FACTORS INVOLVED IN THE USE OF ORGANIC SOLVENTS AS PRECIPITATING AND DRYING AGENTS OF IMMUNE SERA

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In a preliminary report (1) we have given a method by which immune serum can be prepared in dry form by precipitation at room temperature with acetone followed by ether extraction. Since the work of Mellanby reported in 1907 (2), it appears to have been accepted that precipitation of serum proteins by ethyl alcohol or acetone at a temperature above 14°C. results in varying degrees of denaturation of the serum proteins. Mellanby found that when precipitation is carried out at temperatures below 14°C. the precipitate is completely resoluble. He designated this temperature the critical temperature. In addition to being a dividing point between denaturation and no alteration of solubility of the proteins, it was also the point of minimum precipitation, any concentration of alcohol exerting least precipitating effect at this temperature. Mellanby (3) later (1908) extended his work to diphtheria antitoxin and found antitoxic value was not affected by alcohol as long as no coagulation occurred. Felton (4) recently reported similar findings. Later work by Hardy and Gardiner (5) and Hartley (6) has shown that the destructive effect of alcohol and acetone upon antibodies is related to the denaturation of the serum proteins. From the work of Mellanby these later workers devised methods by which serum proteins could be precipitated in the cold and subsequently extracted with ether, dried, and a powder obtained. This material was completely soluble and possessed its original antibody activity excepting that precipitating action on specific antigen was diminished or lost (6). These workers have attributed lack of denaturation to the low temperature.

Mellanby did not report the effect of ethyl alcohol in concentrations above 75 per cent. The effect of temperature, hydrogen ion concentration, and of time in these higher concentrations has apparently not been reported. As we have previously noted (1) there exists a critical concentration of the organic solvents, ethyl alcohol, and acetone in the zone of 70 to 75 per cent concentration at which the coagulating effect is maximal. As the concentration is increased from this point there is progressively less coagulation until at concentrations exceeding about 87 per cent precipitation can be accomplished at room temperature and yet the proteins remain completely resoluble. The following report deals with our studies of this phenomenon and its relationship to known facts concerning the action of organic solvents upon serum proteins and antibodies.

Relation of Concentration of Organic Solvents to Precipitation of Serum Proteins and Resolubility of the Precipitate

In order to determine the rôle of concentration of the organic solvents studied, two general methods have been employed. In the first of these methods, the organic solvent in increasing quantities was added to a constant volume of a given dilution of serum. This gave increasing percentage concentration of the solvent but since the volume increased also the serum concentration became progressively less. The results of one such experiment are shown in Fig. 1. Working at room temperature (22°C.) horse serum was diluted 1:5 with 0.85 per cent NaCl solution and 0.5 cc. added to each of eleven tubes. Absolute ethyl alcohol was then added quickly followed by immediate shaking. The precipitate was approximated after 5 minutes; all tubes were then centrifuged, and the supernatant decanted. The solubility of the precipitates was approximated by adding 2 cc. of saline to each tube followed by immediate shaking.

It will be noted that precipitation was complete in concentrations of 60 per cent[1] or above and that precipitates formed between 60 to 70 per cent concentration was least soluble while precipitates formed in the presence of greater than 87 per cent alcohol were completely resoluble. The agglutinin loss, when agglutinating serum was employed, approximately paralleled the loss in solubility.

[1] All reference to concentration used in this report refers to final concentration of the organic solvent in the mixture.

Sufficient alcohol was added to each of the decanted supernatants to bring the total volume to 5.0 cc. The precipitates were centrifuged down and the solubility determined as before. It was noted that the proteins not precipitated by lower concentrations of alcohol are completely resoluble after precipitation in the presence of 90 per cent alcohol.

Certain precautions are necessary in order to thus demonstrate the rôle of concentration upon precipitation and resolubility. Even very brief exposure of the serum to the action of 60 to 70 per cent alcohol at room temperature results in considerable loss in solubility. Therefore when precipitation is accomplished in high concentrations of

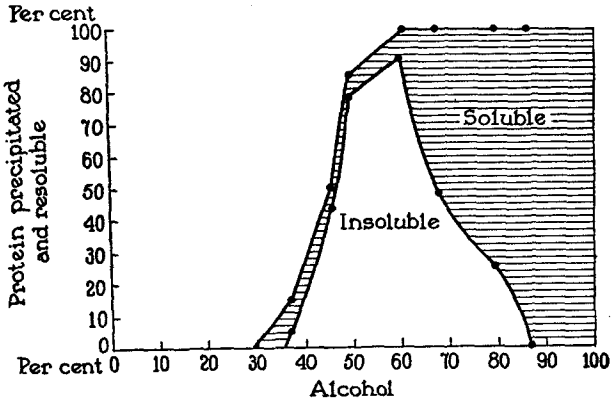

FIG. 1. Alcohol concentration and precipitation and resolubility of serum proteins. Serum diluted 1:5, plus increasing amounts of alcohol. Temperature 22°C. Duration of exposure 5 minutes.

alcohol the mixing must be instantaneous. Slowly raising the concentration through 60 to 70 per cent up to 90 per cent results in some loss in solubility. Furthermore, the solubility of precipitates formed in 75 per cent alcohol is not enhanced by subsequent extraction with 95 per cent absolute alcohol. Similarly if precipitation is accomplished in 90 per cent alcohol and the concentration lowered to 70 per cent by adding water there is a loss in solubility, the resulting precipitate being equally as insoluble as if precipitation had been accomplished in 70 per cent alcohol. Hence in determining solubility of the precipitate immediate shaking is necessary. If saline is carefully added to the precipitate of a tube containing 90 per cent alcohol,

and allowed to stand for 30 minutes the precipitate does not dissolve and upon subsequent shaking the solution is incomplete. If shaken immediately after the addition of the saline the precipitate completely dissolves.

The second general method that has been used for determining the rôle of the concentration of the organic solvent was to employ constant volumes of solvent-water mixture to give the desired concentration of solvent after the addition of serum. By this method all factors could be kept constant and the effect of varying concentration of the organic solvent more accurately determined. For example 0.25 cc. of serum was added to 4.75 cc. of alcohol-water mixtures of such proportions that the final alcohol concentration was 10, 20, 30, etc., per cent. The results by this method were essentially the same as in the experiments discussed above. As will be shown below some variation in the degree of precipitation by any given alcohol or acetone concentration is introduced by varying the concentration of the serum. All subsequent results are on determinations made employing constant volumes and constant serum concentration for any one set-up.

By either method ethyl, methyl, and propyl alcohols gave practically the same results. Acetone in general was found to be a slightly better precipitating agent and at the same time the precipitates formed were slightly more soluble. The critical concentration, or point of greatest loss of solubility was in the same range with all four solvents.

Rôle of Temperature and Duration of Exposure upon Precipitation and Resolubility of the Precipitate

Mellanby (2) has shown that up to 70 per cent concentration of ethyl alcohol the precipitating effect and coagulating effect of ethyl alcohol increases rapidly during the first few minutes of exposure and more slowly as the duration is prolonged. At all temperatures he reports having investigated, complete precipitation occurred immediately in 70 per cent alcohol.

The influence of duration of exposure to 95 per cent ethyl alcohol at various temperatures upon the resolubility of the precipitate is shown in Table I. These results were obtained by adding 0.25 cc. of beef serum to 4.75 cc. of absolute ethyl alcohol, both components being at the temperatures indicated before mixing.

The duration of exposure was the time elapsing after mixing before centrifuging so that the actual time elapsing between mixing and dilution with water to determine solubility was in each case about 4 minutes more than the figures would indicate. All determinations were centrifuged at room temperature, and solubility determined by adding to each tube 2.5 cc. of distilled water followed by immediate shaking.

The results indicate that there is no loss in solubility following precipitation by and exposure to 95 per cent alcohol for 24 hours at

TABLE I

Influence of Duration of Exposures to 95 Per Cent Alcohol at Various Temperatures upon Resolubility of the Precipitated Serum Protein

Duration of exposure	Temperature			
	5°C.	25°C.	35°C.	50°C.
1 min.	C.s.	C.s.	C.s.	P.s. Ca. 75 per cent
10 min.	C.s.	C.s.	C.s.	P.s.
60 min.	C.s.	C.s.	Slight precipitate left	P.s.
4 hrs.	C.s.	C.s.	More precipitate left	P.s.
24 hrs.	C.s.	Very slight flocculent precipitate undissolved	More precipitate left	P.s. Ca. 25 per cent

C.s. = completely soluble
P.s. = partially soluble

5°C. There is beginning loss in solubility in 24 hours at 25°C., in 60 minutes at 35°C., and immediately at 50°C.

The effect of temperature on precipitation and resolubility at various alcohol concentrations is shown graphically in Fig. 2. Again 0.25 cc. of beef serum was added to 4.75 cc. of alcohol-water mixture in such proportions that the final alcohol concentration was 10, 20, 30, etc., per cent up to 95 per cent. All components were brought to the desired temperature before mixing. After 30 minutes the degree of precipitation was approximated, the tubes centrifuged, and the solubility of the precipitates determined in each case in 2.5 cc. of distilled water. The 5°C. determinations were carried out in their entirety in

the cold room at 5°C. The shaded area indicates the approximate amount of the precipitated material that was resoluble.

On the first graph (upper left) is given the comparative precipitations at 5°, 22°, 37°, and 50°C. together with the resolubility of the 50°C. precipitate. It will be seen that precipitation occurs in definitely lower concentrations of alcohol at 5°C. the precipitating effectiveness decreasing as the temperature is raised. The difference

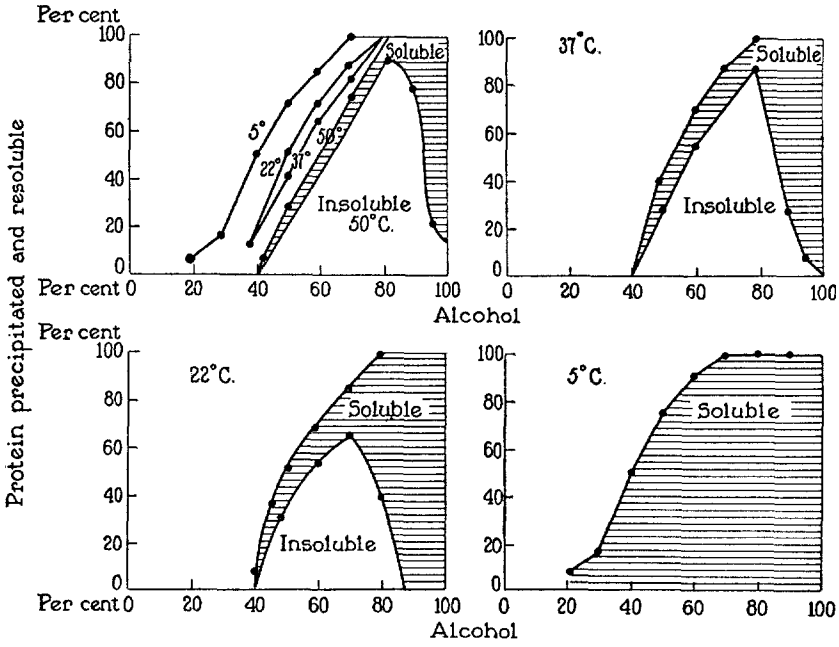

FIG. 2. Effect of temperature on precipitation and resolubility. Alcohol and beef serum.

between the three higher temperatures is not so pronounced but nevertheless is present. This is contrary to the findings of Mellanby (2) who found minimum precipitating effect occurred at 14°C. and that precipitating effect increased as the temperature was increased.

The graphs show clearly the effect of temperature upon resolubility. At 5°C. precipitation is greatest and the precipitate produced by all concentrations of alcohol were soluble. There was definite loss in solubility in concentrations up to 95 per cent at 37°C. and 50°C.

The difference in the 20°C. curves here shown and that in Fig. 1 is attributed to the difference in the concentration of the serum proteins in the two cases and the duration of exposures. As previously stated the precipitating effectiveness of these solvents increases with increasing concentration of the serum proteins and with increasing duration of exposure.

These same experiments have all been repeated using acetone in place of ethyl alcohol with essentially the same results. Slight differences were encountered due to the fact that acetone is a slightly better precipitating agent and also the acetone precipitates tend to be a little more soluble. The general phenomena, however, appear to be the same in the two cases.

Rôle of Hydrogen Ion Concentration upon Precipitation and Resolubility of the Precipitate

In the preceding experiments the hydrogen ion concentration of the serum has not been changed. Mellanby (2) observed that slight acidification of serum rendered it more readily precipitated by alcohol, while greater acidification decreased precipitation. It is generally stated that alcohol precipitation is inhibited by acids and alkalies.

In Fig. 3 is given a comparison of precipitation and resolubility of the precipitate by various alcohol concentrations at pH 6.0 and 7.5. Precipitation at pH 6.0 is as good in 20 per cent as in 40 per cent alcohol at pH 7.5. The precipitates show little variation in solubility, both being completely soluble where precipitation occurred in concentrations of alcohol exceeding 87 per cent.

The effect of pH upon precipitation is more clearly shown in Fig. 4 in which pH is plotted as abscissa against per cent of proteins present which are precipitated as ordinates. The results for both ethyl alcohol and acetone, each at various concentrations, are shown. A sample of serum was adjusted to each of the desired pH values and then an equal portion of 0.25 cc. added to a series of tubes each containing 4.75 cc. of organic solvents so diluted that the final concentrations would range from 10 to 95 per cent. The results were read on a basis of the approximate per cent of proteins precipitated after 30 minutes. The precipitates were then thrown down by centrifuging and the solubilities determined in 2.5 cc. of distilled water.

It will be seen from the curves that maximum precipitation occurs in the range of pH 5.0 to 6.0. Ethyl alcohol caused no precipitation in any concentration at pH 4.2 and only slight precipitation in high

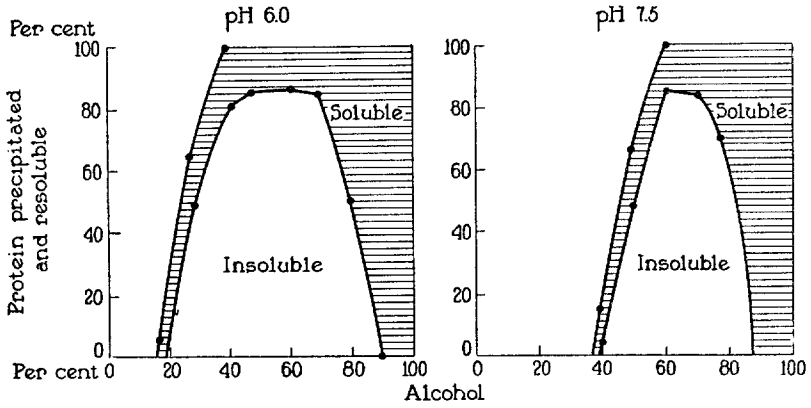

FIG. 3. Comparison of precipitation of serum protein at pH 6.0 and 7.5. Resolubility of precipitate.

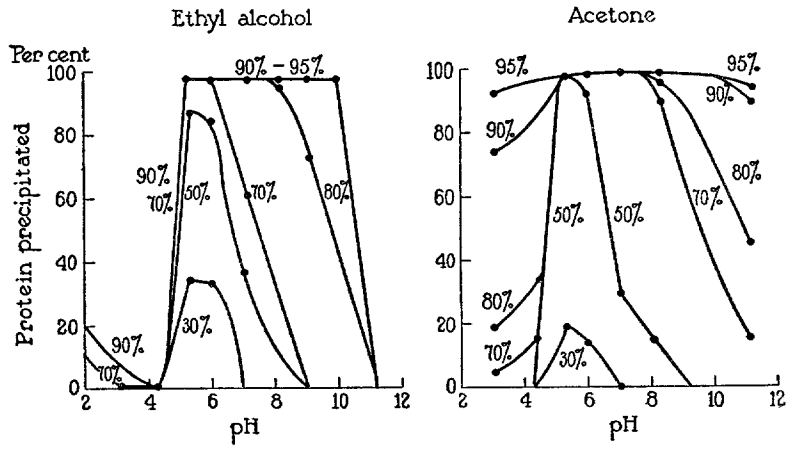

FIG. 4. Relation of pH and precipitation of serum proteins by ethyl alcohol and acetone.

alcohol concentrations in the more acid range. As the alkalinity increases beyond pH 6.0 the amount of precipitation decreases, there being no precipitate in concentrations of less than 70 per cent

beyond pH 9.0. As the concentration of alcohol is increased the pH range through which precipitation occurs becomes broadened.

The same features are found in acetone precipitation, except that acetone is a more active precipitating agent and especially in the acid range precipitation is more complete. In lower concentration of acetone (*e.g.*, 30 to 50 per cent) the zone of maximum precipitation is the same as for ethyl alcohol.

The solubilities of the precipitates are not shown in the figure. The zone of maximum loss of solubility (critical concentration) is evident regardless of the pH but in general the solubility of the precipitates produced by high concentrations of these solvents becomes progressively less with changes in hydrogen ion concentration either way from pH 6.0.

In the above experiments the serum was adjusted to a specified pH prior to adding the alcohol or acetone. Quite different results are obtained if the acid or alkali is added to the organic solvent and the unadjusted serum added. There is uniformly more precipitate produced under the latter conditions. Furthermore, the precipitate is less soluble. If unadjusted serum is added to the alcohol or acetone and then acid or alkali is added to the mixture there is partial resolution of the precipitate, the end result being almost identical with results obtained when the solvent is acidulated or alkalinized prior to the addition of the serum.

Effect of the Concentration of the Serum upon Precipitation of Serum Proteins by Organic Solvents

It was indicated above that the serum concentration very materially alters the alcohol precipitation curve. With increasing concentrations of serum a lower concentration of alcohol has been found necessary to yield a given percentage of the proteins precipitated.

This phenomenon has been studied in its relation to precipitation of serum proteins by acetone at 5°C. Felton (4) has suggested that some fractionation of serum proteins by precipitation with acetone in the cold is possible. The rôle of serum concentration in such fractionation has been investigated with the results given in Table II.

In each of these experiments the serum was added in the desired dilutions to a test-tube and acetone added to give the indicated final

concentrations. The results are given in terms of approximate percentage of the total proteins present that are precipitated assuming 8 plus to be complete precipitation. The plus signs therefore do not indicate the absolute amount of precipitate. 4 plus precipitation almost quantitatively separates the globulins and albumins, the globulins being precipitated. It is seen that this is accomplished in 33 per cent, 36 per cent, 40–45 per cent, and 50 per cent in serum undiluted, diluted 1:2, 1:5, and 1:10 respectively.

In these lower concentrations of the organic solvents, then, the concentration of the serum plays an important rôle in determining the proportion of the total proteins present that will be precipitated. Apparently the same phenomenon occurs in the case of ethyl alcohol.

TABLE II

Influence of Concentration of Serum on Precipitation by Acetone at 5°C.

(Complete precipitation of protein denoted by 8 plus)

Serum dilution	Acetone						
	25 per cent	30 per cent	33 per cent	36 per cent	40 per cent	45 per cent	50 per cent
Undiluted	+	2+	4+	6+	7+	−	
Dilution 1.2	+	+±	3+	4+	6+	−	
Dilution 1.5	±	+±	−	3+	4+	4+	6+
Dilution 1.10	?	±	+	−	2+	3+	4+

Effect of Extraction of Dried Immune Serum by Various Reagents

Rabbit *B. coli* immune serum in 0.25 cc. portions was precipitated in 4.75 cc. of absolute alcohol, extracted with alcohol followed by three washings with ether, and the resulting precipitate dried at 37°C. These samples of dried serum were used to determine the effect of various organic substances upon first, the subsequent solubility of the serum proteins in water; and second, the effect upon the agglutinin titer. The substance being tested was added directly to the tube containing the dried serum followed by sufficient shaking to thoroughly mix and allowed to stand in most cases for 1 hour. The serum constituents were then centrifuged down, the supernatant poured off, and the residual organic extractant then extracted from the protein mass by two washings with ether. The precipitate was finally dried at

37°C. to drive off the ether and the precipitate suspended in 2.5 cc. of saline. The agglutinin titer of the dissolved or partially dissolved material was then determined.

The following substances were employed with the results given:

1. Amyl alcohol—does not extract antibodies or render proteins insoluble.
2. Chloroform—definite decrease in agglutinin titer and some loss in solubility. No agglutinin activity demonstrable in residue of chloroform extract after evaporation to dryness.
3. Pyridine—no antibody decrease and no loss in solubility.
4. Ethylene dichloride—no antibody decrease and no loss in solubility.
5. Benzene—no antibody decrease and no loss in solubility.
6. Ethyl acetate—no antibody decrease and no loss in solubility.
7. Ethylene glycol—no antibody decrease and no loss in solubility.
8. Aniline—does not extract agglutinins; causes some decrease in solubility and agglutinating activity.
9. Glycerine—proteins completely soluble. No loss in antibody activity. Precipitated from glycerine solutions by alcohol in almost the same alcohol concentrations as from watery solutions.

Effect of Alcohol Precipitation and Ether Drying upon the Antibody Titers of Immune Sera

To each of twelve small test-tubes was added 0.2 cc. of rabbit *B. coli* immune serum followed in each case by 5 cc. of 95 per cent ethyl alcohol. The heavy flocculent precipitates were each washed with 5 cc. of 95 per cent alcohol, 5 cc. of alcohol-ether 1:1, and finally two times with ether. The final precipitate was dried for 1 hour at 37°C.

The tubes of dry powder were stoppered with cotton plugs. Tubes 1–4 inclusive were placed in the incubator (37°C.); Tubes 7 and 8 were left at room temperature; Tubes 9–12 inclusive were placed at 5°C. Tubes 5 and 6 were heated to 165°C. for 1 hour. 2 cc. of saline were then added to each of the latter two tubes. There was only slight solution and complete loss of agglutinating activity.

After 1 week incubation 2 cc. of saline were added to each of Tubes 1, 7, and 9. There was complete solution in each case. The agglutinin titers of each redissolved precipitate was compared with untreated serum which had been kept at 5°C. The agglutinin titers were the same in each case.

After 6 months 2 cc. of saline were added to each of Tubes 4, 8, 11. Solution was not complete in Tubes 4 and 8 (37°C. and room temperature respectively) there remaining a slight flocculent precipitate in each. The solution of Precipitate 11 was equally as active as the untreated serum kept at the same (5°C.) temperature. There was approximately a 15 per cent reduction in the agglutinating activity in 8 and 25 per cent in Tube 4.

After 11 months Tubes 3 and 12 were similarly tested. Precipitate 12 was completely soluble and had a titer equal to that of the untreated serum. The precipitate in Tube 3 was incompletely soluble and there was a loss of approximately 50 per cent in agglutinating activity.

Thus there was complete retention of solubility and agglutinating activity of the dried serum after 11 months when kept at 5°C. At higher temperatures there was a loss in solubility and agglutinating activity. It should be noted that the conditions to which these dried preparations were subjected were somewhat extreme. No effort was made to protect the materials from the air. Possibly somewhat less loss in solubility would occur if the material were placed in partially evacuated air-tight containers.

The method would seem to offer distinct possibilities as a method for preservation of immune sera for routine laboratory tests and possibly even therapeutic sera.

In preliminary tests upon hemolytic antisera and antitoxic sera there has resulted no loss in solubility or of antibody activity as a result of preparation of the dried material as listed above.

Preparation of Immune Sera in a Dry State

As stated above we have applied the findings reported for the preparation of antisera in a dry state. The method which has been found most applicable is as follows:

To 10 or more volumes of acetone add slowly with shaking 1 volume of serum. Collect the precipitate on a filter, wash once with acetone followed by three washings with anhydrous ether, the precipitated mass being stirred with a wooden spatula after each ether addition. Approximately 5 volumes of ether to each original volume of serum are required for each washing. The final white mass is spread out on the filter paper and placed in the 37°C. incubator for about 1 hour. The resulting dry mass is readily pulverized with the wooden spatula to an extremely light, white, fluffy powder. This powder (due to slow wetting) is slowly though completely soluble in distilled water or saline (0.85 per cent NaCl). If the acetone washing is replaced by absolute ethyl alcohol the final product is a little more quickly dissolved.

Absolute ethyl alcohol may be substituted for acetone as precipitating agent in the above procedure. Also 95 per cent alcohol may be used but 19 volumes of alcohol to one of serum are necessary so the final alcohol concentration does not fall below 90 per cent.

It is essential to use anhydrous ether for washing. Washing with either U.S.P.

or anesthesia ether produces a final product which is granular in nature and slightly brown in color. It is, however, completely soluble.

The results reported in Fig. 2 and Table I indicate the necessity of working at temperatures below 35°C. and of carrying out the filtration and washing with minimal loss of time. We have encountered no loss in solubility or in antibody activity when the dried material is thus prepared at room temperature (20–25°C.) and the duration of exposure does not exceed 1 hour.

DISCUSSION

The phenomenon of precipitation of serum proteins in high concentration of organic solvents at room temperature has much practical and theoretical significance. From a practical standpoint it provided a rapid and effective method of reducing immune sera to a dry powder. This simplifies preservation and indications are that it increases keeping qualities. In addition it opens up a new field of approach to the purification and concentration of therapeutic sera.

From a theoretical standpoint it is interesting that such a critical concentration exists. There is undoubtedly some relationship between this coagulation phenomenon and the greater germicidal effect of 70 per cent alcohol than 95 per cent alcohol.

The results reported above indicate that denaturation does not necessarily occur at the time of precipitation, since proteins precipitated in 95 per cent alcohol are rendered insoluble by diluting the alcohol with water to 70 per cent. Furthermore proteins precipitated at 5°C. in 70 per cent alcohol are resoluble, but if the temperature of the precipitate is allowed to rise to 25°C. without altering the alcohol concentration a large part of the precipitate becomes insoluble.

SUMMARY AND CONCLUSIONS

1. In concentrations of 70 to 75 per cent the organic solvents methyl, ethyl, and propyl alcohols, and acetone cause complete precipitation of serum proteins and produce maximum loss in solubility. We have referred to this concentration range as the critical concentration.

2. As the concentration of the solvents is increased from about 75 per cent precipitation continues complete but loss in solubility progres-

sively decreases until at all concentrations above about 87 per cent the precipitates formed at room temperature are completely soluble.

3. The degree of resolubility of the precipitates formed even in these high concentrations of the organic solvent decreases as the temperature is raised and as the duration of exposure is increased.

4. At 5°C. the precipitates formed in all concentrations of these organic solvents are completely resoluble. Also these solvents exert maximum precipitating effect at lower temperature.

5. Maximum precipitating effect by these organic solvents occurs at about pH 6.0 precipitation becoming progressively less as the pH value is altered either way from this point.

6. The more concentrated the serum, the greater the proportion of protein present that will be precipitated by any given concentrations of organic solvent.

7. A method for preparing dry immune sera has been given. Such dried sera have been extracted with a number of organic compounds without loss in solubility or antibody activity.

BIBLIOGRAPHY

1. Merrill, M. H., and Fleisher, M.S., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 799.
2. Mellanby, J., 1907, *J. Physiol.*, **36**, 288.
3. Mellanby, J., 1908, *Proc. Roy. Soc. London, Series B*, **80**, 399.
4. Felton, L. D., 1930, *J. Immunol.*, **21**, 357.
5. Hardy, W. B., and Gardiner, E., 1910, *Physiol. Soc.*, **40**, p. lxviii.
6. Hartley, P., 1925, *Brit. J. Exp. Path.*, **6**, 180.