X. THE SPECIFICITY OF CASEINOGENS. A COMPARATIVE STUDY OF THE CASEINOGENS OF THE COW AND THE SHEEP.

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For many years the question as to whether the caseinogens derived from different species of mammals are one and the same chemical substance has remained undecided.

The general physical and chemical characteristics of the caseinogens precipitated from various milks by dilute acid are so similar that no satisfactory conclusions can be drawn regarding the question of their chemical identity, although some earlier investigators have either supported the hypothesis of chemical identity on the strength of the general similarity of the substances or opposed it on account of observations of slight differences.

Abderhalden and Schittenhelm [1906] compared the amounts of amino-acids contained in the caseinogens of cow's and goat's milk with the result that a striking similarity was demonstrated and the assertion of differences in the quantitative amino-acid composition of the two proteins was not possible.

Elementary analyses of caseinogens coincide so closely that they have been adduced as evidence in favour of the view that the caseinogens of various mammals are identical. The comparison of cow's and sheep's caseinogens, with which this paper is especially concerned, serves to show how closely the analyses agree.

Cow ..	..	C 52.96 %	H 7.05 %	N 15.65 %	S 0.758 %	P 0.847 %
						[Hammarsten, 1883, 1885]
Sheep	..	C 52.92	H 7.05	N 15.60	S 0.771	P 0.809
						[Tangl, 1908]

Nevertheless it has long been suspected that as a matter of fact there may be differences in the structure of various caseinogens, not necessarily in respect to the relative amounts of amino-acids present, but possibly in the actual arrangement and order of these acids within the molecules. This is a reasonable assumption on biological grounds, and biological experiments—the formation of specific precipitins on injection into the blood-stream of pure caseinogen preparations—have given support to this view.

In this paper, however, we bring forward for the first time definite chemical evidence in support of the general assumption of specificity in caseinogens, and proof in particular of a difference in intramolecular structure between cow's and sheep's caseinogens. We have been enabled to do this by a comparative study of the amino-acids derived from the "racemised" caseinogens. If caseinogen be dissolved in dilute alkali and the solution allowed to stand at 37° for about three weeks the value of the optical rotation falls to a practically constant value. The protein is then said to be "racemised" and the amino-acids obtained on subsequent hydrolysis are found to be in some cases optically inactive, in others partially active and in others fully active. Dakin [1912] has shown that in all probability the optical properties of the amino-acids depend upon their positions in the peptide chains. According to his view amino-acids whose carboxyl groups are free and which therefore may occupy terminal positions at the ends of peptide chains, are incapable of racemisation, whilst those whose carboxyl groups are united in actual peptide linkages, and are therefore probably situated within the chains, may undergo racemisation. The optical activity or inactivity of the amino-acids among the hydrolytic products of a "racemised" protein will therefore give some clue as to the original positions of these acids in the protein molecule, although the full interpretation of the significance of these findings is not yet possible.

Accordingly we "racemised" a quantity of sheep's caseinogen with dilute alkali under precisely the same conditions as those employed by Dakin and Dudley [1913] in an investigation of the racemisation of cow's caseinogen. By a comparison of the optical properties of the corresponding amino-acids derived from "racemised" cow's and sheep's caseinogens we hoped possibly to be able to detect structural differences relating to the positions of the respective amino-acids in the molecules of the two proteins. In this we were successful; the following table compares the optical properties of the amino-acids from racemised cow's and sheep's caseinogens respectively:

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

It will be seen that, as is to be expected, most of the amino-acids from the two caseinogens display identical optical properties, indicating a general similarity in the molecular structures of the two proteins. But a sharp difference is found in the case of tyrosine. The tyrosine derived from "racemised" cow's caseinogen is completely inactive, that from "racemised" sheep's caseinogen is fully active.

Again, the lysine derived from cow's caseinogen is totally inactive, but the specimen isolated from sheep's caseinogen displayed two-thirds of the full activity. The lysine fraction was originally contaminated with some *l.* proline, which was only removed with difficulty by fractional precipitation of the phosphotungstates. Bearing in mind the possibility that traces of *l.* proline might still cling to the lysine, together with the fact that lysine is not a very stable substance, it seems probable that no racemisation of lysine took place before hydrolysis. Further, we find that a small quantity (about 15 %) of the total glutaminic acid obtained from "racemised" sheep's caseinogen is optically active, while that derived from cow's caseinogen is completely inactive.

Although the significance of these differences cannot yet be fully elucidated, they may be taken at least as direct proof that cow's and sheep's caseinogens are two distinct proteins. Their general structure is undoubtedly very similar and might even be, with regard to amino-acid content, quantitatively practically identical, but small definite differences in the arrangement of the amino-acids within the molecules of the two substances must occur.

We incline to the view that each species of mammal may synthesise its own specific caseinogen, and therefore that every caseinogen is a distinct member of a class of very similar proteins.

One point of difference in the comparisons of the amino-acids from the two caseinogens lies in the fact that in the investigation of cow's caseinogen the "racemised" protein was precipitated from the alkaline solution after

"racemisation" and hydrolysed separately (except in the isolation of tyrosine), whereas the alkaline solution of sheep's caseinogen was hydrolysed as it stood, thus including the products of the hydrolysis which proceeds simultaneously with "racemisation." We do not, however, consider that this procedure vitiates our present results because it has been shown [Dakin and Dudley, 1913] that the amino-acids from "racemised" caseose, the main hydrolytic product during the process of "racemisation," are identical with those of "racemised" caseinogen, and that the quantity of free amino-acids formed under the experimental conditions of "racemisation" is very small.

Fortunately our most definite finding, the difference between the two specimens of tyrosine, is in any case not affected by this possible criticism, since in the case of both caseinogens the entire alkaline solutions were hydrolysed as they stood after digestion, and worked up in exactly the same way.

We propose to extend these investigations to the study of the relationships between other proteins.

In conclusion we desire to thank Dr H. D. Dakin for permission to apply the method of protein racemisation to problems of this nature.

EXPERIMENTAL.

Preparation of sheep's caseinogen.

The caseinogen was precipitated from the "separated" milk by means of dilute acetic acid. The best conditions for precipitation were found to be as follows.

A 0.06 % (vol.) solution of acetic acid was prepared in a large glass vessel and for each litre of this solution 50 cc. of milk were added with vigorous stirring. The caseinogen, which flocked out almost immediately, was allowed to settle and the clear liquid was siphoned off. The caseinogen precipitate was washed with distilled water until free from soluble proteins. If tap water is used the preparation becomes discoloured. After filtering on a large Buchner funnel the caseinogen was ground up in a mortar with an alcohol-ether (4 : 6) mixture, filtered, ground up again with ether, filtered and dried in vacuo over sulphuric acid.

Racemisation of sheep's caseinogen.

This was carried out exactly as in the case of cow's caseinogen [Dakin and Dudley, 1913], 10 % solutions of the caseinogen in $n/2$ sodium hydroxide being digested at 37° for 20–24 days. The fall in rotation and the amount of rotation were of a similar order to those recorded for cow's caseinogen.

Isolation of Amino-acids.

Tyrosine. 50 g. sheep's caseinogen were racemised. The alkaline solution was then hydrolysed with hydrochloric acid, the solution filtered and evaporated in vacuo at 45°. The residue was then taken up in water, boiled with animal charcoal and filtered. The solution, rendered slightly alkaline with ammonia, was concentrated and tyrosine was allowed to crystallise. The crude tyrosine was filtered off, washed with small quantities of hot glacial acetic acid, and then dissolved in hydrochloric acid. The solution, after treatment with animal charcoal, was neutralised with ammonia and on cooling tyrosine crystallised in characteristic form. It was found to be fully *laevo*-rotatory. It was compared with a specimen of pure *l.* tyrosine prepared by the pancreatic digestion of cow's caseinogen. In 4 % HCl solution the two specimens showed identical specific rotations: -11.60° and -11.65° .

400 g. sheep's caseinogen were racemised in the usual way and then hydrolysed with hydrochloric acid for investigation of the amino-acids by the esterification method. Levene's modification of Fischer's method [Levene and van Slyke, 1909] was employed.

Alanine. A specimen of pure *i*-alanine was obtained by fractional crystallisation. Other fractions containing alanine and valine were recovered which possessed a *dextro*-rotation. They may have been mixtures of *d.* alanine and *d.* valine, or possibly of *i.* alanine and *d.* valine.

Valine was isolated in mixtures of *i.* and *d.* valine containing over 50 % of the active form. The *dextro*-variety was also found in leucine-valine mixtures and in valine-alanine fractions.

Leucine was obtained in the pure state almost inactive, and fractions of *l.* leucine containing only very small amounts of *d.* valine were also separated.

Phenylalanine was readily obtained in the pure state and was found to be totally inactive.

Proline was isolated and displayed the partial racemisation which always occurs as a result of the process of separation. Its activity was such as to render it probable that no racemisation had occurred before hydrolysis.

Aspartic acid was isolated in the inactive form. None of the active variety could be detected.

Glutaminic acid was obtained in large quantity and proved to be a mixture of the inactive acid with about 15 % of the *dextro*-form.

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

Kutscher. 150 g. sheep's caseinogen were racemised for this purpose. Arginine and histidine were both inactive. The lysine fraction displayed a definite *laevo*-rotation when first isolated. By fractional precipitation of the phosphotungstates a specimen of *l.* proline was removed and found to be responsible for the original *l.* rotation. The lysine, examined as the dihydrochloride, now displayed a specific rotation of $+9.4^{\circ}$. It was identified by the preparation of the characteristic picrate.

SUMMARY.

By comparing the optical properties of the amino-acids derived from "racemised" cow's and sheep's caseinogens it is shown that the intramolecular arrangement of the amino-acids in these two proteins is not identical, and it is considered probable that each milk-producing species may synthesise its own specific caseinogen.

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