Short Communications

Action of rennin on casein: the function of the neuraminic acid residues

Casein contains small amounts of hexose, hexosamine and neuraminic acid[1] and one of the results of the action on it of rennet is to split off a fragment, termed glycomacropeptide[2], which is rich in these components. Some experiments designed to determine whether the saccharide constituents of casein, in particular the neuraminic acid, are concerned in the clotting reaction are reported. κ-Casein has been used throughout since this appears to be the casein fraction which is the primary substrate for rennet action[3].

Materials and methods: κ-casein was prepared from the α-casein obtained from casein by the urea fractionation method of HIPP *et al.*[4]. (Details of preparation to be published.) The material appeared almost homogeneous by free electrophoresis and by paper electrophoresis in presence of urea. It was stable in solution at pH 6.6 in presence of 0.2 *M* $CaCl_2$ and gave a precipitate after addition of rennin. Rennin released 20% of the N of this preparation in a form which remained soluble after the residue had been precipitated by calcium. This compares favourably with 23% obtained by WAKE[3] from his preparation.

Crystalline rennin was fractionated on DEAE-cellulose by the method described by JIRGENSONS, IKENAKA AND GORGURAKI[5]. α-Rennin was obtained from the refractionated major enzyme peak. Neuraminidase was prepared from *Vibrio cholerae* filtrates as described by SCHRAMM AND MOHR[6]. Proteolytic activity could not be detected.

For measurement of the clotting reaction, amounts of 2 mg casein in 0.4 ml

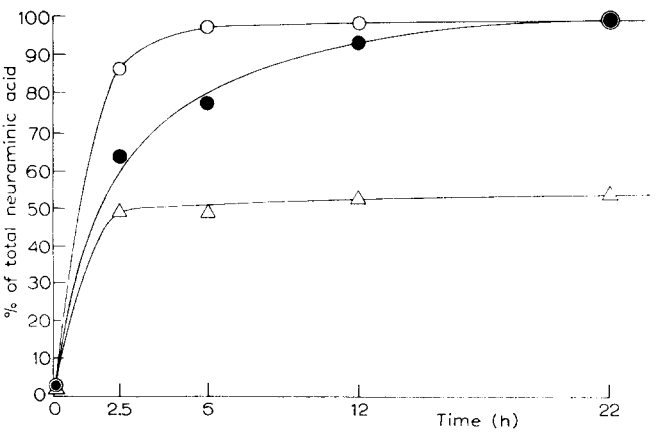

Fig. 1. Rate of release of neuraminic acid by neuraminidase (pH 5.5). ○—○, κ-casein, 1.0%; ●--●, neuramin-lactose, 0.025%; △—△, bovine submaxillary mucoid, 0.05%.

acetate buffer (pH 5.5, I 0.1) containing 10^{-4} M Ca^{2+} were treated with 0.01 ml of a 0.7% solution of α-rennin at 37° for 30 min. The precipitate was removed by centrifugation, washed twice with 0.4 ml of buffer and nitrogen was estimated in the combined supernatants. Neuraminic acid was estimated in an aliquot of the supernatant and in the washed precipitate.

Neuraminic acid was released by adding to 5 ml of a 1% solution of κ-casein in acetate buffer (see above), 0.5 ml neuraminidase solution and 1 drop of toluene. The enzyme reaction was allowed to proceed for 24 h at 37°. Bovine submaxillary mucoid[7] and neuramin-lactose[8] were similarly incubated at concentrations of 0.05% and 0.025%, respectively.

Free electrophoresis was carried out in a Perkin Elmer apparatus at a concentration of 0.7% in Veronal–HCl buffer (pH 7.3, I 0.15). Electrophoresis on paper was carried out in the presence of urea[12].

Results: The rate of release of neuraminic acid from κ-casein and two other substrates by the action of neuraminidase is shown in Fig. 1. After incubation, the κ-casein was isolated by dialysis and freeze drying. Analyses of the saccharide constituents of the material before and after enzyme action are shown in Table I,

TABLE I

	Hexose (as galactose) %	*Hexosamine (as free base)* %	*Fucose* %	*Neuraminic acid (as N-acetyl derivative)* %	*Electrophoretic mobility of main component (95% of the whole) Barbiturate buffer (I 0.15, pH 7.3)*
κ-Casein,	1.00*	0.39*	0.11*	0.79**; 0.80***	$5.73 \cdot 10^{-5}$
Neuraminidase-treated κ-casein	1.02*	0.36*	—	0	$5.70 \cdot 10^{-5}$

* Estimated as described by GIBBONS[9].
** Thiobarbituric acid method[10].
*** Resorcinol method after resin-column separation[11].

from which it is clear that the saccharide components other than neuraminic acid are largely unaffected. The κ-casein showed no sign of forming a clot or precipitate during neuraminidase action. Under the conditions of electrophoresis employed no change could be detected after neuraminidase action, either in the electrophoretic pattern or in the mobility of the main component.

On addition of rennin at 37° the neuraminidase-treated κ-casein became opalescent within a few seconds and thereafter deposited a precipitate. Subjectively the reaction appeared to be slightly more rapid than that of undegraded κ-casein. After rennin action on 2 mg of sample, 81 μg N was found in the supernatant from κ-casein and 83 μg N in that from the neuraminidase-treated κ-casein. The glycomacropeptide released from undegraded κ-casein by rennin contains virtually all (97%) of the neuraminic acid originally present. The washed precipitated para-κ-casein is devoid of neuraminic acid. Rennin fails to liberate free neuraminic acid from κ-casein or from either of the other two substrates described; it fails to liberate detectable amounts of free amino or free reducing groups from bovine submaxillary or cervical mucoid; furthermore, it has no effect on the viscosity of their solutions.

N-glycolyl neuraminic acid, though present in some fractions isolated from colostrum[13] was not found in the neuraminic acid derived from the κ-casein preparation.

It is clear that it is possible to remove all the neuraminic acid in κ-casein without affecting significantly its behaviour towards rennin. Furthermore, the neuraminic acid is present ketosidically linked as a terminal residue. Rennin is devoid of neuraminidase or glycosidase activity, and the theoretical possibility that the neuraminic acid in κ-casein, by virtue of its potentially bifunctional character, forms a link between the glycomacropeptide and the remainder of the κ-casein molecule may be ruled out.

The release of neuraminic acid from κ-casein by neuraminidase is somewhat more rapid than from the 2 → 3-linked neuramin-lactose. The rate is comparable with that at which part of the neuraminic acid in bovine submaxillary mucoid is hydrolysed. The latter contains 2 → 6 neuraminosidic linkages, although about half the neuraminic acid in the preparation used is resistant to neuraminidase. The difference in rate is probably not sufficiently great to warrant any firm conclusion as to the linkage of neuraminic acid in κ-casein.

An important point arises with regard to the molecular weight of κ-casein. Glycomacropeptide is reported to contain 14.3% N-acetyl neuraminic acid and to be of molecular weight of about 8000 to 10000 (ref. 14). Since the κ-casein preparation contains only 0.79% neuraminic acid it must have a molecular weight in the region of 160000–200000. If the recently reported[15] molecular weight of about 26000 for a preparation of κ-casein having similar properties to that used in this investigation is correct then the κ-casein used in this work must be grossly inhomogeneous in that about 20% of the molecules present must contain all the neuraminic acid. This is considered rather unlikely as it is possible to remove the neuraminic acid without affecting the electrophoretic behaviour of the preparation appreciably. The molecular weight of 280000 given by PAYENS[16] would appear to be more consistent with the observations reported here.

One of us (R.A.G.) gratefully acknowledges the support of the Population Council, Inc., Rockefeller Institute, New York, who also provided the electrophoresis apparatus used.

National Institute for Research in Dairying, Shinfield, Reading, Berkshire (Great Britain)

R. A. GIBBONS
G. C. CHEESEMAN

1 B. JOHANSSON AND L. SVENNERHOLM, *Acta Physiol. Scand.*, 37 (1956) 324.
2 H. NITSCHMANN, H. WISSMAN AND R. HENZI, *Chemica*, 11 (1957) 76.
3 R. G. WAKE, *Australian J. Sci.*, 20 (1957) 147.
4 N. J. HIPP, M. L. GROVES, J. H. CUSTERS AND T. L. MCMEEKIN, *J. Dairy Sci.*, 35 (1952) 272.
5 B. JIRGENSONS, T. IKENAKA AND V. GORGURAKI, *Makromol. Chem.*, 28 (1958) 96.
6 G. SCHRAMM AND E. MOHR, *Nature*, 183 (1959) 1677.
7 R. HEIMER AND K. MEYER, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 728.
8 R. KUHN AND R. BROSSMER, *Chem. Ber.*, 89 (1956) 2013.
9 R. A. GIBBONS, *Biochem. J.*, 73 (1959) 209.
10 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
11 L. SVENNERHOLM, *Acta Chem. Scand.*, 12 (1958) 547.
12 E. A. ZHDANOVA AND I. N. VLODAVETS, *Biokhimiya*, 24 (1959) 371.
13 R. A. GIBBONS, unpublished observations.
14 P. JOLLES AND C. ALAIS, *Compt. rend.*, 251 (1960) 2605.
15 H. A. MCKENZIE AND R. G. WAKE, *Australian J. Chem.*, 12 (1959) 734.
16 T. A. J. PAYENS, *Biochim. Biophys. Acta*, 46 (1961) 441.

Received August 2nd, 1961