protein as a lyophilized product free of TCA was made.

This presented certain difficulties. In one attempt the recovery was made as given in the ex-

perimental part under run 2. A product was obtained with good physical properties, but on analysis it still contained 6.3% chlorine, indicating that a considerable amount of TCA was still bound. Dr. Henry Kunkel found this material to have certain modified solubility properties as compared to native serum albumin in that it did not entirely dissolve in barbital buffer at *p*H 8.6. He found the part in solution to be indistinguishable from native HSA by starch electrophoresis and by dye binding.

However, a re-run shown in Fig. 3 clearly indicated that it was different from the starting material. If the transformation had occurred during the initial run the partition ratios at a given concentration across the main band would not have been constant, a criterion well established in countercurrent distribution.[6,17] This was not the case and the contrast of Fig. 2 and Fig. 3 clearly shows the difference. It was thought, therefore, that the transformation process had begun during the isolation from the system and attention was turned toward finding a better isolation procedure. A longer dialysis time as tried in Run 3 reduced the TCA content further but failed to remove it entirely. It was then found that the protein in the upper phase of the system could be transferred almost entirely to the lower phase by bringing up the *p*H to 7 with dilute sodium hydroxide using phenol red as indicator. The lower phase was then dialyzed at 4° against a running stream of a 0.02 *M* solution of the sodium salt of acetyl-*d,l*-tryptophan overnight.

Lyophilization now gave a product which contained no chlorine on analysis. Countercurrent distribution of material recovered in this way gave a result entirely comparable with that of Fig. 1. This method of isolation was used thereafter. Recovery of the albumin from the run gave a product with a specific rotation of −65°. Jirgensons[18] reported a rotation of −70° at *p*H 3 and −65° at *p*H 4.5 for native HSA.

The electrophoretic mobility of material recovered from the protein band of another run by dialysis against 0.02 *M* sodium acetyltryptophanate was compared with the starting material by zone electrophoresis with starch[19] as the supporting medium in two half cylindrical glass troughs. The electrophoresis was carried out for 26.5 hours at +4° in a barbital buffer, *p*H 8.54 and Γ/2 0.1, with a potential gradient of 4.3 v./cm. The sample of recovered protein used weighed 12 mg. but somewhat more than this was in the aliquot of the Cohn V fraction taken. At the end of the run the starch was cut into even sections and each eluted with a constant volume of water. Each point on the curve shown in Fig. 9 comes from the analysis by the modified Folin–Ciocalteu method[20] of the eluates of the consecutive fractions.

A preparation of HSA obtained by fractionation of human serum in the ultracentrifuge was studied in System 1. The result is shown in Fig. 4. The recovered main fraction again was indistinguishable from the original material as tested by zone electrophoresis at *p*H 8.5, immunologically and by dye binding. Bands of at least four other non-dialyzable components, presumably proteins, were widely separated from the albumin.

One of the most striking characteristics of the distribution of serum albumin in TCA systems concerned the width of the band. It was usually much more narrow than the calculated curve. Curves somewhat skewed and too narrow have often been observed before with larger molecules which tend to deviate from ideality but the exact cause has not heretofore been determined. It has been postulated[21] to arise from a particular type of partition isotherm, one which deviated from linearity in one direction at lower concentrations only to change its direction of deviation as the concentration is increased.

In the systems used here serum albumin was found not to give a constant partition ratio at different concentrations of protein. *K* was usually lower at high concentration, higher at low concentration. In order to see if this was directly connected with the width of the band, a run was made with a load about one tenth that used in Fig. 1. This gave a pattern with the main band approximating the theoretical width much more closely as shown in Fig. 5. The narrow band is thus a concentration effect.

Further investigation of the effect of *p*H and TCA concentration on the partition ratio showed that the ability of TCA to partition the protein in the alcohol phase was in general sharply reduced if the *p*H of the lower phase was raised above 3. This would seem to suggest that the reduction in polarity of the protein is connected with the ability of TCA to complex or hydrogen bond with the un-ionized carboxyl groups. Moreover, the observed partition is amazingly sensitive to the concentration of the TCA, an effect noted in the insulin work[2] and also in the A.C.T.H. separations.[22]

Since serum albumin is known to bind a variety of solutes to an outstanding degree its presence in solution in a countercurrent process could alter both the *p*H in certain tubes and the concentration of TCA with unpredictable results. By way of inquiry into this possibility, the lower phases of tubes 11, 12, 16 and 17 of a run which was essentially a duplicate of that shown in Fig. 1 were analyzed for chlorine. The TCA equivalents calculated from these results showed 1.24, 1.4, 0.71 and 0.57 mg., respectively, of TCA to be present per ml. When an analysis of this type was made on the system used for the run, 0.85 mg./ml. of TCA was found in the upper and 0.89 mg./ml. was found in the lower phase. Obviously the tubes at the front of the band had been depleted of their TCA content but those in the trailing part had been enriched. This would create a state of affairs where the partition ratio at the front would be low but that at the trailing edge would be higher, thus slowing down the migration of the front and speeding up the migration of the trailing edge.

(17) W. Hausmann and L. C. Craig, *J. Biol. Chem.*, **198**, 405 (1952).

(18) B. Jirgensons, THIS JOURNAL, **77**, 2289 (1955).

(19) H. G. Kunkel, "Zone Electrophoresis" in Methods of Biochemical Analysis, Vol. I, Interscience Publishers, New York, N. Y., 1953, p. 141.

(20) O. H. Lowry, N. J. Rosebrough, L. A. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

(21) L. C. Craig, *Anal. Chem.*, **23**, 1263 (1951).

(22) R. G. Shepherd, *et al.*, THIS JOURNAL, **78**, 5051 (1956).

Such a self sharpening effect has not clearly been shown before in countercurrent distribution but can be very helpful. Without this effect, for instance, the HSA could not have been so well separated from the acetyltryptophan band in 31 transfers (Fig. 1). It bears a certain analogy to gradient elution in chromatography but clearly is caused only by a favorable deviation from ideality due in part to overloading.

Perhaps in chromatography a self sharpening effect enters more frequently than is realized. In C.C.D. the number of transfers or "plates" applied is always known, but if this were not the case and the numbers of plates had been calculated on the basis of the effective partition ratio and width of the band in Fig. 1 a number approximating fifteen times that actually used would have been obtained.

Irrespective of the value of this comparison, it has now been shown that bands far too narrow in C.C.D. can have a real and logical basis and may be due to a favorable deviation from ideality. One should thus not necessarily avoid overloading in separations dealing with naturally occuring mixtures.

A point of considerable interest concerns the stabilizing effect of the TCA in solution. It was first found that System 1 gave a consistent result in that it gave reproducible partition ratios and a discrete reproducible band in a distribution. But when an attempt was made to remove all the TCA by prolonged dialysis, it was found that this was impossible and that the lyophilized protein had changed with regard to its partition behavior. This was clearly shown by the pattern given in Fig. 3.

When, however, the TCA was removed by *p*H adjustment to 7 and dialysis against a very dilute solution of sodium acetyltryptophanate, a residue was obtained by lyophilization which now could be redistributed with no sign of change as regards partition behavior, starch electrophoresis, optical rotation, dye binding or immunochemical tests.

This would indicate that excess TCA can offer some stabilizing effect on HSA in solution. The small amount remaining after prolonged dialysis does not prevent denaturation on lyophilization. If, however, prior to lyophilization, the TCA is replaced by Na-acetyl-tryptophanate, the latter stabilizes the protein and permits satisfactory lyophilization.

Evidence that the protein remained in its native state during the distribution was obtained by measuring the optical activity of each phase in a tube in the center of the protein band at the end of a distribution, *e.g.*, tube 12 of Fig. 1. Thus $[\alpha]^{25}D$ $-66°$ (*c* 0.182) in the upper phase and $[\alpha]^{25}D$ $-67°$ (*c* 0.29) in the lower phase. Jirgensons found the specific rotation of normal human serum albumin to approximate $-65°$ at *p*H 4.5 and to be somewhat higher at a lower *p*H.

A sample of crystalline bovine serum albumin gave a rotation somewhat lower, -50 to $-54°$ in the system. Even in a 0.5% solution of TCA with the *p*H at 2.11 it still gave a rotation of $-53°$ at 1% concentration. In contrast to this a 1% solution

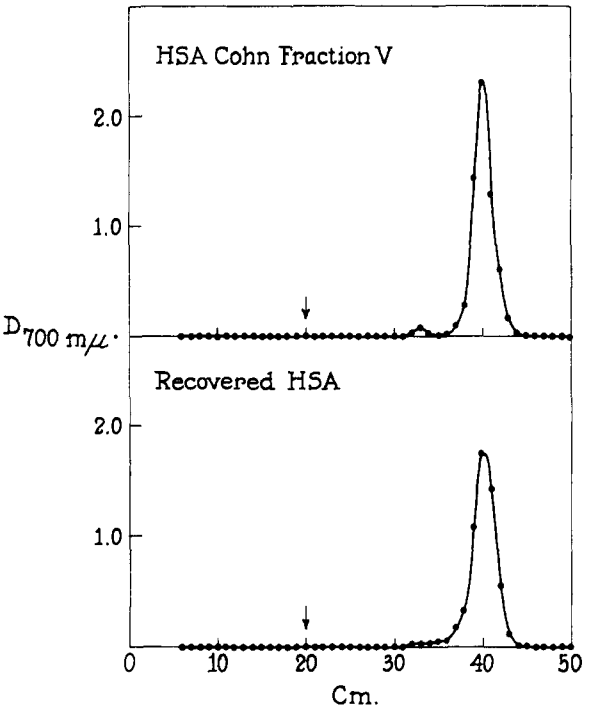

Fig. 9.—Starch electrophoresis patterns of the albumin fraction used and of that recovered after distribution.

of bovine serum albumin in 0.03 *N* HCl at *p*H 2.19 gave a rotation of $-69°$ thus indicating a conformational change. These results also supported the thought that an excess of TCA provided a protective action against the denaturation of serum albumin at low *p*H. In this connection Klotz and Heiney[23] found that a detergent, sodium dodecyl sulfate, also protected serum albumin against the increase of levorotation which would otherwise have occurred at low *p*H.

The stabilizing effect of the halogen acids on serum albumin has been reported before.[10,12,13] Mackay and Martin[24] have also recently made a study of stabilizers and reconfirmed the stabilizing effect of acetyltryptophan.

Distribution of BSA gave a pattern, Fig. 7, similar to that of HSA after 31 transfers. However, it contained an impurity with $K = 0.19$ and a distinct asymmetry of the main band might indicate beginning resolution into several components.

In spite of the fact that in these systems a discrete narrow band is obtained, such a distribution does not constitute evidence that serum albumin is a single substance. Evidence of resolution into two or more components by electrophoresis below *p*H 4 has been reported[25-32] but these forms may

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in part represent interconvertible species. Tiselius, Hjerten and Levin have obtained three components by chromatography.[33] Hughes[34] found that serum albumin does not contain a full equivalent of SH on the basis of a molecular weight of 68,000, but that a fraction can be separated which does show a full equivalent of SH. At a *p*H under 4 boundary spreading is observed in the ultracentrifuge[29] but this may be due to reversible denaturation.

In addition to this incomplete bibliography on the inhomogeneity, work now in progress in this Laboratory with an entirely different system recently discovered and soon to be reported, has shown that both human and bovine serum albumin can be resolved by C.C.D. into several components. Even artificial mixtures of the two can be separated with the separate components of each still retaining their respective separate partition positions. Thus while the TCA systems are not suited to the resolution of the very closely related components of serum albumin they do clearly separate several other protein components as Figs. 4, 6 and 7 show.

The run which gave Fig. 6 was made on a fraction obtained electrophoretically from serum. It had the green color of bilirubin and was known to have a certain amount of α_1-globulin in it. At the start of the run a moderate amount of insoluble material separated at the interface and was discarded. Three bands aside from the albumin one are obvious. One of them is the α_1-globulin. Recovery of the albumin band gave a white solid without the green color of the bilirubin but in about 72% of the amount expected, had the same original weight of Cohn V fraction been taken.

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Immunochemical Study of a Bacterial DNA[1]

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Evidence has been obtained that DNA contributes to the antigenic specificity of a 0.5% phenol-extracted "deproteinized" bacterial DNA preparation. It is based primarily upon the appearance of a Feulgen-positive zone among several bands of precipitation in gel-diffusion tests, the disappearance of antigenic components from the DNA preparation following DNAase treatment as shown by quantitative precipitin analysis, and the occurrence of cross-reactions of DNA from calf thymus and salmon sperm with antiserum produced against bacterial DNA. Preliminary evidence suggests that the extent of these cross-reactions increases with limited depolymerization of the antigenic material by DNAase.

Introduction

A number of earlier studies[3-6] have suggested a serological reactivity for deoxyribonucleic acid (DNA), but the supporting evidence has been indirect and disputable. For example, the precipitations observed by Lackman, *et al.*,[3] could be ascribed to charge effects rather than antigen–antibody reactions, since they proved sensitive to *p*H and ionic strength in the range in which the precipitin reaction is stable. Similarly, the studies by Blix, *et al.*,[4] failed to reveal whether their complement-fixing antibodies were specific for DNA or some other unrelated antigen present. Also, the indirect method employed is questionable when either the antigen or antibody is anticomplementary, as was found to be the case. Medawar's[5] observation regarding effects of DNAase upon a factor controlling tissue transplantability, though strongly suggestive of DNA antigenicity, has not lent itself to direct quantitative measurements. In view of the potential significance of the availability of antibodies specifically directed against DNA or the DNA moiety of a nucleoprotein, and the availability of a different procedure for the isolation from bacterial cells[7,8] of a highly polymerized DNA with transforming activity, the problem has been reinvestigated. The results of these studies, reported here, strongly indicate the existence of an antigenic specificity associated with DNA and the availability of procedures which should lend themselves to further quantitative analyses of this phenomenon.

Experimental

Preparation of Immunizing Antigens.—The material was obtained from cells of the bacterium *Brucella abortus*, strain 19, by extraction with 0.5% phenol in citrate-saline at 37° for 48 hr. and subsequent deproteinization by repeated treatments (2–5 times) with chloroform and amyl alcohol.[8,9] The resulting material appeared to consist principally of DNA on the basis of its absorption at 260 mμ in saline, its viscosity, its susceptibility to DNAase action, a positive Dische–Stumpf reaction and its purine and pyrimidine content as shown by chromatography following hydrolysis. However, on the basis of the biuret test and N/P ratio it was estimated to contain approximately 25% protein which could not be dissociated by repeated treatments with chloroform–amyl alcohol.[8]

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