CII. STUDIES ON LIPIN-PROTEIN COMPLEXES.

I. LECITHIN-CASEINOGEN COMPLEXES.

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It has long been known that lipins and proteins form loose "compounds" with each other, but up to the present no certainty seems to have been reached as to whether the constituents of these compounds are united merely by surface forces or whether their association is of a more intimate chemical nature. Galeotti and Giampalmo [1907] attempted to decide between these alternatives. They found on mixing lipins and proteins in various widely differing proportions that the composition of the resulting precipitates (as judged by the nitrogen content) although varying between only relatively narrow limits was not sufficiently nearly constant to enable them to deduce with certainty the formation of any definite chemical compound. In the same year, however, Mayer and Terroine [1907] studied the mutual interaction of lecithin and albumins and as a result stated definitely that the precipitates obtained on acidifying mixtures of these substances are colloidal complexes. Later Feinschmidt [1912] investigated the precipitation of mixtures of lecithin and various proportions of serum-proteins in solutions of graded acidity, and showed that the optimum hydrogen ion concentration at which the maximum precipitation of such mixtures occurs lies between the respective optimum values observed for the separate constituents alone. Meanwhile Hardy and Gardiner [1910] and Handovsky and Wagner [1911] pointed out the occurrence of lipin-protein complexes in blood-serum, or at least in the precipitates obtained therefrom by the addition of acids or salts, and following this Chick [1914] showed that the euglobulin precipitated by these reagents from serum is itself such a lipin-protein complex. Michaelis [1926] quotes a typical experiment of the kind described by Feinschmidt, and on repeating this we noticed that the precipitates produced in the relatively more acid solutions tended to float to the surface of the liquid, and so appeared to contain a larger proportion of the lighter lipin constituent than those produced in the less acid range, which were seen to settle more quickly to the bottom. As we had already been confronted with the question [Parsons and Parsons, 1927] as to the extent to which the lipins and proteins of serum are united into complexes, and as

to the conditions of precipitation of these complexes by salts, it seemed desirable to undertake a detailed investigation of the formation and properties of such complexes in general, and it is the object of the present paper to record the results of the first experiments made to this end.

In these experiments it was decided to use caseinogen as the protein constituent, on account of the ease with which it can be obtained pure (and, in particular, fat-free), while Merck's lecithin from eggs was chosen as the lipin component. In order to render possible the completion of the determinations on any one series of mixtures within a practicable length of time it was necessary to employ colorimetric methods of analysis. In order to obtain such a method for the estimation of the amount of lecithin present in a precipitated complex we stained this substance before use with a lipin-soluble but water-insoluble dye. When a precipitate was to be analysed it was first treated with a suitable volume of absolute alcohol in which the lecithin and the dye were at once dissolved. A measure of the amount of lecithin in this solution was then obtained by comparing the depth of its colour with that of an alcoholic solution of a known amount of the original stained lecithin preparation. Sudan III proved to be a suitable dye for this purpose. In order to obtain a uniformly stained lecithin preparation the lipin and a suitable small quantity of the dye were dissolved together in a little ether which was then allowed to evaporate. The resulting stained lecithin was then employed for the preparation of the emulsions used in the experiments described below. Of course it is admitted that the presence of the dye might to a certain extent influence the colloidal behaviour of the lecithin; but that such an influence is of appreciable magnitude in these experiments is hardly to be expected, on account of the smallness of the quantity of dye used, and this expectation is further justified by our observation that the point of maximum precipitation of the stained lecithin in the absence of protein corresponds with the hydrogen ion concentration at which the unstained lecithin itself is most readily precipitated from its emulsions. We intend to investigate further the question of the possible small effect of the dye by comparing the quantitative behaviour of deeply stained with lightly stained preparations and by the use of various staining materials. But meanwhile the experimental results recorded below must be understood to refer to lecithin containing the small quantity of Sudan III requisite for its estimation by the method outlined above.

For the colorimetric estimation of the caseinogen in the precipitates the method devised by Wu [1922] for the estimation of serum-proteins was found to be applicable. In this a phosphomolybdotungstate reagent is used which gives a blue colour with phenols generally, and so with the tyrosine of the protein molecule. The protein remaining after the complete alcohol extraction of the lipin from the original precipitate was dissolved in an appropriate volume of saturated sodium carbonate solution. After suitable dilution of this liquid the phenol reagent was then added, and when the resulting colour had

acquired a suitable and practically constant intensity it was compared with that obtained in a standard caseinogen solution treated in the same way simultaneously with the unknown. As a practical detail it may be mentioned that for a quantity of caseinogen of the order of 0.002 g. it was convenient to use 1 cc. of saturated sodium carbonate solution, to dilute then with 5 cc. of distilled water and to add 0.1 cc. of Wu's reagent.

In the first place it was decided to obtain information as to the variation of composition of the lecithin-caseinogen complexes precipitated from mixed colloidal solutions of these substances at hydrogen ion concentrations intermediate between their respective isoelectric points. The starting materials were a 0.4 % solution of caseinogen made up in 0.1 *N* sodium acetate according to the directions given by Michaelis [1926] and a 1 % emulsion in distilled water of Merck's lecithin stained with Sudan III. The necessary range of hydrogen ion concentration was obtained by the use of lactic acid-sodium lactate buffer solutions also made up according to Michaelis, and contained in 30 cc. portions in centrifuge tubes. To each of one series of tubes were added 3 cc. of a mixture of 1 volume of the lecithin emulsion with 2 volumes of the caseinogen solution. For comparison, each tube of a second similar series received 3 cc. of a mixture of 1 volume of lecithin emulsion with 2 volumes of 0.1 *N* sodium acetate solution while a third series was made up with 2 cc. of caseinogen solution + 1 cc. of distilled water in each tube. Thus the total volume of fluid and the concentration of sodium acetate were identical for all the tubes in the three series.

After standing for a period of 48 hours the precipitates of lecithin-caseinogen complex in tubes p_H 3.6 to 1.4 were aggregated by light centrifuging and the supernatant liquids were cautiously sucked off. Each precipitate was then extracted with two successive portions of 5 cc. of absolute alcohol, the alcoholic extracts were carefully decanted from the caseinogen residues and the depths of colour of the combined extracts were compared in tubes of equal diameters with those of a series of standards prepared by suitable dilution of an alcoholic solution containing in 10 cc. the same amount of stained lecithin as had been added to each of the buffer solutions. The caseinogen residues were dissolved each in 2 cc. of saturated sodium carbonate solution + 10 cc. of distilled water, 0.2 cc. of Wu's phenol reagent was added to each tube and after an hour's standing the resulting blue colours were compared in a similar way with those produced in a series of suitable dilutions of the original caseinogen solution simultaneously treated in the same fashion. The results of these determinations, together with those of the similar ones carried out on the pure lecithin and caseinogen precipitates obtained in those tubes that received these substances alone, are represented graphically in Figs. 1 and 2, of which the first shows the actual amounts of lecithin and caseinogen precipitated together at the various hydrogen ion concentrations and the second the increase in the percentage of lecithin in the precipitate with increasing acidity of the solution. It will be seen that the change in composition of the

precipitated complex is really very considerable over the range investigated, the percentage of lecithin increasing from 27 at p_H 4.2 to 100 at p_H 1.4. The increase, however, is not regular, for over the intermediate portion of the range—from p_H 3.6 to p_H 2.9—the percentage of lecithin remains practically constant at a value of about 55. By comparing the two figures it will be seen that this range of constant composition of precipitate corresponds with the range over which all the lecithin and caseinogen present are thrown out of solution. It is conceivable, therefore, that over this range the caseinogen was capable of combining with more lecithin than was actually present, and that

Fig. 1.

Fig. 2.

if we had started with a rather larger proportion of the lipin constituent this region of constant composition of precipitate, and with it the inflexion in the curve of Fig. 2, would have been absent.

It is probable that we are dealing here with a case of the mutual precipitation of two oppositely charged colloids, a phenomenon that has been familiar since the investigations of Biltz [1904]. In this case all the solutions which we used possessed hydrogen ion concentrations greater than that corresponding to the isoelectric point of the caseinogen so that this carried a positive charge, while the lecithin in the same solutions was on the alkaline side of its isoelectric point and so carried a negative charge. Presumably when the oppositely charged colloids are present together in solution the charge on the particles of one tends to be neutralised by the attraction of oppositely charged particles

of the other, and, when this process of neutralisation has resulted in the formation of complexes whose charge, although not necessarily zero, is too small to keep them in solution by mutual repulsion, precipitation occurs. In order to arrive at a quantitative explanation of the results of this experiment it is necessary to possess also some information as to the manner in which the charges on the two colloids which we have employed vary with the hydrogen ion concentration of the dispersion medium. In the case of the caseinogen it may be supposed that the charge on its particles is derived by a process of ionisation and that this ionisation is the more complete (or the activity of the caseinogen ions is the greater) the more remote the hydrogen ion concentration of the solution from the isoelectric point. But we should expect that once a certain hydrogen ion concentration has been reached the ionisation would be complete and that further increase of acidity would be without appreciable effect on the charge on the caseinogen particles. With regard to the lecithin there seems to be less evidence as to how the charge on its particles varies in relation to the reaction of the medium of dispersion. In this case the charge is possibly determined to a great extent by factors other than ionisation so that it might be expected to increase continuously with diminution of hydrogen ion concentration of the solution in a manner somewhat similar to that recently demonstrated by Michaelis and Damboviceanu [1924] in the case of mastic particles. If we assume that such relationships apply to the two colloids here under investigation it is possible to explain the proportions in which they mutually precipitate one another at various reactions. For in the less acid solutions the caseinogen particles will be but feebly positively charged while the lecithin will carry a relatively high negative charge. In this case, therefore, the adsorption of a comparatively small amount of the lipin will suffice to reduce the charge on the caseinogen particles to a point at which the complex will be precipitated. Thus the complex precipitated at these reactions will be rich in caseinogen and correspondingly poor in lecithin. On the other hand in the more acid solutions, the charge on the lecithin being less and that on the caseinogen being greater, relatively more lecithin will be required for neutralisation to the precipitating point and the complex obtained under these conditions will consequently contain a high proportion of lecithin. And this is indeed what is actually found to be the case. At first sight it would seem that on this theory the change of composition of the precipitated complex must be continuous over the range of hydrogen ion concentration between the isoelectric points of the two constituent colloids but since exact neutralisation of charge is probably not essential to mutual precipitation it is easy to account for the precipitates of practically constant composition obtained over a certain range of hydrogen ion concentration on the ground that not all of them were exactly isoelectric. In the more acid solutions the lecithin is too feebly charged to bring down more than a small quantity of the caseinogen; in fact in these solutions the lipin is precipitated when present alone and the protein appears

to exert no protective action on it. On the other hand in the least acid solutions the lecithin appears to be so strongly adsorbed by the caseinogen that a reversal of charge takes place and the resulting complex is therefore sufficiently negatively charged to remain in dispersion, for less of the protein is precipitated in the presence of the lipin than would be precipitated in its absence, a protective action being observed in this case.

We obtained further information regarding the mutual precipitation of these colloids by studying the composition of the complexes obtained when they were added in widely differing proportions to a buffer solution whose hydrogen ion concentration was so far intermediate between those corresponding to the isoelectric points of lecithin and caseinogen respectively that in it neither of these constituents was precipitated if present alone. For this purpose a lactic acid-sodium lactate mixture of p_H 2.7 (standardised electrometrically) was chosen. As before we used a 1 % emulsion of stained lecithin in water and a 0.4 % solution of caseinogen in 0.1 *N* sodium acetate solution. 1 cc. of a mixture of 1 volume of the lecithin emulsion + 2 volumes of the caseinogen solution was first prepared: this gave a mixture denoted by the symbol $\frac{L}{1}$ which has the same composition as that used for the experiment just described. A second mixture, $\frac{L}{2}$, was made by adding 1 cc. of a twofold dilution of the original lecithin emulsion to twice its volume of caseinogen solution, and by using further successive twofold dilutions of the emulsion other mixtures, $\frac{L}{4}$, $\frac{L}{8}$ and $\frac{L}{16}$, were prepared containing the same concentration of caseinogen throughout but diminishing quantities of lecithin equal respectively to $\frac{1}{4}$, $\frac{1}{8}$ and $\frac{1}{16}$ of the amount present in mixture $\frac{L}{1}$. A second series of mixtures, $\frac{C}{2}$, $\frac{C}{4}$, $\frac{C}{8}$, $\frac{C}{16}$, was also prepared using a constant amount of lecithin and twice its volume of successive twofold dilutions with 0.1 *N* sodium acetate solution of the original caseinogen dispersion, so that a total series was obtained in which the relative ratio of lecithin to caseinogen varied from $\frac{1}{16}$ to 16. Of each mixture in the middle of the series 1 cc. was added to 10 cc. of buffer solution, but of the more extreme mixtures, $\frac{L}{8}$, $\frac{L}{16}$, $\frac{C}{8}$ and $\frac{C}{16}$, 3 cc. were added to 30 cc. of buffer solution in order to obtain a larger amount of precipitate for analysis. It was noticed that the mixtures containing the greatest proportion of lecithin precipitated first. After a period of 48 hours the precipitates were aggregated by light centrifuging and analysed by the methods already described, care being taken to treat the standards identically with the unknowns and to obtain as accurate readings as possible by making the comparisons by means of a colorimeter.

From the results so obtained we calculated the amounts of caseinogen and lecithin precipitated in the various complexes per unit quantity (0.0033 g.) of total lecithin originally added to the mixture: these quantities are plotted in Fig. 3 in which positive ordinates represent g. of caseinogen, and negative

ordinates g. of lecithin, while the proportions in which these two constituents were added to the buffer solution are indicated by equally-spaced abscissae, *i.e.* by distances proportional to the logarithms of the actual numerical ratios concerned. The total amounts of the constituents either originally taken or appearing in the precipitated complexes are expressed by the correspondingly labelled curves.

Fig. 3 furnishes additional evidence in support of the view previously put forward that precipitation of the lecithin-caseinogen complex takes place only when the two components have combined in such proportions that mutual neutralisation of their opposite electric charges occurs to an extent sufficient to reduce the final charge on the particles of the total complex, if not neces-

Fig. 3.

sarily to zero, at least to a point below a certain critical value. The first feature to be noted is that at the highest caseinogen concentrations (on the left of the figure) no precipitation at all of either component occurs. In this region we may suppose that the complex formed in solution contains so high a proportion of the positively charged caseinogen that the mutual electrostatic repulsion even of those complex particles that are relatively most feebly charged is powerful enough to maintain the condition of permanent dispersion. We are evidently dealing here with the same phenomenon encountered by Mayer and Terroine [1907] when they found that their lecithin-albumin complexes were soluble in excess of the protein constituent. As the amount of caseinogen present is diminished we come to a point at which precipitation of complex just begins. Here we must suppose that while most

of the particles of the colloidal complex are still too rich in caseinogen, and therefore too highly positively charged to be precipitated, a certain small fraction of them, containing a relatively smaller proportion of the protein than the others, carry less than the critical intensity of charge and so are precipitated. As we proceed further we encounter the, at first sight surprising, circumstance that as the total amount of caseinogen present is still further reduced the amount of the protein in the precipitate steadily increases, which evidently means that the reduction of the percentage of caseinogen in the complex formed over this range is more than compensated by the extra amount of precipitation permitted by the concomitant reduction of charge. This increased precipitation of caseinogen continues until a certain maximum amount is reached in a mixture which is the first from which the whole of the lecithin present appears in the precipitate. With this particular ratio of the constituents we therefore obtain the maximum total amount of precipitate. Beyond this point further reduction in the total amount of caseinogen present leads to a reduction in the amount of this component precipitated, until, in the smallest caseinogen concentrations we at last arrive at a point at which there is not a sufficient proportion of the protein in all of the complex particles to reduce the negative charge due to the lecithin to below the negative critical precipitation value, so that only the particles relatively poor in lecithin are precipitated and a portion of this substance now again remains in solution. Finally we approach the condition in which practically no caseinogen is present, and no lecithin is precipitated.

Somewhere between the point at which all the added lecithin just begins to appear in the precipitate combined with the maximum amount of caseinogen, and that at which, in the mixtures poor in caseinogen, the whole of the lecithin just ceases to be precipitated, there must occur the isoelectric complex in which the charge, although not necessarily zero for all particles has a minimum average value. But from the results of this experiment alone it is not possible to discover which this ratio is. We are not justified in assuming that the isoelectric ratio is that in which the constituents are combined when the total weight of the resulting complex precipitate is a maximum, *i.e.* in the precipitate in which the whole of the lecithin appears associated with the maximum amount of caseinogen. For, as we show in Fig. 5, this precipitate does not contain a maximum *percentage* of caseinogen; it is but one of a series of precipitates whose caseinogen percentages diminish in a regular continuous fashion, so that all we can say is that the particles of this particular complex are, on the whole, less powerfully positively (or more powerfully negatively) charged than the particles of the complex obtained from mixtures richer in caseinogen, and conversely more strongly positively (or less strongly negatively) charged than those given by succeeding mixtures poorer in caseinogen.

An experiment was also carried out in which lecithin-caseinogen mixtures in the same ratios as those used in the experiment just described in detail were added to a less acid (lactic) buffer solution of p_H 3.7 in which caseinogen

itself is precipitated when present alone. The results of this experiment in general confirm the deductions already drawn from a study of the behaviour of these constituents in the more acid solution. Attention is directed to the differences in detail produced by the circumstance that the caseinogen in the medium of p_H 3.7 is less powerfully positively, and the lecithin more powerfully negatively, charged than in the solution previously used. In the first place it was noted that at p_H 3.7 the mixtures containing the greatest proportion of caseinogen precipitated first. Further, the precipitate obtained from any given mixture in the less acid medium contained a lower percentage of lecithin, and therefore a higher percentage of caseinogen than the complex given by the same mixture in the more acid solution, as would indeed be expected from the results of the analyses of the precipitates obtained from the same lecithin-caseinogen mixture over a wide range of hydrogen ion concentration. In the mixtures richer in lecithin less of this substance was carried down in the complex than was the case with the corresponding mixtures in the more acid medium; on the other hand from the mixtures poorer in lecithin a relatively greater proportion was precipitated, but the effect of this on the final

Fig. 4.

percentage of lecithin in the precipitate was more than counterbalanced by the much greater proportion of the added caseinogen that was also carried down. All these differences are readily explained when it is remembered that in the less acid solution a relatively much larger quantity of the now feebly charged caseinogen is required to reduce the high charge on the lecithin particles to the critical precipitation value.

Fig. 4 presents these relationships. Here the ordinates express the charge on the colloidal complex, positive or negative, as the case may be, the zero line indicating the completely discharged or isoelectric condition. The lines drawn parallel to and respectively above and below this represent the critical values of positive or negative charge which are just sufficient to keep the colloidal particles in permanent dispersion. In other words precipitation can occur only of particles carrying a charge whose magnitude is expressed by some point occurring between these two lines. The black portions of the rectangles are imagined as being proportional in area to the amounts of lecithin, and the white portions to the amounts of caseinogen, associated in the formation of complexes that may either be precipitated or remain in dispersion. It is not necessarily the case, nor indeed is it likely, that the

whole quantity of each substance added to a given mixture is used for the formation of such complexes; some of either constituent may remain free, but for the sake of simplicity of representation it is assumed that the whole of the lecithin and regularly progressing quantities of caseinogen are so combined. This assumption merely simplifies but does not invalidate our argument. The height of each rectangle is intended to express the (supposedly constant) range of variation of charge on the complex particles which it is regarded as containing. By following the figure from left to right it is easy to see how the complexes richest in caseinogen are devoid of particles whose charge is below the positive critical precipitation value (rectangle 1); how the first complex particles to be precipitated are still relatively rich in caseinogen (rectangle 2); and how it comes about that the greatest total amount of precipitate and of caseinogen carried down are associated with a complex whose caseinogen percentage is relatively smaller (rectangle 3). Finally by comparing rect-

Fig. 5. The arrows indicate the compositions of the mixtures that are precipitated unchanged at $p_H 2.7$ and $p_H 3.7$ respectively.

angles 3, 4 and 5 it will be seen how complete precipitation of all the lecithin present may occur in complexes of different compositions, although only one of these (represented by rectangle 4) is really isoelectric, having the range of charge of its particles equally distributed about the zero line. Rectangle 6 represents the partial precipitation of a complex carrying a high negative charge on account of the presence of a large percentage of lecithin. The corresponding relationships for the less acid medium of $p_H 3.7$ are represented by rectangles 1' to 6' in which a much larger percentage of the now feebly charged caseinogen is represented as being necessary for the reduction of the high charge on the lecithin to the critical precipitation value.

We pass now to a consideration of Fig. 5 in which the changes of composition of the precipitated complex observed in these experiments are plotted, in terms of percentages of lecithin as ordinates, against the corresponding ratios in which the constituent substances were mixed expressed numerically (not logarithmically) as abscissae. From the resulting curves it is seen that after

the first rapid increase in the percentage of lecithin in the precipitate, further increase in the total amount of lecithin present in the solution produces less and less increase in the proportion of this substance in the precipitated complex. The occurrence of this long range over which the composition of the precipitated complex varies but little in response to very wide variations in the ratio in which its constituents are mixed readily accounts for the tendency on the part of some authors to regard this complex as a definite chemical compound, the small variations in the composition of the actual precipitate being ascribed to admixture of small and variable quantities of its constituents. But our observations show that this range of almost constant composition is but part of the continuous and in other ranges more pronounced variation in the composition of the complex that would be expected on purely colloid-chemical grounds.

SUMMARY.

1. The percentage of lecithin in the precipitates obtained by the addition of a mixture of lecithin and caseinogen to buffer solutions of graded acidity increases with increasing hydrogen ion concentration of the medium.
2. When mixtures of caseinogen and progressively increasing quantities of lecithin are added to the same buffer solution the increase, at first rapid, in the percentage of lipin in the several precipitates becomes more and more gradual until a stage is reached at which very considerable further increase of the total proportion of lecithin added produces but slight increase in the percentage of this constituent in the precipitated complex.
3. These results are explained in terms of the mutual precipitation of two colloids whose opposite charges vary in intensity with the hydrogen ion concentration of the dispersion medium.

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