results are expressed as micrograms of enzyme protein in mitochondria per gram of mammary tissue (Table III). The enzyme concentrations found for all four goats were of the same order of magnitude and averaged 11.09 μg/g of tissue.

The fact that acetyl-CoA synthetase has been purified and antibodies to the protein have been produced in rabbits will provide an opportunity for extensive characterization of the enzyme. At present the approximate molecular weight of the enzyme is known. The protein has been shown to contain carbohydrates and is a glycoprotein (Stamoudis and Cook, 1975).

LITERATURE CITED

Cook, R. M., Liu, S.-C. C., Quraishi, S., *Biochemistry* **8**, 2966 (1969).
Davis, B. J., *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
Martin, R. B., Ames, B. N., *J. Biol. Chem.* **236**, 1372 (1961).
Niswender, G. D., Reichert, L. E., Jr., Midgley, A. R., Jr., Nalbandov, A. V., *Endocrinology* **84**, 1166 (1969).
Qureshi, S., Cook, R. M., part V of the series, in this issue.
Stamoudis, V., Cook, R. M., part VII of the series, in this issue.

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Utilization of Volatile Fatty Acids in Ruminants. VII. Acetyl-Coenzyme A Synthetase. A Glycoprotein

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Acetyl-CoA synthetase purified from mitochondria of lactating cow and goat mammary gland was tested for the presence of carbohydrates. Polyacrylamide gel electrophoresis followed by PAS staining was positive. Sulfuric acid hydrolysis or preincubation with neuraminidase gave a difference in anodic migration on polyacrylamide gel electrophoresis suggesting the presence of *N*-acetylneuraminic acid. The thiobarbituric acid test for neuraminic acid was positive. GLC analysis showed that the cow enzyme contains fucose, glucose, and *N*-acetylneuraminic acid. The goat enzyme contains fucose, galactose, glucose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid. These observations were confirmed using mass spectrometry. The role carbohydrates play in determining structural and catalytic properties of acetyl-CoA synthetase is not clear at this time. Other work in our laboratory has shown that acetyl-CoA synthetase is more active on propionate than on acetate in liver and lung, but is equally active on both substrates in heart and kidney. Also, the enzyme is not active in the nonlactating mammary gland but activity begins to develop near the time of parturition. The activity in the mammary gland decreases with advancing lactation. These differences in substrate specificity and other phenomena may be explained by differences in the carbohydrate composition of the enzymes in different tissues and under different physiological states.

We consider the acetate activation reaction catalyzed by acetyl-CoA synthetase to be an important rate-limiting step in acetate utilization by ruminant tissues (Cook et al., 1969; Quraishi and Cook, 1972). In order to study this reaction further, considerable effort was devoted to purification of the enzyme from mitochondria of lactating ruminant mammary gland (Qureshi and Cook, 1975; Cook et al., 1975). During these studies the apparent aggregation phenomena and difficulties in purification suggested to us that the enzyme might be a glycoprotein.

Consequently, the purified enzyme was tested for carbohydrates and was found to be a glycoprotein. The details of these experiments are reported in this paper.

EXPERIMENTAL SECTION

Enzyme Purification. The enzyme from lactating bovine mammary gland was purified as described by Qureshi and Cook (1975). The enzyme from lactating goat mammary gland was purified as described by Cook et al. (1975).

Enzyme Assay. Acetyl-CoA synthetase activity was determined by the acetate-dependent disappearance of the free sulfhydryl group of coenzyme A as described by Cook et al. (1969). Protein was determined by the method of Lowry et al. (1951).

Polyacrylamide Gel Electrophoresis. The general method of Davis (1964) was used for these studies. The buffer used was 0.025 *M* Tris-HCl–0.20 *M* glycine (pH 8.3). The electrophoresis was carried out with a current of 6 mA/tube for 30 min. After electrophoresis the gel columns were stained for proteins with Coomassie Blue according to the procedure of Chrambach et al. (1967). Staining for glycoproteins was performed according to Hotchkiss (1970) and Kaschnitz et al. (1969).

Determination of Neuraminic Acid. The thiobarbituric acid assay was used to determine neuraminic acid (Warren, 1959). Neuraminic acid was removed from the protein by hydrolysis for 60 min at 80° in 0.1 *N* H_2SO_4.

Gas–Liquid Chromatography. Monosaccharides were determined as the Me_3Si derivatives of the methyl glycosides by GLC as described by Sweeley et al. (1972). The chromatograph was equipped with a flame ionization detector. The column used was 6 ft × ⅛ in. glass packed with Chromosorb W containing 3% OV-1. Nitrogen was used as carrier gas at a flow rate of 30 ml/min. Isothermal GLC was conducted at two different temperatures, 160 and 190°.

Mass Spectrometry. The presence of carbohydrates was confirmed using a LKB-9000 gas chromatograph–mass spectrometer. The ionizing energy was 70 eV. The GLC column was 4 ft × ⅛ in. glass packed with Chromosorb G containing 3% SE-30. The temperature was programmed

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Table I. Thiobarbituric Acid Assay for Neuraminic Acid Content of Acetyl-CoA Synthetase

Source of acetyl-CoA synthetase	Protein/ assay, mg	Neuraminic acid, μg	μg of neuraminic acid/mg of protein
Goat MG 6/8/71	0.77	0.6	0.85
Goat MG 11/12/71	0.72	1.8	2.50
Goat MG 1/19/72	1.20	1.0	0.83
Goat MG 2/25/72	0.88	0.7	0.79
Goat MG 8/1/72	1.65	1.8	1.10
Cow MG 6/28/72	0.31	2.2	7.00
Cow MG 6/13/72	0.72	6.3	8.00
Cow MG 10/29/71	1.44	11.0	7.60
Cow MG 8/30/71	3.10	16.2	5.20

Table II. Effect of Neuraminidase on the Electrophoretic Mobility of Acetyl-CoA Synthetase

Incubation period, hr	Mobility rel to control
0.5	0.80
3	0.84
5	0.76
11	0.64

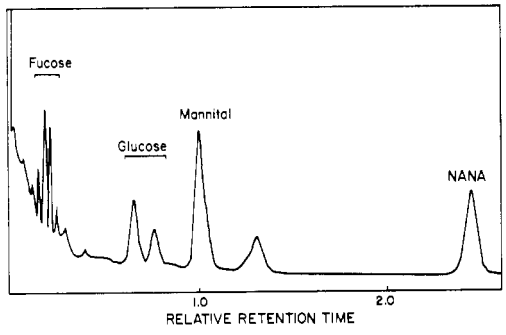

Figure 1. Gas chromatogram of the Me_3Si derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase at 160°.

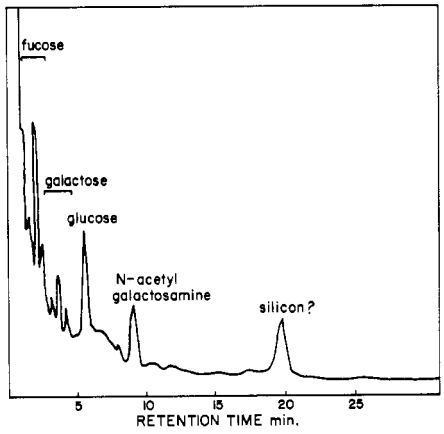

Figure 2. Gas chromatogram of the Me_3Si derivatives of methyl glycosides prepared from goat mammary gland acetyl-CoA synthetase at 190°.

Table III. Relative Retention Times of Standard Me_3Si Derivatives of Methyl Glycosides Chromatographed at 160°

Me_3Si deriv. of Me glycoside of	Rel retention time
L-Fucose	0.16
	0.18
	0.20
D-Mannose	0.43
	0.51
D-Galactose	0.53
	0.62
D-Glucose	0.66
	0.76
Mannitol	1.00
N-Acetylgalactosamine	1.30
	1.50
N-Acetylglucosamine	1.70
N-Acetylneuraminic acid	2.70

[a] Time of reference peak, 25 ± 2 min. Column used was 3% OV-1.

Table IV. Relative Retention Times of Standard Me_3Si Derivatives of Methyl Glycosides Chromatographed at 190°[a]

Me_3Si deriv. of Me glycoside of	Rel retention time
L-Fucose	0.23
	0.28
	0.34
D-Mannose	0.50
	0.57
D-Galactose	0.50
	0.57
	0.64
D-Glucose	0.74
	0.80
Mannitol	1.00
N-Acetylgalactosamine	1.10
	1.35
N-Acetylglucosamine	1.30
	1.60
N-Acetylneuraminic acid	5.50

[a] Time of reference peak, 8.1 ± 2 min. Column used was 3% OV-1.

from 160 to 240° with 5°/min increases. The protein samples were prepared as described for the GLC analysis.

Neuraminidase Treatment. Neuraminidase modified acetyl-CoA synthetase was prepared by adding 5 μl (0.02 unit) of neuraminidase solution to the protein samples (0.5 mg of protein in 1.0 ml of acetate buffer (pH 5.5), 0.10 *M* CH_3COOK, 0.02 *M* $CaCl_2$, and 0.28 *M* KCl). The mixture was incubated at 37°. Serial samples were removed from the incubation mixture and stored frozen until examined by polyacrylamide gel electrophoresis.

RESULTS

The periodic acid Schiff (PAS) staining method to detect glycoproteins following electrophoresis on polyacrylamide gel gave positive results for both the cow and goat enzyme. Bovine serum albumin and ovalbumin were used as positive controls for the PAS staining and chymotrypsinogen and lactaglobulin were used as negative controls.

Neuraminic acid was determined in the cow and goat

Table V. Gas-Liquid Chromatography of the Me_3Si Derivatives of Methyl Glycosides Prepared from Acetyl-CoA Synthetase[a]

Monosaccharide found	Goat enzyme rel retention of peaks at 190°	Cow enzyme rel retention of peaks at 190°	Cow enzyme rel retention of peaks at 160°
D-Fucose	0.28	0.22	0.16
	0.33	0.25	0.19
		0.28	0.22
D-Galactose	0.48		
	0.57		
	0.64		
D-Glucose	0.83	0.73	0.66
		0.80	0.76
Mannitol	1.00	1.00	1.00
N-Acetylgalactosamine	1.35	1.10	1.30
N-Acetylneuraminic acid			2.40

[a] The retention times of the derivatives relative to mannitol were determined at 190° for the goat enzyme and at 160° and 190° for the cow enzyme.

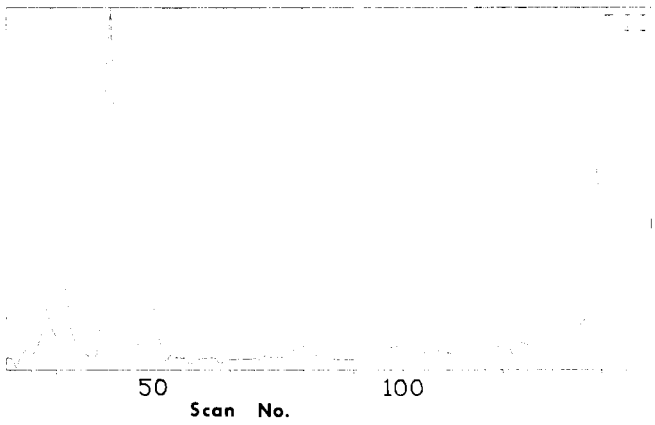

Figure 3. The gas chromatogram of the Me_3Si derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase that was used for the mass spectral analysis.

enzyme using the thiobarbituric acid assay (Table I). The cow enzyme contained 0.7% neuraminic acid and the goat enzyme contained 0.1%. Neuraminic acid forms formylpyruvic acid upon periodate oxidation. Formylpyruvic acid then reacts with 2-thiobarbituric acid to yield a strongly chromagenic compound with an absorption maxima at 549 nm. The chromagen is destroyed upon the addition of strong alkali in contrast to the chromagen formed by the product of oxidation of polyunsaturated fatty acids and 2-deoxy sugars. Strong alkali treatment of the samples destroyed the color within a few minutes. Consequently, it can be concluded that the color development was due only to neuraminic acid in the protein.

When the protein was either hydrolyzed with 0.1 *N* sulfuric acid or treated with neuraminidase there was a decrease in anodic migration when examined using polyacrylamide gel electrophoresis. The effects of neuraminidase treatment are shown in Table II. This decrease in anodal migration is evidence that covalently linked neuraminic acid is present in a terminal position.

The carbohydrates present in both the cow and goat enzyme were identified using GLC. Typical chromatograms are presented in Figures 1 and 2. In Tables III-V are presented the retention times relative to mannitol of the Me_3Si derivatives of the methyl glycosides used as standards and the Me_3Si derivatives of methyl glycosides obtained from the cow and the goat enzymes. A comparison of the relative retention times shows the presence in the cow enzyme of fucose, glucose, and *N*-acetylneuraminic acid. The compound that emerges immediately after mannitol corresponds to the minor peaks from *N*-acetylgalactosamine. However, it cannot be concluded that this compound is *N*-acetylgalactosamine because the major peak is not present.

The carbohydrates present in the goat enzyme were fucose, glucose, galactose, and *N*-acetylgalactosamine. *N*-Acetylneuraminic acid was not detected using GLC. However, both the thiobarbituric acid assay and neuraminidase treatment were positive for this compound. The Me_3Si derivative of *N*-acetylneuraminic acid is known to be unstable if stored for 1 or 2 days. The sample from the goat enzyme was analyzed for *N*-acetylneuraminic acid by GLC about 24 hr after the Me_3Si derivatives were prepared. This may explain why the compound was not detected using GLC. The last peak (Figure 2) from the goat enzyme does not correspond to any of the standards and mass spectrometry shows a fragmentation pattern characteristic of silicon.

The data from the GLC analysis were confirmed using mass spectrometry. All the peaks observed by GLC analysis of the Me_3Si derivatives from both the cow and goat enzyme gave fragmentation patterns characteristic of the pyranoside ring of the sugars (DeJongh et al., 1969). Figure 3 shows the gas chromatogram of the cow enzyme that was used for mass spectral analysis. Figures 4 and 5 show the fragmentation patterns from scans 33 and 99 of Figure 3. The mass spectrum of scan 33 contains fragments at *m/e* 73, 103, 117, 129, 133, 147, 191, 204, 217, 305, 361, and 407. All of these fragments are characteristic of methyl 2,3,4,6-tetra-*O*-trimethylsilyl-D-glucopyranoside. Scan 99 corresponds to the mass spectrum of the Me_3Si derivative of *N*-acetylneuraminic acid (methyl labeled at the carboxyl group, Figure 6).

It is concluded that the cow enzyme contains fucose, glucose, and *N*-acetylneuraminic acid. The goat enzyme contains fucose, galactose, glucose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid.

DISCUSSION

Fatty acid activation in mammalian cells has generally been considered to be catalyzed by three different acyl-CoA synthetases. Acetyl-CoA synthetase activates acetate and propionate, a medium-chain acyl-CoA synthetase activates C_4 to C_{12} fatty acids, and a long-chain acyl-CoA synthetase activates C_8 to C_{20} fatty acids. Our earlier studies of short-chain fatty acid activation by ruminant tissues indicated that two different enzymes may activate acetate and propionate (Cook et al., 1969). For example, ruminant liver preferentially activates C_3 to C_8 straight fatty acids. However, ruminant kidney readily activates C_2 to C_8 fatty acids. Ruminant lung preferentially activates propionate and not acetate. Rumen epithelium preferentially activates butyrate and not acetate. Only three ruminant tissues, heart, mammary gland, and testis, activate just acetate and propionate. Yet, the acyl-CoA synthetase in ruminant fetal heart activates propionate but not acetate. The young calf heart activates propionate but does not readily activate acetate. The ability of ruminant heart to activate acetate develops as the animal becomes a ruminant (Marinez and Cook, 1972).

These observations suggest to us that acetyl-CoA synthetase actually exists as more than one molecular species. These multiple forms could differ in substrate specificity. Different forms could predominate in different ruminant tissues resulting in the differences in substrate

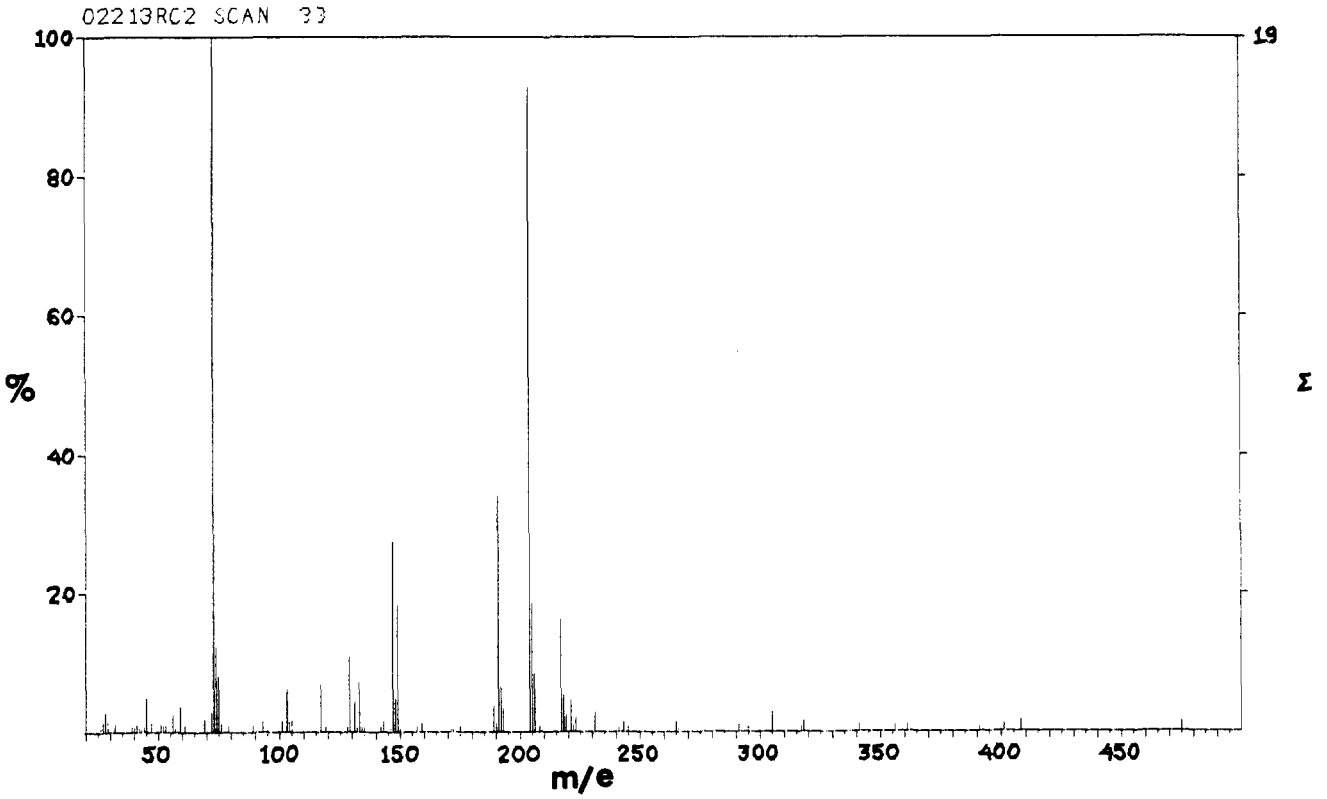

Figure 4. The mass spectrum from scan 33 (Figure 3) of the Me_3Si derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

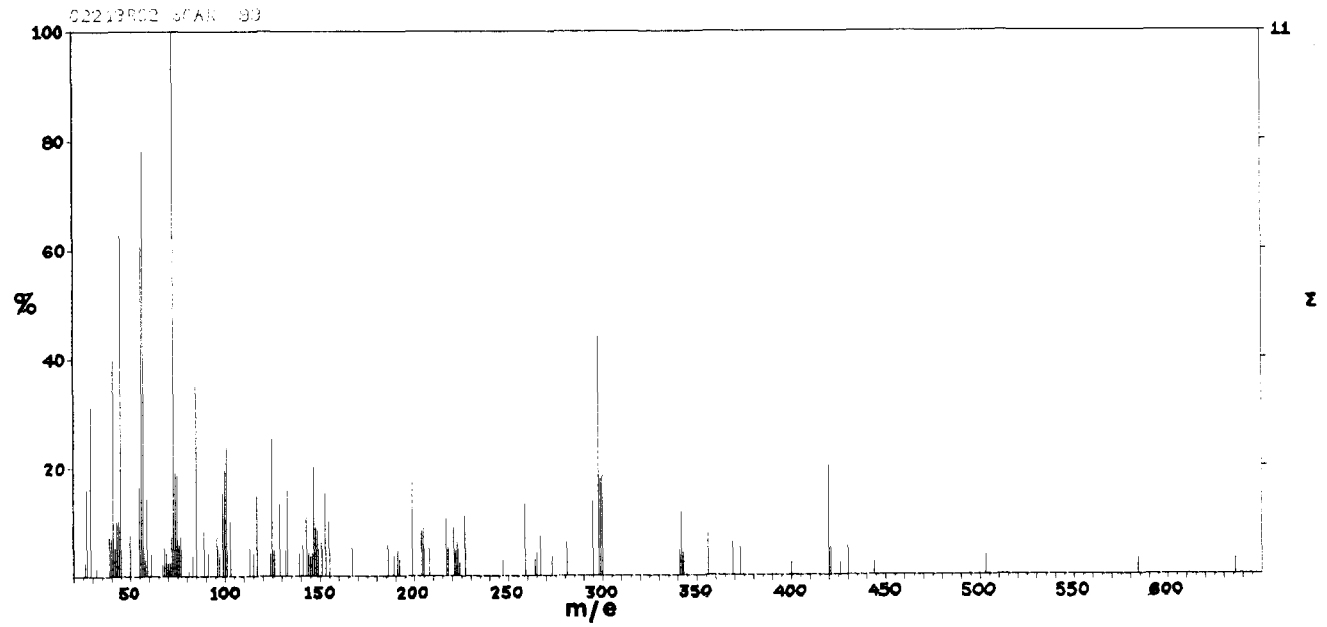

Figure 5. The mass spectrum from scan 99 (Figure 3) of the Me_3Si derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

specificity observed between ruminant tissues. Huang and Stumpf (1970) reported that acetyl-CoA synthetase from potato tuber was made up of isozymes. DeVincenzi and Klein (1970) reported that yeast acetyl-CoA synthetase consisted of isozymes. Our work on purification of acetyl-CoA synthetase from ruminant mammary gland has provided evidence that the enzyme exists as more than one molecular form (Qureshi and Cook, 1975).

The discovery that acetyl-CoA synthetase is a glycoprotein provides an explanation for the observations above. The short-chain fatty acid activating enzymes apparently exist in multimolecular forms, differing only in the carbohydrate portion of the molecule which may determine substrate specificity.

Hence, different forms of the glycoenzyme predominate in the different tissues. For example, one form may predominate in lung that preferentially activates propionate. This may be the same glycoenzyme in fetal heart that preferentially activates propionate. Yet, one or more glycoenzymes may be present in liver that preferentially ac-

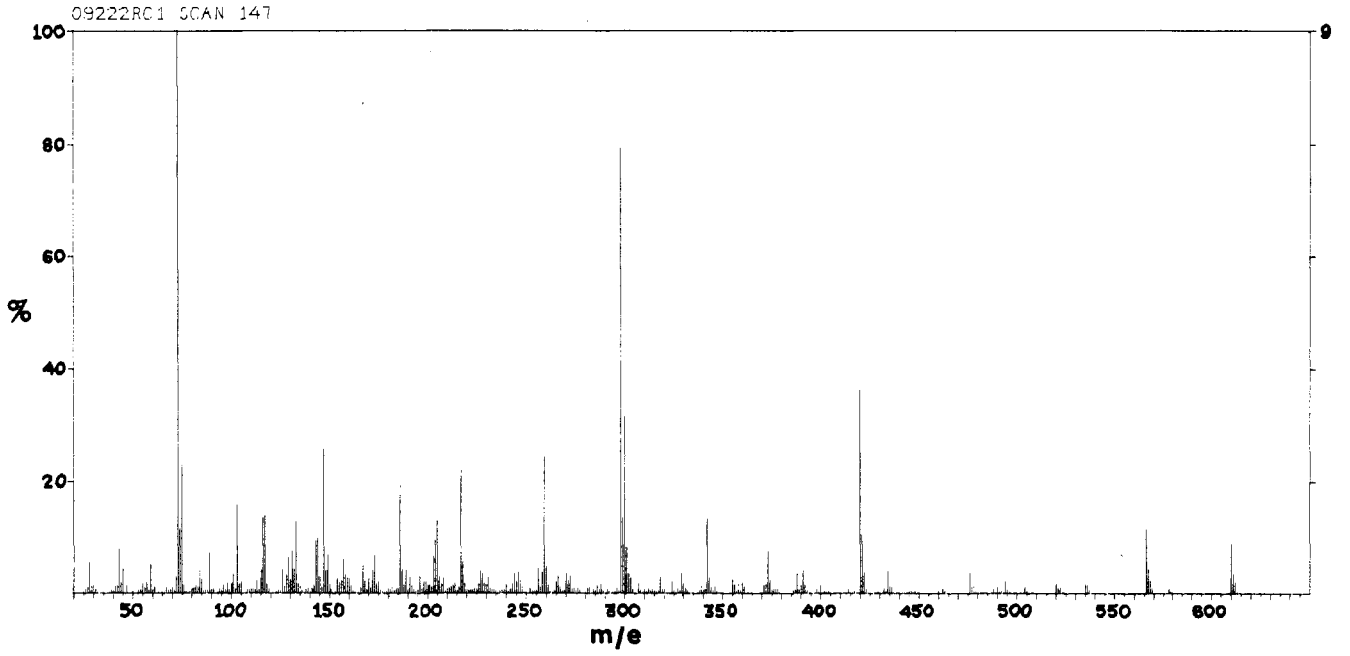

Figure 6. The mass spectrum of the Me_3Si derivative of the methyl glycoside of *N*-acetylneuraminic acid.

tivates C_3 to C_8 fatty acids. The glycoenzyme that activates acetate is absent in ruminant liver but present in ruminant kidney because the latter tissue readily activates acetate as well as C_3 to C_8 fatty acids (Cook et al., 1969).

An example of the effects of carbohydrates on the properties of enzymes is shown by ribonuclease A and ribonuclease B from bovine pancreas (Plummer and Hirs, 1963). Ribonuclease B contains an appreciable proportion of carbohydrates whereas the A form does not. The two enzymes possess similar catalytic properties and the same amino acid composition. The differences in their electrophoretic and chromatographic properties are due to carbohydrates.

Carbohydrates have a significant effect on several physical properties of glycoproteins. The intrinsic viscosity, frictional ratio, diffusion coefficient, and solubility are all affected by the presence of carbohydrates (Jackson and Hirs, 1970). Through their effects on various physical properties it is very likely that carbohydrates affect the catalytic activity of acetyl-CoA synthetase in the ruminant.

Although the significance of carbohydrates in acetyl-CoA synthetase must remain open to conjecture at this point, the knowledge our laboratory has developed continues to point to this enzyme as being important in regulating the utilization of acetate and propionate by ruminant tissues.

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LITERATURE CITED

Chrambach, A., Reisfield, R. A., Wyckoff, M., Zaccari, J. J., *J. Anal. Biochem.* **20,** 150 (1967).
Cook, R. M., Liu, S.-C. C., Quraishi, S., *Biochemistry* **8,** 2966 (1969).
Cook, R. M., Simon, S., Ricks, C. A., part VI of the series, in this issue.
Davis, B. J., *Ann. N.Y. Acad. Sci.* **121,** 404 (1964).
DeJongh, D. C., Radford, T., Hubour, J. D., Hanessian, S., Bieber, M., Dawson, G., Sweeley, C. C., *J. Am. Chem. Soc.* **91,** 1728 (1969).
DeVincenzi, D. L., Klein, H. P., *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **27,** 872 (1970).
Hotchkiss, R. D., *Arch. Biochem. Biophys.* **16,** 131 (1970).
Huang, K. P., Stumpf, P. K., *Arch. Biochim. Biophys.* **140,** 158 (1970).
Jackson, R. L., Hirs, C. H. W., *J. Biol. Chem.* **245,** 624 (1970).
Kaschnitz, R., Peterlik, M., Weiss, H., *Anal. Biochem.* **30,** 148 (1969).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
Marinez, D. I., Cook, R. M., Proceeding of the 9th International Congress on Nutrition, Mexico City, Mexico, 1972.
Plummer, T. H., Hirs, C. H. W., *J. Biol. Chem.* **283,** 1399 (1963).
Quraishi, S., Cook, R. M., *J. Agric. Food Chem.* **20,** 91 (1972).
Qureshi, S., Cook, R. M., part V of the series, in this issue.
Sweeley, C. C., Laine, R. A., Esselman, W. J., *Methods Enzymol.* **28,** 159 (1972).
Warren, L., *J. Biol. Chem.* **234,** 1971 (1959).

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