XXVIII. THE PROTEINS OF COW'S COLOSTRUM. PART I. THE RELATION BETWEEN THE EUGLOBULIN AND PSEUDOGLOBULIN OF COW'S COLOSTRUM.

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IN a previous communication [1915] the authors have brought forward data which demonstrate that the intramolecular arrangements of the amino-acids in the caseinogens of the cow and the sheep are not identical. This conclusion was arrived at from a comparison of the optical properties of the amino-acids derived from "racemised" cow's and sheep's caseinogens.

In subsequent work the method of protein racemisation has been extended successfully to the study of other proteins in an investigation which had for its object the elucidation of the relationships existing between the proteins of cow's colostrum and the corresponding compounds of ox-serum. In the present communication we give the results of the first part of this work, in which the relations between the globulins of cow's colostrum have been determined.

It has long been known that globulin, both in colostrum and in serum, consists of a mixture of two proteins, namely, euglobulin and pseudoglobulin. These compounds are characterised by widely different physical properties and solubilities. If a solution of globulin be submitted to dialysis, the euglobulin separates out, whilst the pseudoglobulin remains in solution.

The chemical relationship between the euglobulin and pseudoglobulin of serum has formed the subject of an investigation by Chick [1914], who concluded that the two proteins were very closely related. She found that pseudoglobulin preparations always contained, even at the end of a long dialysis, large quantities of a water-insoluble protein resembling euglobulin, and that the latter was largely derived from the pseudoglobulin by a gradual process of "denaturation." She suggested that this artificial euglobulin was

a mechanical complex originating from the interaction and mutual precipitation of two colloidal systems, viz. pseudoglobulin solution and lipoid emulsion. It appeared probable that serum euglobulin was a protein-lipoid complex of similar origin.

Further work by Hartley [1914] on this subject lends support to this view. The figures obtained by him in the analysis of serum euglobulin and pseudoglobulin by the method of van Slyke indicated that these substances were very similar in chemical composition.

Hitherto, little work of a comparative nature has been carried out with euglobulin and pseudoglobulin of cow's colostrum. In this paper we bring forward evidence in support of the view that the globulins of colostrum, just as of serum, are very closely related, if not actually identical, in chemical structure. Our experiments have indeed failed to reveal any structural differences at all between colostrum euglobulin and pseudoglobulin.

This conclusion has been arrived at by two independent methods of investigation, viz.

1. A comparison of the optical rotatory properties of euglobulin and pseudoglobulin in alkaline solution.
2. A comparative study of the optical properties of the amino-acids derived from the hydrolysis of "racemised" euglobulin and pseudoglobulin.

Graph showing progress of racemisation in $N/2$ alkali

If either of the globulins be dissolved in dilute alkali and the solution allowed to remain at 37° for several days, the initial rotation gradually diminishes until it reaches a practically constant value. The course of such a "racemisation" is represented graphically in the accompanying diagram.

The ordinates represent specific rotation values and the abscissae the number of hours the reaction had been allowed to proceed. The solutions were made in semi-normal sodium hydroxide and contained about 7 per cent. of protein (see experimental part).

The points indicated by small circles represent a series of specific rotations, determined on a solution of euglobulin in $N/2$ alkali (7.35 g. protein per 100 cc. solution) at different intervals during the period of racemisation. A second series of readings on a solution of pseudoglobulin in $N/2$ soda (7.04 g. protein per 100 cc. solution) is represented by the crosses. The diagram shows clearly that the optical behaviour of the globulins in $N/2$ alkali is identical. One curve can be drawn to satisfy equally both sets of points. Not only are the initial and final rotations the same in both cases, but the rate of diminution of the optical rotation, which in all probability is conditioned by intramolecular structure, is identical for both globulins.

If $N/4$ instead of $N/2$ alkali be employed as a solvent for the proteins, a rather different optical behaviour becomes apparent. It is found that in each case, using solutions containing about 7 per cent. protein, the specific rotation sinks from an initial rotation of about -75° to a final constant value of about -66° , as compared with a final value of about -37° in $N/2$ alkali. In other words, the reaction does not proceed to anything like the same extent in $N/4$ alkali as it does in $N/2$ alkali. The significance of this difference is not quite clear as yet; it is possible, however, that this behaviour may indicate the existence of a definite stage in the racemisation process. Acidification at this stage causes the separation of a flocculent precipitate, whereas in the case where the stronger alkali has been employed, addition of dilute acid only occasions a very slight flocculation. It will be interesting to study in greater detail the process of racemisation under a variety of conditions and to note the influence of differing protein and alkali concentrations on the course of the reaction. For the present, however, it is sufficient to note that the behaviour of the two globulins in respect of their optical rotations is identical in $N/4$ just as it is in $N/2$ alkali.

An explanation of the behaviour of such protein solutions in suffering a gradual diminution of their specific rotations has been put forward by Dakin [1912]. According to this view, the racemisation of proteins by dilute alkali at low temperatures is due to a keto-enol tautomerism of the —CH—CO— groups in the protein complex.

Wherever this change occurs, the asymmetry of that particular grouping will be destroyed. Consequently, if the racemised protein be hydrolysed, it will be found that certain of the amino-acids are totally inactive, whilst others are fully or partially active. It is extremely probable that the optical properties of the amino-acids thus isolated depend upon their positions in the peptide chains. Those amino-acid components of the protein molecule, whose carboxyl groups are free and which therefore may occupy terminal positions at the ends of peptide chains, will be incapable of racemisation, whilst those which are attached to the protein molecule by condensation of their carboxyl groups with the amino-groups of other constituents, and are therefore probably situated within the chains, may undergo racemisation, since with such an arrangement the keto-enol tautomeric change is possible, this change being accompanied by loss of asymmetry of the grouping in question. On the grounds of the absolute stability of racemised caseinogen and caseose against the common proteolytic enzymes, Dakin and Dudley [1913] concluded that the racemisation due to this tautomeric change must be complete for every such group affected in the protein complex. It thus becomes possible to obtain some evidence as to the original positions of the amino-acids within the protein molecule by a study of the optical properties of these acids among the hydrolytic products of the racemised protein.

In the accompanying table are given the optical properties of the amino-acids derived from the hydrolysis of racemised euglobulin and pseudoglobulin. The results obtained in a similar manner by the authors [1915] for the caseinogens of the cow and the sheep are also tabulated for purposes of comparison.

	Racemised colostrum euglobulin	Racemised colostrum pseudoglobulin	Racemised cow's caseinogen	Racemised sheep's caseinogen
Glycine...	—	—	—	—
Alanine ...	inactive and <i>d</i>	inactive and <i>d</i>	inactive (and <i>d</i> ?)	inactive (and <i>d</i> ?)
Valine ...	<i>d</i> and inactive	<i>d</i> and inactive	<i>d</i> and inactive	<i>d</i> and inactive
Leucine ...	<i>l</i> and inactive	<i>l</i> and inactive	<i>l</i> and inactive	<i>l</i> and inactive
Tyrosine ...	inactive	inactive	inactive	<i>l</i>
Phenylalanine...	inactive	inactive	inactive	inactive
Proline ...	<i>l</i>	<i>l</i>	<i>l</i>	<i>l</i>
Aspartic acid ...	inactive	inactive	inactive	inactive
Glutamic acid ...	<i>d</i> and inactive	<i>d</i> and inactive	inactive	<i>d</i> and inactive
Arginine ...	inactive	inactive	inactive	inactive
Histidine ...	inactive	inactive	inactive	inactive
Lysine ...	inactive	inactive	inactive	<i>d</i>

It will be noted that the amino-acids from racemised euglobulin and pseudoglobulin exhibit no differences whatever. This is regarded as a further confirmation of the structural identity of the two proteins.

The inactivity of the "hexone bases" is noteworthy; it would seem that these diamino-acids in the globulins (as also in cow's caseinogen) are situated entirely within the peptide chains. This fact would appear to lend support to the view sometimes put forward, that they represent the actual "foundation stones" on which the protein molecule is erected. Their basic character would naturally fit them for filling such a function.

It is also curious to notice that, though globulin contains about 3.5 per cent. of glycine, yet in neither case was this amino-acid found among the products of hydrolysis of the racemised globulins. This may possibly be due to the glycine being split off during the racemisation with loss of ammonia, since a certain amount of hydrolysis always occurs simultaneously with racemisation. If this be true, it would seem to indicate that the total glycine in globulin is so situated within the molecule, that it is split off with extreme ease by the action of hydrolysing agents. In this respect, globulin exhibits a markedly different behaviour from gelatin, as Dakin [1912] was able to recover about 17 per cent. of glycine from the products of hydrolysis of racemised gelatin. Eu- and pseudo-globulin behave identically in this respect; so that this fact is also consistent with the idea of their structural similarity.

In comparing the optical properties of the amino-acids derived from the hydrolysis of racemised cow's caseinogen and colostrum globulin, one is struck by the close similarity displayed, suggestive, it may be, of a near structural relationship between racemised caseinogen and racemised globulin. Such a kinship is not altogether impossible, in view of the fact that globulin appears to lose glycine during racemisation, whereas caseinogen, in like circumstances, suffers a loss of phosphate content.

In conclusion, reference may be made to an interesting confirmation of the results arrived at in this investigation, regarding the identity of the globulins in colostrum, which is furnished by work carried out in this Institution by Crowther and Raistrick [1916]. They analysed euglobulin and pseudo-globulin by the method of van Slyke and were unable to detect any differences in the amino-acid content of the two proteins. The results of their analyses demonstrated further that the total globulin of milk was identical in composition with that of colostrum.

EXPERIMENTAL.

Preparation of euglobulin and pseudoglobulin from cow's colostrum.

Colostrum, which is the fluid secreted by the mammary glands in the earliest stages after parturition, is eminently suited to be the starting point for the preparation of globulin. The colostrum secretion is distinguished by its richness in protein material, which may at times amount to as much as 18 to 20 per cent. of the total secretion. Of this, 4-5 per cent. usually consists of caseinogen, 0.7-1.5 per cent. of albumin and 6-12 per cent. of globulin. This is in striking contrast to the ordinary milk secretion which usually contains about 3.0 per cent caseinogen and 0.4 per cent. albumin, whilst globulin is only present in very small amounts. The preponderance of globulin in the initial secretion rapidly disappears with the development of mammary activity and the colostrum assumes the characteristic composition of milk often within about 48 hours of calving [Crowther and Raistrick, 1916].

The first step in the preparation of the globulins was to precipitate the caseinogen by means of dilute acetic acid. The best conditions for precipitation were found to be as follows.

The colostrum was added to dilute acetic acid containing 2 cc. of glacial acetic acid per litre; for every litre of colostrum, it was found advisable to use 2 litres of the dilute acid. The mixing was carried out in a tall glass jar and the liquid was stirred vigorously during the addition. The caseinogen, carrying along with it the bulk of the fatty material, immediately separated out.

After allowing the caseinogen precipitate to settle, the clear liquid, containing the globulins and albumin in solution, was siphoned off. A further quantity of globulin liquor was obtained by centrifuging the residual slime of caseinogen and fat.

The solution was next passed through a folded filter paper and made exactly neutral by the addition of the requisite amount of dilute soda. The large volume of liquid was then reduced somewhat by concentration in a vacuum pan at a temperature of 40-45°, and a sufficient quantity of magnesium sulphate was then stirred into the concentrated solution to ensure complete saturation. This effected the separation of the globulins, which were filtered off on a large Buchner funnel. The filtrate was preserved with a view to isolating the albumin for further work.

The mass of mixed globulins was dissolved in water and resalted out by means of magnesium sulphate, in order to render the separation from albumin

more complete. The globulin so obtained was once more dissolved in the minimum amount of water and the solution was thoroughly dialysed against running tap water, in the initial stages, and finally against distilled water. During this process, the euglobulin separated out in a gelatinous form from the solution. The pseudoglobulin, which is soluble in distilled water, remained in solution.

The dilute solution of pseudoglobulin, after filtration, was concentrated *in vacuo* at a temperature of 40–45°, until a content of about 17 per cent. protein was attained. Emulsions of euglobulin (after thorough washing with distilled water) were prepared of a similar strength by stirring the protein into water containing a little common salt in solution. The advantages of working with such protein preparations, instead of employing the dried materials, were twofold. Firstly, we were enabled to avoid all processes which might have exerted a "denaturing" effect on the protein (*e.g.* excessive heat, treatment with alcohol, etc.). Secondly, it was possible, after carrying out a nitrogen estimation on such preparations, to make up quickly and without difficulty, solutions of any desired strength in alkali. This was a point of some importance, as it was required to determine the optical rotations of such solutions with as little delay as possible.

Rotatory properties of euglobulin and pseudoglobulin in dilute alkali.

If a 10 per cent. solution of euglobulin or pseudoglobulin be made in $N/2$ caustic soda solution, agglutination occurs almost immediately, the solution thickening to the consistency of a jelly. The jelly-like mass softens after 15 to 20 minutes; but this occurs much more readily if the flask containing the solution be placed in an incubator at 37°. In quite a short time, a clear solution of the globulin is obtained, which can be filtered without difficulty.

It will be noted that both globulins display the same physical changes accompanying their solution in dilute alkali.

In the following series of determinations, solutions of globulin of a 7–8 per cent. concentration in $N/2$ alkali were employed. At higher concentrations than this, it was found that the change from the jelly phase to the clear filterable solution was slower than desirable, in view of the importance of determining the rotation as soon as possible after making the solution.

The method of preparing such a solution was as follows. 10 cc. of $5N/2$ caustic soda solution were run into a 50 cc. graduated flask. After diluting this with distilled water, a measured amount of the globulin preparation, containing about 3.5 g. of protein, was added quickly and the bulk made up

to the 50 cc. mark by the addition of distilled water. During the mixing process, the contents of the flask were shaken vigorously. The flask was then placed in the incubator, and after about two hours, the solution was filtered and the optical rotation determined in a decimetre tube. The exact concentration of the filtered solution was determined by a Kjeldahl estimation on 5 cc.

The rotation of the solution was determined at regular intervals, until it had attained a constant value. The two tables which follow give the results of comparative trials with the two globulins in $N/2$ caustic soda solution. It will be noted that the data, which have been plotted in the diagram already referred to, failed to reveal any differences in the structural make-up of the globulins. Such slight discrepancies as may exist fall well within the limits of experimental error. In both cases, the specific rotation fell rather rapidly in the first stages of racemisation; but the rate of diminution became slower later on, until finally the rotatory value became practically constant.

I. Pseudoglobulin (7.04 g. in 100 cc. $N/2$ alkaline solution).

Time (in hours)	Rotation in 1d tube	$[\alpha]_D = \frac{\text{Rotation}}{\text{Concentration}}$
2.25	-5.22	-74.15
3.5	-5.18	-73.57
5	-5.00	-71.02
22.5	-4.15	-58.95
30.5	-3.83	-54.40
47	-3.45	-49.00
94	-3.03	-43.04
120	-2.93	-41.62
142	-2.87	-40.77
170.5	-2.73	-38.78
194.5	-2.68	-38.07
215.5	-2.65	-37.64
262.5	-2.65	-37.64

II. Euglobulin (7.35 g. in 100 cc. $N/2$ alkaline solution).

Time (in hours)	Rotation in 1d tube	$[\alpha]_D = \frac{\text{Rotation}}{\text{Concentration}}$
2.5	-5.46	-74.28
5	-5.18	-70.48
22	-4.23	-57.55
52	-3.45	-46.94
72.5	-3.20	-43.54
95	-3.08	-41.91
120	-3.02	-41.09
167	-2.86	-38.91
190.5	-2.76	-37.55
336	-2.76	-37.55

Before leaving this part of the subject, it may be pointed out that the method outlined above should prove of value in future investigations, which may be designed with a view to establishing the identity or non-identity of related proteins. Its relative simplicity and the small amounts of material required are points in its favour. Its operation does not involve the length of time and the vast amount of labour, which are required by many of the ordinary methods of protein investigation. Moreover, it may be made a very exhaustive test; since if two proteins are to be pronounced structurally identical, then they must not only display the same optical behaviour under one set of conditions, but must continue to do so when the concentration of the protein and the strength of the alkaline solution are varied.

Isolation of Amino-acids.

For the purpose of isolating the amino-acids, 10 per cent. solutions of the globulins in $N/2$ NaOH were digested at 37° for 8–10 days. The rotation in a decimetre tube fell during this period from about -7.5° to a constant value of about -4° .

Tyrosine. 45 g. euglobulin and an equal weight of pseudoglobulin were racemised separately. The alkaline solutions were hydrolysed with hydrochloric acid, the solutions filtered and evaporated *in vacuo* at 45° . Water was added to the residues, and the solutions were once more evaporated under these conditions. This process was repeated until the bulk of the hydrochloric acid had been removed. The residues were subsequently dissolved in water, boiled with the addition of animal charcoal and filtered. After making the solutions slightly alkaline by means of ammonia, they were concentrated and the tyrosine was allowed to crystallise. The two specimens of crude tyrosine were filtered off, washed with a little hot glacial acetic acid and then dissolved in dilute hydrochloric acid. After treatment with animal charcoal, the solutions were neutralised with ammonia. On cooling, tyrosine separated out in characteristic form. The two samples were further repeatedly crystallised from slightly ammoniacal solutions, in order to remove completely traces of cystine. They were then dissolved in 4 per cent. hydrochloric acid and their optical rotations determined. They were both found to be inactive.

The mother liquor, from which the crude tyrosine had crystallised, was acidified with hydrochloric acid and added to the acid solution of mixed amino-acids for investigation by the esterification method.

Arginine, histidine and lysine were isolated by the method of Kossel and

Kutscher [1900]. For this purpose, 135 g. of euglobulin and 150 g. of pseudoglobulin were racemised separately. Arginine and histidine were in both instances inactive. The lysine fractions displayed definite *laevo* rotations when first isolated, owing to contamination with *l*-proline. It was found possible to remove the latter by fractional precipitation of the phosphotungstates, but a more convenient method was as follows. The copper salts of the mixture were prepared in the dry, powdered form. The latter preparation was then thoroughly extracted with absolute alcohol, in which the proline-copper salt was readily soluble. The residual insoluble lysine-copper salt was dissolved in water, acidified slightly with dilute sulphuric acid and decomposed by means of sulphuretted hydrogen. After such treatment, the lysine preparations from both sources were found to be totally inactive, whereas the fraction obtained from the decomposition of the proline-copper salt possessed a decided *laevo* rotation.

The large volume of acid solution, obtained in the above isolation of the diamino-acids after the filtration of the phosphotungstate precipitates, was made almost neutral by the addition of powdered commercial baryta. After filtering, the solution was evaporated to a small bulk in a vacuum pan at 45°, and the remaining sulphuric acid was removed quantitatively from the concentrated liquid by means of baryta. The solution thus obtained was acidified with hydrochloric acid and utilised to augment the yields in the investigation of the amino-acids by the esterification method.

200 g. of euglobulin, and 240 g. of pseudoglobulin, were racemised separately in the manner already described and then hydrolysed with hydrochloric acid for the amino-acid separation by the esterification method. To the acid solutions were added the solutions of amino-acids recovered from the tyrosine and diamino-acid separations. Levene's modification of Fischer's method was employed [Levene and van Slyke, 1909].

Glycine. As already mentioned, no glycine was found among the amino-acid preparations resulting from the hydrolysis of racemised euglobulin and pseudoglobulin. The fraction of crystals containing alanine and valine, where the glycine, if present, should occur, was fractionally crystallised, and the percentage of nitrogen in the more soluble portion of the crystals was determined. After carrying out this procedure repeatedly, the percentage of nitrogen increased to a constant value of 15.7 per cent., which is the nitrogen content of pure alanine, whereas that for glycine is the much higher value of 18.7 per cent.

The absence of glycine was confirmed by our inability to obtain crystals

of the characteristic picrate from the alanine fractions by the method of Levene [1906].

Alanine. Specimens of pure *i*-alanine were obtained by fractional crystallisation from both globulin sources. In both cases, it was also found possible to recover other fractions containing alanine and valine, in which the rotation was *dextro*. They consisted of mixtures of *d*-alanine with *i*- and *d*-valine.

Valine was isolated in mixtures of *i*- and *d*-valine, containing as much as 70 per cent. of the active form, from both racemised globulins. In addition to this, specimens of *i*-valine were isolated in each case, and the *dextro* variety was found to occur in leucine-valine mixtures and in valine-alanine fractions.

Leucine. Both racemised globulins gave leucine in the pure state almost inactive, whilst in each instance, fractions of *l*-leucine containing only small amounts of *d*-valine were isolated.

Phenylalanine was isolated in the pure state. The specimens from both racemised globulins were inactive.

Proline. In both cases, the proline was strongly *laevo* rotatory, but displayed the partial racemisation which always occurs as a result of the process of separation. It was probable that in neither case had any racemisation taken place previous to hydrolysis.

Aspartic acid was isolated in the inactive form from racemised euglobulin, as well as from racemised pseudoglobulin. In neither case was any of the active variety detected.

Glutaminic acid. A specimen of glutaminic acid containing about 44 per cent. of the *dextro* variety was isolated from the hydrolytic products of racemised euglobulin. Racemised pseudoglobulin yielded an acid, which contained about 37 per cent. of the active form. These results were sufficiently close to be confirmatory of the identity of the two globulins, especially in view of the fact that a partial racemisation of glutaminic acid is brought about as a result of the hydrolysis and the subsequent process of isolation.

SUMMARY.

It has been shown that:

1. Euglobulin and pseudoglobulin of cow's colostrum display the same behaviour in respect of their optical properties, when dissolved in $N/2$ and $N/4$ alkali.
2. The optical properties of the amino-acids derived from the hydrolysis of racemised colostrum euglobulin are identical with those of the corresponding acids from hydrolysed racemised colostrum pseudoglobulin.

These results are in accord with the findings of Hartley [1914] and Chick [1914] on the corresponding globulins of ox-serum, and of Crowther and Raistrick [1916] on the colostrum globulins; they are confirmatory of the hypothesis, that the two globulins are identical, at least as far as the protein part of their molecules is concerned.

This reservation is necessary in view of the observations of Hardy [1905] and Haslam [1913] on the phosphorus content of globulin prepared from ox-serum. Haslam prepared pseudoglobulin free from phosphorus and showed that euglobulin contained about 0.1 per cent. of phosphorus, about half of which was extractable with alcohol and ether.

The results given by Hartley on the one hand, and Crowther and Raistrick on the other, amply demonstrate that such slight differences are limited to the non-protein ingredients of the globulin preparations. Chick [1914], as a result of her investigations on the serum globulins, expressed the opinion that the phosphorus content of euglobulin was due to the association of the protein with a serum-lipoid. The incomplete extraction of the fatty substance containing the phosphorus in the euglobulin preparation was probably due to the state of aggregation of the protein and not to the phosphorus constituting an integral part of the euglobulin protein molecule.

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