ACETYL-CoA SYNTHETASE
A GLYCOPROTEIN

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
VASSILIKI STAMOUDIS
1973

ABSTRACT

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By

Vassiliki Stamoudis

The acetate activation reaction catalyzed by acetyl-CoA synthetase is considered to be an important rate-limiting step in acetate utilization by ruminant tissues. In order to study this reaction further acetyl-CoA synthetase was purified from cow and goat mammary gland mitochondria. During these studies the apparent aggregation phenomena and difficulties in purification suggested that the enzymes might be glycoproteins. Consequently, the enzymes were tested for the presence of carbohydrates. Polyacrylamide gel electrophoresis followed by PAS staining was positive. Sulfuric acid hydrolysis or neuraminidase treatment gave a difference in anodic migration, suggesting the presence of N-acetyl-neuraminic acid. The thiobarbituric acid test for N-acetyl neuraminic acid was positive for both cow and goat acetyl-CoA synthetase. GLC analysis showed that the cow enzyme contains fucose, glucose and N-acetyl neuraminic acid. However, the goat enzyme contained fucose, galactose, glucose and N-acetylgalactosamine. The presence of N-acetyl neuraminic acid, in the goat enzyme, was not detected using GLC. 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. Previous work 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 non-lactating mammary gland but becomes active after parturition. The activity 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.

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Vassiliki Stamoudis

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1973

To

Xenia and Vassilis

ACKNOWLEDGMENTS

I wish to express my gratitude to my academic advisors, Professors Robert M. Cook and John C. Speck for their helpful guidance advice and encouragement during these studies.

I also wish to thank Dr. W. Wells, Dr. H. Knull, Dr. W. Esselman, Dr. D. Marinez and my husband Vassilis for helpful discussions.

The technical assistance provided by Rosemary Parker, Lorene Scholtz and Jack Harten is greatly appreciated.

The financial support provided by Dr. R. M. Cook is also gratefully acknowledged.

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INTRODUCTION

Acetate is an important source of energy in ruminants and plays a major role in lipogenesis comparable to the one played by glucose in non-ruminants. This is because the activity of ATP-citrate lyase is negligible in ruminants. Therefore, glucose can only supply limited amounts of acetyl-CoA for fatty acid synthesis. It is important to understand major factors that control the utilization of acetate by ruminants tissues. Before being utilized by the cell acetate must be activated (covalently linked to the thiol group of coenzyme A). The acetate activation reaction catalyzed by acetyl-CoA synthetase [acetate: CoA ligase (AMP) (6.2.1.1)], according to the following reaction is considered to be an important rate limiting step in acetate utilization by ruminant tissues(2,3).

$$CH_3COOH + ATP + CoA\text{-}SH \overset{Mg^{++}}{\rightleftharpoons} CH_3COSCoA + AMP + P\text{-}Pi$$

The distribution of acetyl-CoA synthetase in several ruminant tissues has been established (2,3). The enzyme is localized predominantly in the cytoplasm in heart and mammary gland and is almost equally divided between mitochondria and cytoplasm in kidney. However, the enzyme is localized predominantly in the mitochondria in lung and in liver (1). Acetyl-CoA synthetase is not very active in the liver

cytoplasm but it is known that ruminant liver uses mostly propionate and butyrate as major precursor for lipid synthesis. The enzyme activity is low also in rumen epithelium and in lung.

Acetyl-CoA synthetase has been generally shown to be active on acetate and propionate. In bovine fetal tissue the enzyme preferentially activates propionate rather than acetate, a situation in contrast to the adult enzyme (4). Another interesting observation is that acetyl-CoA synthetase is not active in non-lactating mammary gland. The enzyme becomes active at parturition in goats and cows and the activity decreases as the lactation progresses (4). The enzyme from bovine mammary gland has been extensively purified (4). The data indicates that acetyl-CoA synthetase exists in multiple molecular forms. The nature of these forms is not clearly understood. Isozymes might exist or there may be aggregated forms of the enzyme. Huang and Stumph (17), report 5 isozymes of acetyl-CoA synthetase in potato tuber, all with similar kinetic properties. Multiple forms of the enzyme were also reported in yeast by DeVincenzi (18). Activity that emerges as a single peak from a DE-52 cellulose column separates into four protein peaks using a calcium phosphate column and different ionic strengths of phosphate buffer. Two of the four protein peaks had enzyme activity. Each of the two protein peaks upon gel electrophoresis gives 4 bands, 2 of which are found to be associated with enzymatic activity. This data suggests the existence of isozymes. Also the fact that the fetal heart enzyme activates propionate but not acetate in contrast to the adult enzyme, can be explained on the basis of isozymes. That is isozymes exist in fetal tissues that preferentially activate propionate whereas an isozyme in adult tissue predominates that activates acetate.

On the other hand, much of the data suggests that the enzyme aggregates to yield multiple forms. Acetyl-CoA synthetase activity decreases in concentrated solutions perhaps as the extent of aggregation increases. The presence of ammonium sulfate known to prevent aggregation has a beneficiary effect on the stability of the enzyme.

Sedimentation equilibrium studies indicate the presence of at least 2 molecular species one with a molecular weight of 84,500 and one with a molecular weight of 49,000. This higher value could represent a dimer form. However, the molecular weight value of the enzyme from sucrose density gradient centrifugation is estimated to be 62,000 (4). The minimum molecular weight reported for acetyl-CoA synthetase from bovine heart mitochondria is 31,000 to 34,000 (47). If it is assumed that the enzymes from both tissues have similar molecular weight, then the mammary gland enzyme could possibly have been aggregated to a dimer form under the conditions of the experiment to give a value to 62,000.

It is known today that some glycoproteins exist as isoglycoenzymes. These isozymes differ only in carbohydrate content. Also it is known that the presence of a small molecule such as sugar, especially when it is charged, can contribute to interactions between subunits.

The thought that the enzyme might be a glycoprotein and that this could explain much of the data already known, led to these studies.

The function at the molecular level of the carbohydrate residues in glycoproteins fall into two categories, biological

and structural. One widely accepted role for carbohydrate residues, is in the transport of glycoproteins through cellular membranes (5). It is believed that sugar is added to many polypeptide chains to facilitate export of the protein from the cell. A large number of extracellular proteins are glycoproteins. Carbohydrates may complex with receptors and other carrier substances in the cellular membrane. This may result in conformational changes allowing the passage of the macromolecule.

The catabolism of some of the serum glycoproteins and hormones may be regulated through their carbohydrate moieties. Removal of sialic acid from orosomucoid, fetuin ceruloplasmin, haptoglobin, human chorionic gonadotropin, follicle stimulating hormone, led in each case to the production of materials which after injection into rats, were removed from the circulation much more rapidly than were the original glycoproteins (6).

In this process neuraminic acid (7) and D-galactose residues (8) appear to be important. Removal of sialic acid leaves galactose in non-reducing terminal position and it is these galactose residues which are involved in that recognition process, which leads to the removal of the molecules from the circulation.

Glycoproteins secreted by the cell often function as antigenic substances (9). But the carbohydrate component appears to play only a minor role in the immunological process (10).

The level of plasma protein bound carbohydrate is elevated in a number of pathological states, but the biological significance of this interesting phenomenon is still not clear. Carbohydrates have

a significant effect on several of the physical properties of the glycoproteins. The intrinsic viscosity, frictional ratio, diffusion coefficient and solubility are all affected by the presence of carbohydrates. Changes in these properties have been noted, especially for porcine ribonuclease (11).

A protective role of the carbohydrate residues is shown by the fact that glycoproteins are resistant to hydrolysis by proteolytic enzymes (12, 13), and they are remarkably stable on storage and at elevated temperatures (14, 15). Carbohydrates function as stabilizers of the tridimensional structure of proteins (14) especially for enzymes having a molecular architecture similar to that of glycoamylase. Here many carbohydrate side-chains are present on the surface of the molecule, positioned in such a way as to minimize molecular transformations. This is the case for pepsinogen (16).

Glycoenzymes often occur in multimolecular forms and evidence is accumulating that such forms are isoglycoenzymes, differing only in the carbohydrate portion of the molecules. Examples are: ribonucleases A and B from bovine pancreas (19). Ribonuclease B contains an appreciable proportion of carbohydrate whereas the A form does not. The two enzymes possess identical catalytic properties and they have the same amino acid composition. The differences in their electrophoretic and chromatographic properties are apparently due to the presence of carbohydrate in one of them.

The monosacharides most often found in glycoproteins are D-mannose and 2-acetamido-2-deoxy-D-glucose. Also D-glucose, D-galactose, D-xylose, L-arabinose, L-fucose and sialic acid are often present. Carbohydrates are attached to the protein by two types of

linkages, the N-glycolyl and the O-glycosyl. The N-glycosyl linkage occurs between the reducing end of the carbohydrate chain and an L-asparagine residue of the protein, whereas the O-glycosyl linkage is found between the reducing end of the carbohydrate chain and the hydroxy group of a serine or threonine residue in the protein. There is evidence showing that the attachment of the bridge carbohydrate residues to the polypeptide chain, occurs while the chain is still attached to the ribosomes (22, 23, 24). Several of the bridge carbohydrates have been identified and some glycosyl transferases have been purified.

For example, 2-acetamido-2-deoxy-D-glucose is one of the bridge carbohydrate residues of glycoproteins. Nucleotidyl transferases are responsible for the activation and transfer of the hexosamine to the polypeptide chain. Other glycosyl transferases are responsible for attachment of carbohydrate moieties to hexosamine (26, 27). For glycoenzymes containing D-mannose and D-xylose as bridge carbohydrates the appropriate enzymes for formation of GDP-mannose and UDP-xylose have been purified (27, 28).

Table 1 gives the carbohydrate components of some glycoproteins (29). It can be seen that the carbohydrate content varies widely from one protein to another. Table 2 gives the structures of carbohydrate moieties of some glycoproteins (30). The asparagine involvement for the N-glycolyl linkage is clearly shown. N-acetylglucosanine is very often the first carbohydrate attached to the polypeptide chain and sialic acid usually is at a terminal position.

This study was conducted to determine whether or not acetyl-CoA synthetase is a glycoprotein.

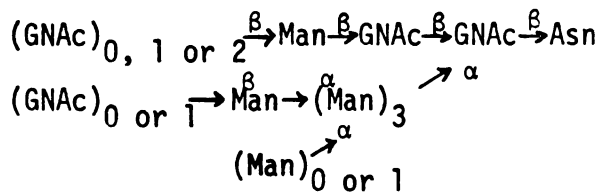
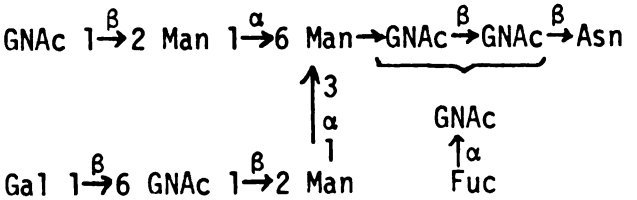
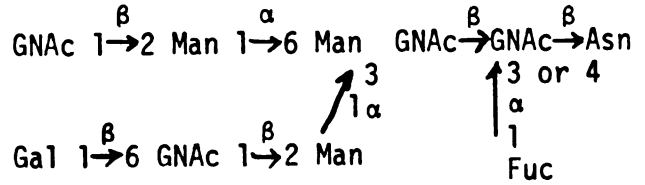
TABLE 1*: The Carbohydrate Components of Some Glycoproteins

					No. of residues per molecule of protein, or percentages									
No.	Class	Protein	Source	Mol. wt.	Man	Gal	Fuc	G	Total Neutral Sugars	GN	GalN	Total Hexo-samine	Sialic Acid	Total Sugar Residues
1	Enzymes	ribonuclease B	ox pancreas	14,700	5	-	-	-	5	2	-	2	-	7
2		deoxyribonu-clease B	ox pancreas	31,000	5	-	-	-	3	2	-	2	-	7
3		proteinase b	snake venom	95,000	10	30	tr	-	42	34	-	34	9	85
4		NADase	Neurospora crassa	36,500	68	66	-	32	136	n.d.	n.d.	4	-	140
5	Hormones	luteinizing	sheep pituitary gland	16,300	4	1	1	-	6	5	2	7	0.25	13
6		follicle-stimulating	pig pituitary gland	30,000	2.3	2.0	3.0	-	7.3	n.d.	n.d.	6.2	n.d.	13.5
7	Immuno-globulins	γG	rabbit serum	140,000	5.8	2.9	1.0	-	10	8	+	8	1.1	19
8		γG	human serum	140,000	5	3	2.0	-	10	9	-	9	1	20
9		γA	human serum	140,000	14	14	2	-	28	n.d.	n.d.	21	9	58
10		γM	human serum	1,000,000	+	+	49		270	n.d.	n.d.	212	59	541
11		MOPC 46	mouse urine (BenceJones)	23,000	2	3	tr		5.8	3.6	-	3.6	0.7	10
12		MOPC 70	"	23,000	n.d.	n.d.	n.d.	n.d.	0.85	n.d.	n.d.	0.75	0.1	1.7
13		MOPC 195	mouse serum (γG-Be2)	140,000	+	+	+	-	11.2	n.d.	n.d.	7.1	0	18
14		MOPC 172	"	140,000	+	+	+	-	1.9	n.d.	n.d.	3.6	0	6
15	Structural proteins	aorta GPI	ox aorta	60,000	+	+	1.8	1.1	17	11	-	11	3.6	33
16		collagen	dog Achilles tendon		-	0.28%	-	0.4%	0.68%	n.d.	n.d.	0.04%	0.06%	0.8%

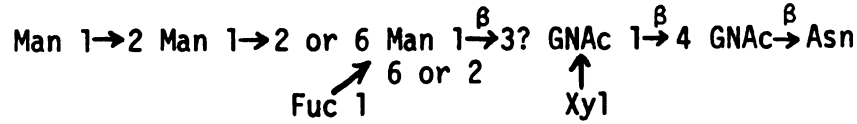
17		basement membrane	ox kidney		0.77%	3.05%	0.22%	2.47%	6.5%	1.5%	0.22%	1.7%	1.2%	9.4%	
18	Mucins	A-substance	ovarian cyst-fluid	1,000,000	-	950	1,100	-	2,050	980	1,090	2,070	72	4,120	
19		gastric GP	human gastric juice				17.3%		51.4%			27.1%	2.5%	83.6%	
20		submaxillary GP	sheep sub-maxillary gland	1,000,000	8	17	24	-	49	tr	800	800	800	1,600	
21	Plant	glycoprotein I	kidney beans		7.8%	-	-	-	11.0%	2.7%	-	2.7%	-	14%	
22		A hemagglutinin	soybeans	110,000	28	-	-	-	28	5	-	5	-	33	

*Adapted by R. D. Marshall, A. Neuberger, Adv. in Carboh. Chem. Biochem. Vol. 25, (1970).

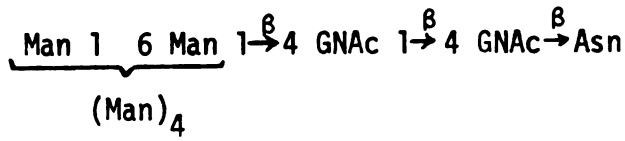
TABLE 2*: Structures of carbohydrate moieties of some glycoproteins

Glycoprotein	Structure
1. Hen egg albumin	
2. γG mycloma protein	
3. γG myeloma protein	
4. Sheep and ox submaxillary glycoproteins	

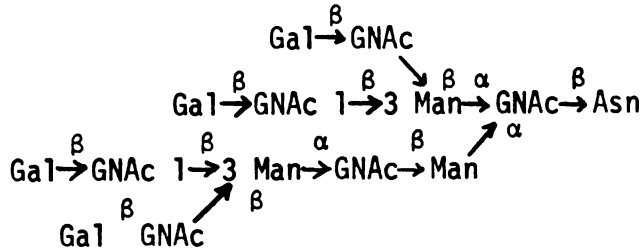
5. Pineapple stem bromelain



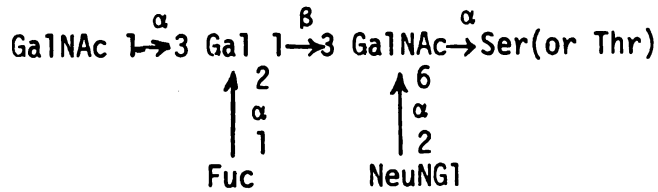
6. α-Amylase (Aspergillus oryzae)



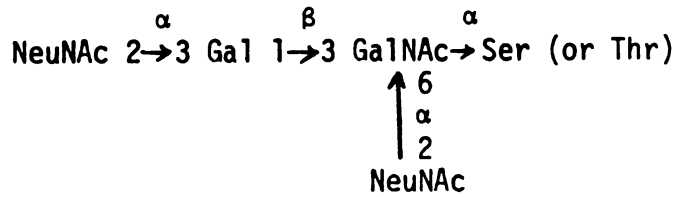
7. Desialized human orosomucoid



8. Pig submaxillary A glycoprotein



9. Human red cell MN antigen



10. "Antifreeze" glycoprotein

*Adapted from R. D. Marshall, Ann. Rev. Bioch. 1972, page 673.

EXPERIMENTAL SECTION

A. Enzyme Assay

Acetyl-coenzyme-A synthetase activity was measured by the acetate dependent disappearance of the free sulfydryl group of coenzyme A as described by Mahler et al. (41).

In a total volume of 0.20 ml the complete reaction mixture contained 5 µmoles of K-acetate, 1.1 µmoles of ATP, 1.5 µmoles of $MgCl_2$, 0.17 µmoles of CoA-SH, 16 µmoles of Tris (hydroxymethyl) amino methane hydrochloride buffer. From 25 to 4 µg of enzyme protein were used.

All tubes were preincubated for one min. at 37°. After the enzyme addition the incubation period was 5 min. at 37°. In some assays the incubation period was 10 min. The reaction was terminated by the addition of 2.8 ml of the nitroprusside color reagent prepared by the method of Grunert and Phillips (42). The optical density was read after 30 seconds at 520 mµ.

The difference in optical density between the blank and the complete reaction mixture is the measure of enzyme activity. Enzyme concentration is adjusted to give a difference in optical density between 0.075 and 0.250. Within this range ΔOD_{520} is proportional to enzyme concentration under the assay conditions. A difference of 0.185 in optical density corresponds to the disappearance of 0.10 µm of CoA. One unit of enzyme activity is defined as the amount

which catalyzes the disappearance of 1 mμmole of CoA per minute.

Specific activity is expressed in units of enzyme activity per mg of ptotein.

B. Protein determination

Protein was determined by the method of Lowry et al. (43) using bovine serum albumin (BSA) as a reference standard.

In some cases protein was determined by the absorbance at 280 and 260 μm according to the method of Warburg and Christian (44).

C. Gel Electrophoresis

Polyacrylamide gel (5% acrylamide) electrophoresis was performed by the method of Davis (45). The gel was prepared by mixing solutions A, B, C and H_2O, using a ratio of 1:2:1:4.

Solutions A contained: 2.42 g Trisma base; 12.10 g glycine; 0.23 ml temed NNN'N' tetramethyl-ethylene diamine and made up to 100 ml with H_2O. Solution B contained: 20.0 g Acrylamide; 0.735 g BIS N N'-methylene; bisacrylamide per 100 ml of H_2O. Solution C contained: 4 mg ribovlavin per 100 ml H_2O.

One end of each gel tube was sealed using parafilm. To each tube was added 1.6 ml of the above solution. The gel solution polymerized within 25 to 30 min. under a fluorescent lamp. The protein sample was made denser by adding a few crystals of sucrose and 20 to 50 μl containing 50 to 200 μg of protein were layered on the gel. 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 mAm/tube for 30'. After electrophoresis the gel columns were stained for proteins with coomassie blue according to the method of Chrombach (46).

The gels were fixed in 10% TCA for 15 min. and then put in the staining solution (0.4 g coomassie blue dye in 100 ml of 20% methanol and 10% TCA) for 12 hours. The gels were then rinsed with 33% methanol and 10% TCA for 6 hours and finally transferred to 10% TCA for 10 to 12 hours.

D. Enzyme isolation procedure (Figure 1)

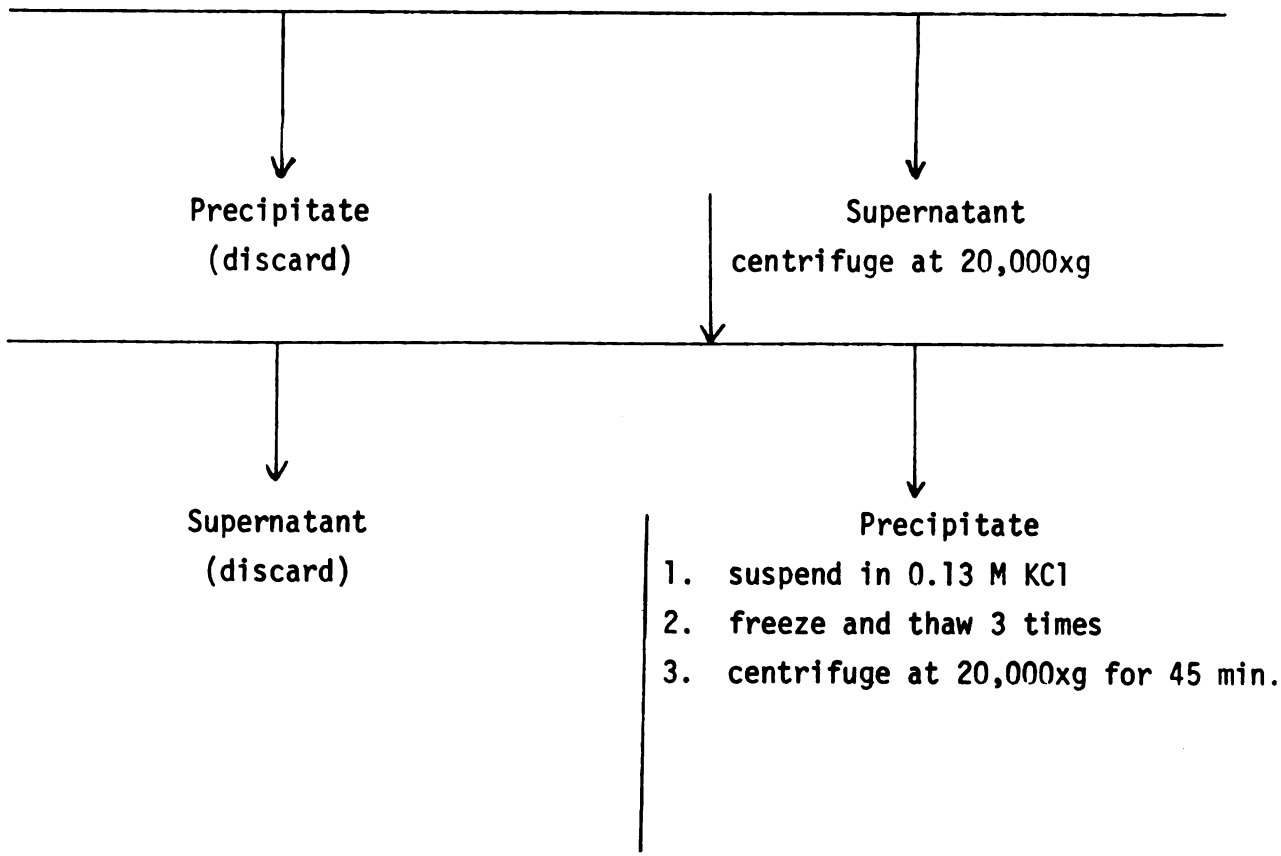
The enzyme isolation procedure used was similar to that described by Qurashi (4).

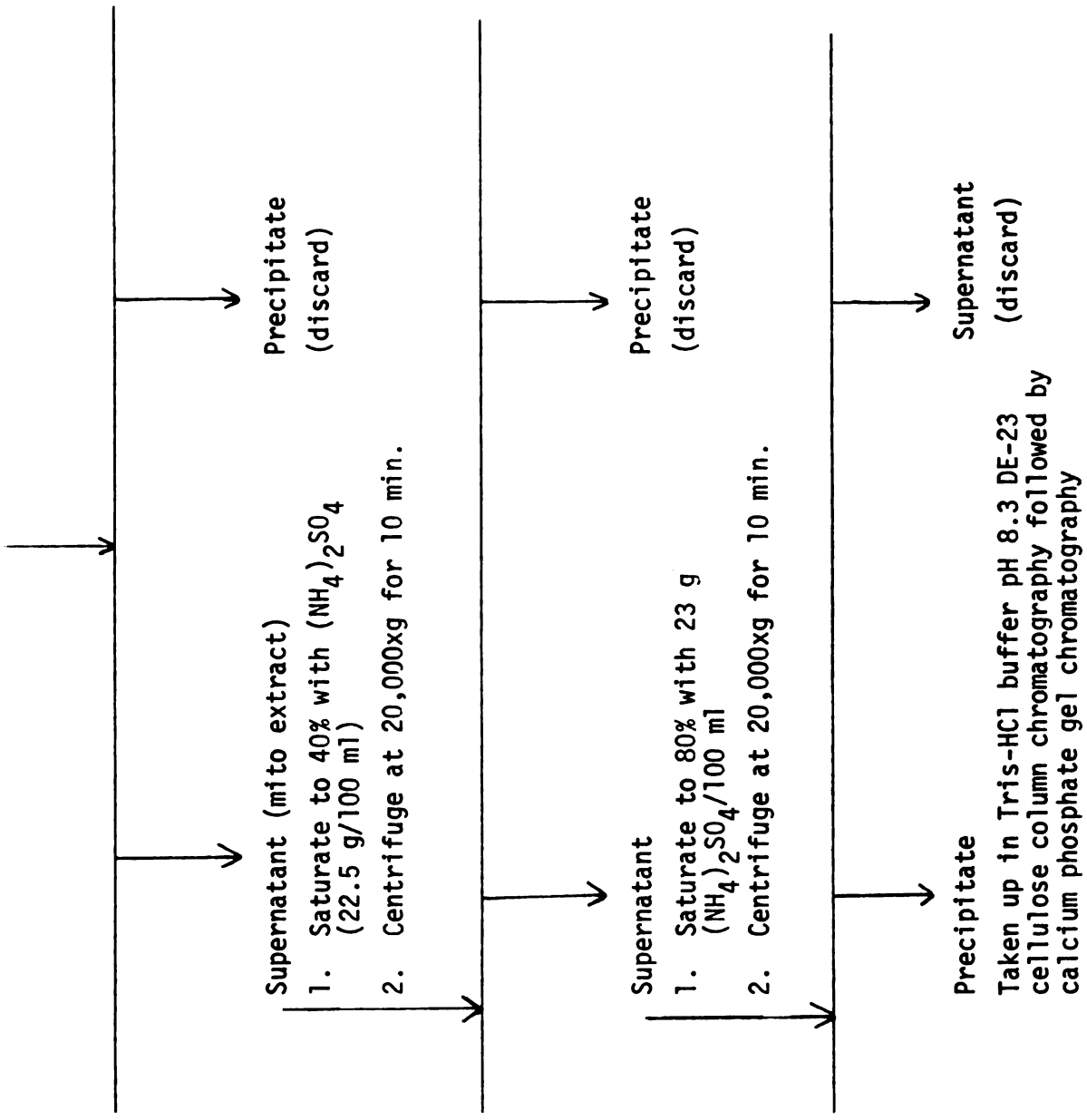
Isolation of Mitochondria: Mammary gland tissue was immediately chilled in ice at slaughter. After removal of fat and connective tissue the tissue was cut into thin long strips and ground in a meat grinder. One kg of the tissue was then homogenized in 2 liters of 0.13 M KCl, (adjusted to pH 8 with KOH), using a one gallon Waring blender at high speed for 10 sec. and then at low speed for 20 sec. The homogenate was then centrifuged in a MSE six liter centrifuge at 1200 x g for 15 min. All steps were carried out at 5°.

The 1200 x g supernatant was filtered through 8 layers of cheese cloth and centrifuged in a Sorvall RC-2B at 20,000 x g for 25 min. The top fluffy layer was discarded and the mitochondrial pellet was taken up in 0.13 M KCl (.1 g (wet weight) per 3 ml of KCl) and briefly homogenized. The resulting suspension was frozen in plastic bottles at -20°C.

Ammonium sulfate fractionation: The mitochondria were thawed rapidly by swirling in a water bath at 35°C. After each thaw the pH was adjusted to 8 with 1 N NH_4OH. This process was repeated three times. After the last thaw the mitochondrial suspension was centrifuged at

FIGURE I: Fractionation of bovine mammary gland tissue. Mammary gland tissue was homogenized in 0.13 M KCl (1 g tissue per 2 ml KCl) and the homogenate was centrifuged at 1000xg for 20 min.




Supernatant (mito extract)
1. Saturate to 40% with $(NH_4)_2SO_4$ (22.5 g/100 ml)
2. Centrifuge at 20,000xg for 10 min.
Precipitate (discard)
Supernatant
1. Saturate to 80% with 23 g $(NH_4)_2SO_4$/100 ml
2. Centrifuge at 20,000xg for 10 min.
Precipitate (discard)
Precipitate
Taken up in Tris-HCl buffer pH 8.3 DE-23 cellulose column chromatography followed by calcium phosphate gel chromatography
Supernatant (discard)

20,000 x g for 45 min. The supernatant was adjusted to pH 8 (and to 0.1 M in 2-mercaptoethanol).

To the fraction thus obtained, the mitochondrial extract, 21 g of ammonium sulfate per 100 ml were added slowly and with stirring. The solution was adjusted to pH 8 with 1 N NH_4OH, stirred for one hour and then centrifuged at 20,000 x g for 10 min. An additional 23.5 g of ammonium sulfate were added to each 100 ml of the supernatant. The precipitate obtained was recentrifuged to remove excess ammonium sulfate, and was then taken up in Tris-buffer pH 8.5 and stored at -60°C.

E. DE-23 cellulose chromatography

DE-23 cellulose was washed according to Whatman's procedure (31) and then equilibrated in 0.005 M Tris-HCl buffer pH 7.5. The column dimensions were 1.7 cm x 42 cm. The flow rate was 15-20 ml/hour. The ammonium sulfate precipitate was dialyzed for 30 min., diluted to 10 mg of protein/ml with 0.005 M Tris-HCl and added to the column. The column was then washed with 140 ml of 0.005 M Tris-HCl buffer and then with 160 ml 0.01 M Tris-HCl buffer pH 7.5. The activity was eluted with 600 ml of a linear KCl gradient of 0 to 0.6 M in 0.01 M Tris-HCl buffer pH 7.5.

Six ml fractions of eluate were collected. The tubes with the highest specific enzyme activity were pooled and concentrated in a dia-flow cell. The concentrated enzyme protein was stored at -60°C.

F. Chromatography of acetyl-CoA synthetase on calcium phosphate gel

The calcium phosphate gel was made by slowly adding equal volumes of 1 M K_2HPO_4 and 1 M $CaCl_2$ to a large beaker. Mixing was accomplished

FIGURE 7: Chromatography of acetyl-CoA synthetase on DE-23 cellulose.

The column was washed first with 140 ml of 0.005 M Tris HCl buffer pH 7.5. Then with 160 ml of 0.01 N Tris HCl buffer pH 7.5. The activity was eluted with 600 ml of a linear KCl gradient of 0 to 0.6 M in 0.01 M Tris HCl buffer pH 7.5.

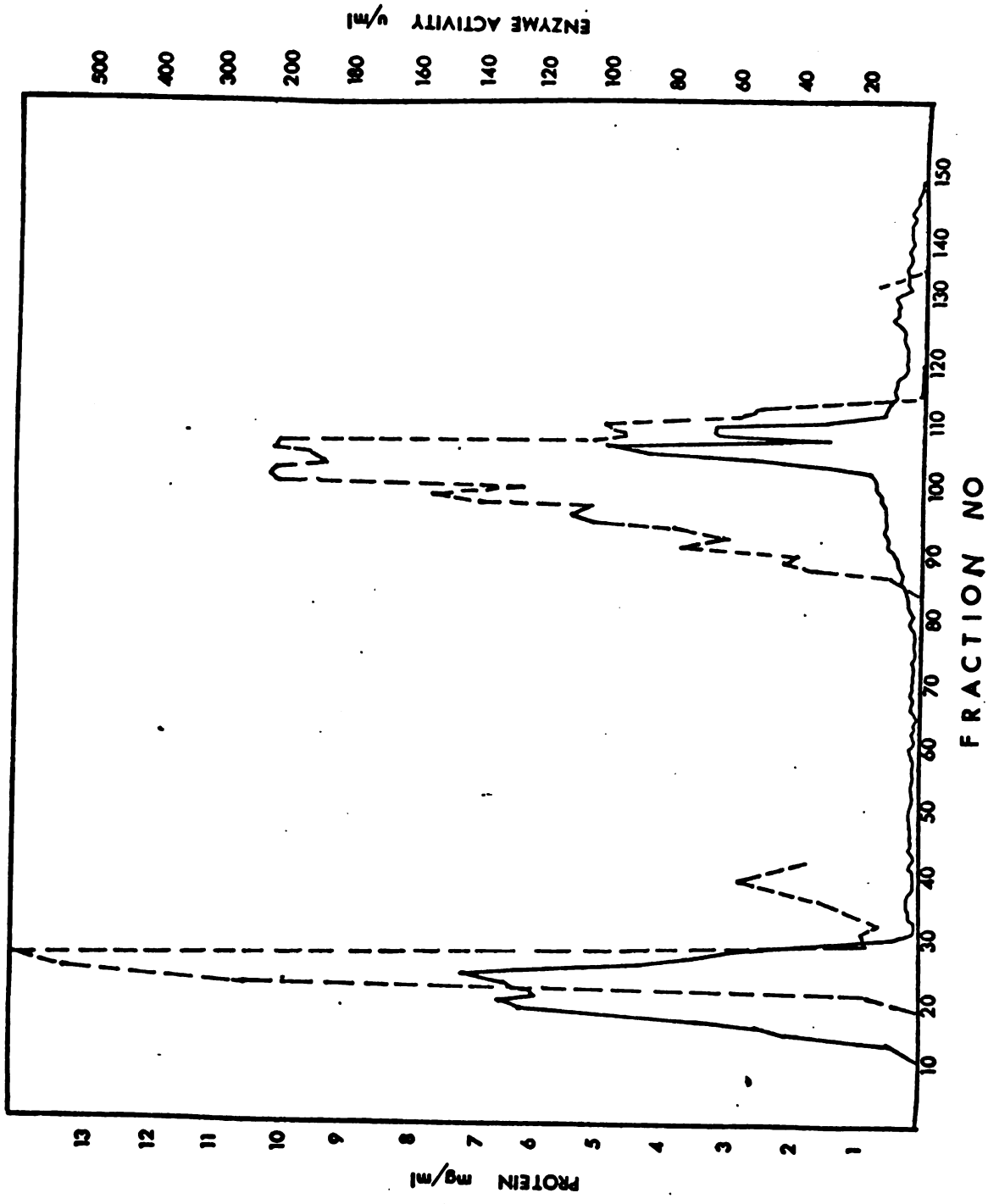

ENZYME ACTIVITY u/ml
500
400
300
200
180
160
140
120
100
80
60
40
20
FRACTION NO
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
PROTEIN mg/ml
13
12
11
10
9
8
7
6
5
4
3
2
1

FIGURE 8: Chromatography of acetyl-CoA synthetase on calcium phosphate gel.

The column was eluted with a stepwise gradient of increasing concentration of potassium phosphate buffer pH 7.0.

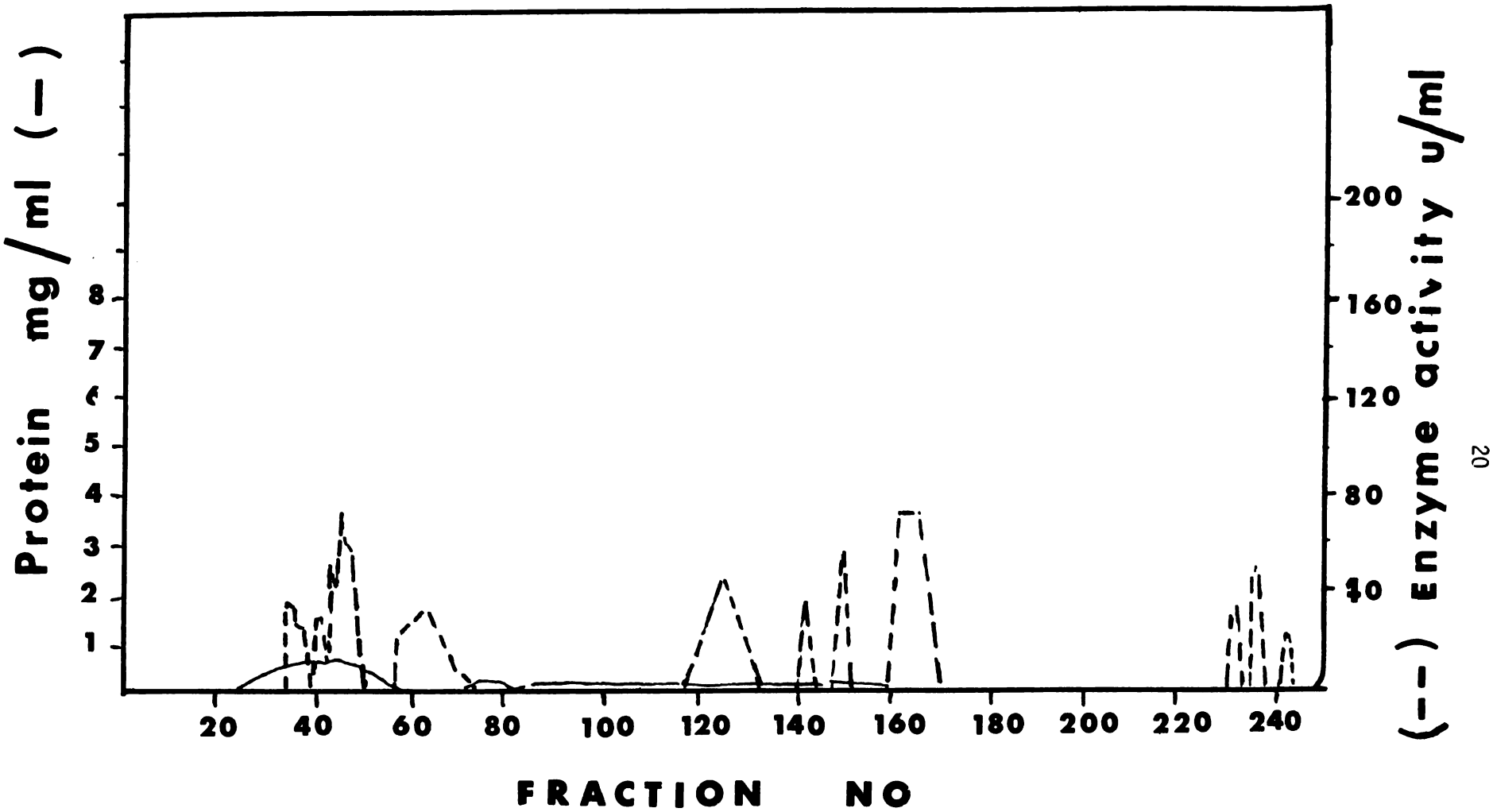

Protein mg/ml (—)
(--) Enzyme activity u/ml
FRACTION NO
1
2
3
4
5
7
8
40
80
120
160
200
20
40
60
80
100
120
140
160
180
200
220
240

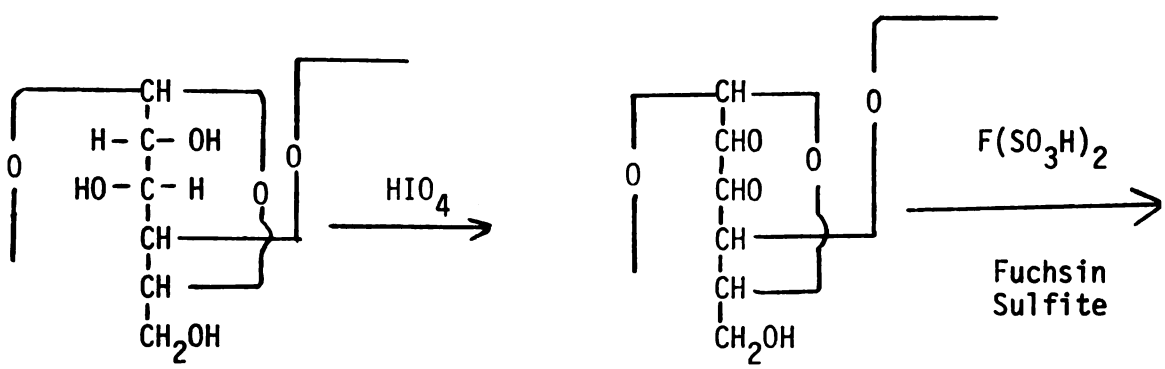
TABLE 3: Elution of the calcium phosphate column

Molarity of potassium phosphate buffer pH 7.0	Amount used for elution, mls
0.001	100
0.010	100
1.030	100
0.050	100
0.065	100
0.070	100
0.080	100
0.100	100
0.200	150
0.500	200

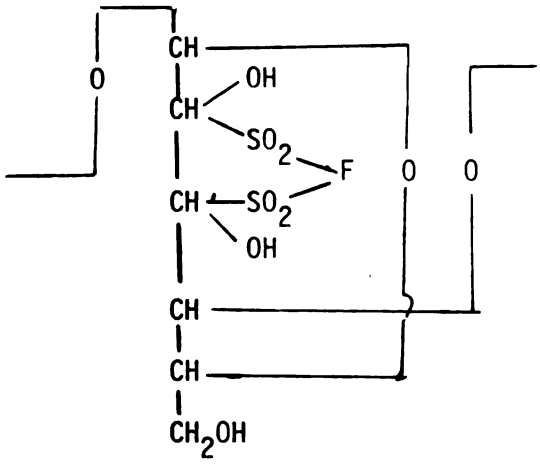
using a magnetic stirrer. About 50 mg of the protein, purified from the DE-23 column were diluted to 150 ml with 0.001 M potassium phosphate buffer. The protein solution was equilibrated for 30 to 60 min. in the cold room and then was layered on the column. The column dimensions were 3 x 12 cm. A buffer schedule of increasing concentration of potassium phosphate buffer pH 7.0 was used to elute the protein. Table 3 gives the molarity and the amount used of each buffer. The eluate was collected in 5 ml fraction.

G. Glycoprotein staining following electrophoresis on acrylamide gels

The principle of the periodic acid - Shiff (PAS) technique for detection of glycoproteins following electrophoresis on acrylamide gels is the oxidation by periodic acid of the carbohydrate components of the protein to give polyaldehydes which yield violet-red compounds with Schiff's reagent, fuchsin sulfate, according to the following scheme:



polyaldehyde



poly-substituted dye-compound

The protein samples were stained according to the procedures of Hotchkiss (34) and Kaschnitz et al. (32). The details are outlined in Table 4 and 5 respectively. Basically the two procedures differ only as to the agent used for fixation of the protein on the gel. The solutions used for the staining according to Hotchkiss (34) were prepared as follows.

Periodic acid solution: 400 mg periodic acid, dissolved in 10 cc distilled water, 5 cc of M/5 sodium acetate (equivalent to 135 mg of the hydrated crystalline salt) and 35 cc ethyl alcohol. This solution may be used for several days if protected from exposure to light.

Reducing rinse: One g potassium iodide and 1 g sodium thiosulfate pentahydrate were dissolved in 20 cc distilled water. 30 cc ethyl alcohol were added and then 0.5 cc 2N HCl. A precipitate of sulfur slowly forms and is allowed to settle out, the solution may be used immediately. (This is designed to be an iodide thiosulfate

TABLE 4: Staining of glycoproteins according to Hotchkiss (34)

Step	Gel treatment	Time interval min.
1	Bring the gel into 70% alcohol	10
2	Leave at room temperature in periodic acid solution	5
3	Flood with 70% alcohol, transfer to reducing rinse	5
4	Flood with 70% alcohol, leave in fuchsin-sulfite	15-45
5	Wash 2-3 times with SO_2-water	
6	Store in 3-5% acetic acid	

TABLE 5: Staining of glycoproteins according to R. Kaschnitz, et al. (32)

Step	Gel Treatment	Time interval min.
1	Immerse in 12.5% TCA (25-50 mg/gel)	30
2	Rinse lightly with distilled water	1
3	Immerse in 1% periodic acid (made in 3% acetic acid)	50
4	Wash 6 times for 10 min. each in 200 ml distilled water/gel with stirring or shaking or wash overnight with a few changes If 60 min. washing was used check last wash with 0.1 N $AgNO_3$ and when test is negative for IO_3^-; continue washing with 2 more changes	60 or overnight
5	Immerse in fuchsin-sulfite stain; store in the dark	50
6	Wash with freshly prepared 0.5% metabisulfite 3 times for 10 min. each (25-50 ml/gel)	30
7	Wash in distilled H_2O with frequent changes and motion until excess stain is removed	overnight
8	Store in 3 to 5% acetic acid	

solution containing the maximum amount of mineral acid compatible with the thiosulfate; when it ceases to be acidic, it should be re-acidified or replaced. Sulfite wash water was prepared by adding 0.5 ml of concentrated HCl and 0.2 g of potassium metabisulfite to 50 ml of distilled water.

The fuchsin-sulfite solution that was used for both procedures was prepared according to McGuckin and McKenzie. To 1 liter of water 8 g potassium metabisulfite and 10.5 ml of concentrated HCl were added. When solution was obtained 4 g of basic fuchsin were added and the mixture was stirred gently with a mechanical stirrer for 2 hours at room temperature. At this time the dye was in solution. After standing for 2 hours a small amount of charcoal (Darco-G-60) was added and the solution was filtered within 15 min. The resultant colorless reagent was stored at 5°C. It was stable for several months.

H. Thiobarbituric acid assay (36)

This method determines only free sialic acid, consequently when employed for the measurement of this sugar in glycoproteins sialic acid must first be released by weak acid hydrolysis or neuraminidase.

Reagents: 1) Sodium metaperiodate, 0.2 M in 9 M phosphoric acid stored in an amber glass bottle. 2) 10% sodium arsenite in a solution of 0.5 M sodium sulfate and 0.1 N H_2SO_4. 3) Thiobarbituric acid, 0.6% in 0.5 M sodium sulfate.

Procedure: Sialic acid must first be released from the protein by hydrolysis in 0.1N H_2SO_4 at 80° for 1 hour. The samples and

standards should contain 2 to 18 μg of sialic acid dissolved in 0.2 ml of water. To each tube as well as to 0.2 ml water blank, is added 0.1 ml of the periodate solution. The tubes are shaken and allowed to stand at room temperature for 20 min. One ml of the arsenite wolution is then added. The tubes are shaken until the yellow-brown color has disappeared. Then 3 ml of the triobarbituric acid solution is added to each tube, the contents are mixed by shaking, the tubes are capped with glass bulbs and heated in a vigorously boiling water bath for 15 min. They are cooled in a water bath for 5 min. Then the entire 4.3 ml of aqueous solution is extracted with 4.3 ml of cyclohexanone. The tubes are vigorously shaken and then are centrifuged. The top cyclohexanone phase is transferred to cuvettes and the optical density is determined at 549 mμ. (Table 6). Color production varies linearly with increasing concentrations of N-acetyl neuraminic acid (NANA) over the range usually used, 0.01 to 0.06 μmole (Figure 2). A strongly acidic environment is required to obtain a maximal molecular extinction coefficient. Strong acid is probably required to remove the acetyl group before oxidation. The error is considerable if the sample to be assayed is in a volume greater than 0.2 ml. This is because the acidity of the reaction mixture drops.

I. Preparation of 1 N HCl in CH_3OH

Methanol was dried by heating 500 ml with magnesium turnings (3.0 g) and iodine (0.1 g) under reflux for 2 hours. The dry methanol was then distilled using a system to exclude moisture and collected in a clean dry flask. Dry HCl gas (36.46 g per liter)

TABLE 6: N-acetyl neuraminic acid standard curve

N-acetyl neuraminic acid µg	Optical density at 549 mµ
2.5	.080
5.0	.145
7.5	.226
10.0	.258
12.5	.340
15.0	.400
17.5	.495
20.0	.530
22.5	.630
25.0	.725
30.0	.820
35.0	.900

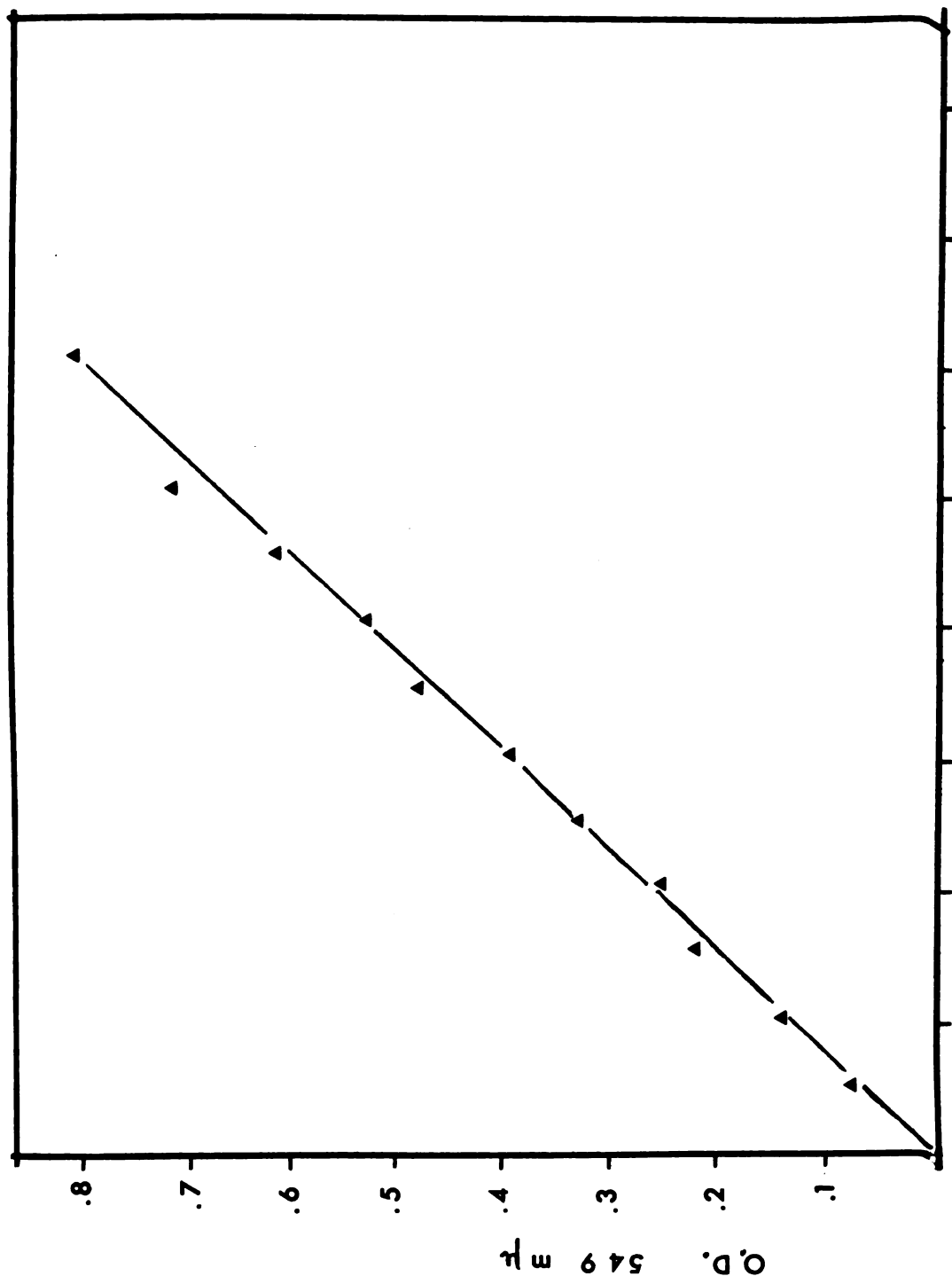


FIGURE 2 NANA STANDARD CURVE

was slowly bubbled through a dispersion tube into the methanol.

J. Methanolysis procedure and preparation of methyl glycoside standards (28, 39, 40).

The purified enzyme samples were freeze dried. Using 3, 1 ml aliquots of 1 N methanolic HCl the sample was transferred with a pasteur pipette to an open ampule. The tubes were gassed with N_2 for 30 sec., sealed and heated in an oil bath at 85°C for 4 hours. The total protein was 2 to 5 mg. L-fucose, D-mannose, D-galactose, D-glucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and N-acetyl neuraminic acid were used as control standards. The amount of each carbohydrate used was 1 to 5 mg. Mannitol was used as internal standard (0.4 mg mannitol in 0.3 ml methanolic HCl). In addition, a control mixture of sugars was prepared having 0.25 mg of each carbohydrate component and 0.4 mg mannitol. Also, a tris-buffer control and a potassium phosphate buffer control was prepared. After heating at 85° all ampules were cooled to room temperature and neutralized with powdered silver carbonate (∿0.5 g). Re-N-acetylation was performed in the same tube with 0.3 ml of acetic anhydride. The reaction was allowed to take place at room temperature for 6 hours with occasional mixing. The samples were than centrifuged and the supernatant was transferred to a plastic stoppered vial. The centrifugation step was repeated 3 times using 1 ml of methanol. The pooled supernatants were taken to dryness under a stream of nitrogen. The samples were finally dried for 12 hours in a vacuum dessicator over P_2O_5.

K. Gas liquid chromatography

The methyl glycosides were separated as the trimethylsilyl

ethers. To the material dried over P_2O_5, 0.3 to 1 ml of trimethylsilylating agent consisting of pyridine, trimethylchlorosilane and hexamethyldisilazane (5:1:2, by volume) was added. After thorough trituration, the tube was stoppered and left at room temperature for 15 to 30 min. From 1 to 5 µl of the supernatant were injected into the gas chromatograph. Isothermal rather than temperature programmed chromatography was employed in order to obtain exact retention time data. At 160°C better separation of fucose, galactose, mannose and glucose is obtained but sialic acid eluted at ∿65 min. So, for shorter retention times, isothermal chromatography at 190°C was conducted. The chromatograph was equipped with a flame ionization detector. The column was 6 ft. x 1/8 inch glass packed with chromosorb W containing 3% OV-1. Nitrogen was used as carrier gas at a flow rate of 30 ml/minute.

L. Sulfuric acid hydrolysis for removal of sialic acid

Protein samples were treated with sulfuric acid at a final concentration of 0.1 N, at 80° for 1 hour to release sialic acid. After sulfuric acid hydrolysis, the pH of each sample was adjusted to 8.3 (pH of the unhydrolyzed control sample). Gel electrophoresis was then performed simultaneously on both hydrolyzed and unhydrolyzed samples for comparison of the electrophoretic mobility.

M. Removal of the sialic acid by the use of neuraminidase

Neuraminidase (1.82 mg, 2 units) was dissolved in 0.5 ml water, 1 unit of neuraminidase liberates 1 µmole of NANA (309µg) per minute at pH 5.0 at 37°.

To the protein sample (about 0.5 mg/ml in Tris buffer pH 8.3)

was added 0.5 ml acetate buffer, pH 5.5 (0.1 M CH_3COOK, 0.02 M $CaCl_2$ and 0.28 M KCl). The pH was adjusted to 5.5 with a few drops of 2N HCl. 100 µl of the resulting mixture were saved as control for the gel electrophoresis. Five µl (0.02 units) of the sialidase solution were added to the reaction mixture and the tube was incubated in a 37° water bath for a total of 20 hours. Samples were taken for gel electrophoresis after 1/2, 1, 3, 5, 11, 15 and 20 hours incubation period.

RESULTS AND DISCUSSION

A. Experiments using gel electrophoresis

Gel electrophoresis of acetyl-CoA synthetase under conditions already described, gives 4 bands for the goat enzyme and 3 for the cow enzyme. Ninety percent of the protein is found at the faster moving protein band. Samples (200 μg/gel) of the cow enzyme, goat enzyme, BSA, ovalbumin, chymotrypsinogen, and β-lactoglobulin were electrophorized in duplicate and one set was stained with coomassie blue and the other with fuchsin sulfite according to Hotchkiss (34). The same experiment was performed using the PAS staining technique of Kaschnitz et al. (32). In both cases acetyl-CoA synthetase, BSA and ovalbumin gave distinct pink bands characteristic of glycoproteins. Chymotripsinogen and β-lactoglobulin were negative for the test. A schematic representation of the experiment is given in Figure 3.

Acetyl-CoA synthetase samples, purified from both cow and goat mammary gland were then tested using the thiobarbituric acid assay (36) for their sialic acid content. Sialic acids form β-formyl pyruvic acid upon periodate oxidation; β-formyl-pyruvate then reacts with 2-thiobarbituric acid to yield a strongly chromogenic compound with absorption maximum at 549 mμ. 2-deoxy sugars and polyunsaturated fatty acids that might be present in a preparation as contaminants, produce malonaldehyde upon periodate oxidation. The latter reacts also with 2-thiobarbituric acid and yields a

FIGURE 3: Polyacrylamide gel electrophoresis of acetyl-CoA synthetase. Schematic presentation of the coomassie blue or PAS staining.

a: Bovine mammary gland acetyl-CoA synthetase

b: Goat mammary gland acetyl-CoA synthetase

c: BSA

d: Ovalbumin

e: Chymotrypsinogen

f: β-lactoglobulin

a, b, c, d - gave positive PAS staining.
e, f - gave negative PAS staining.

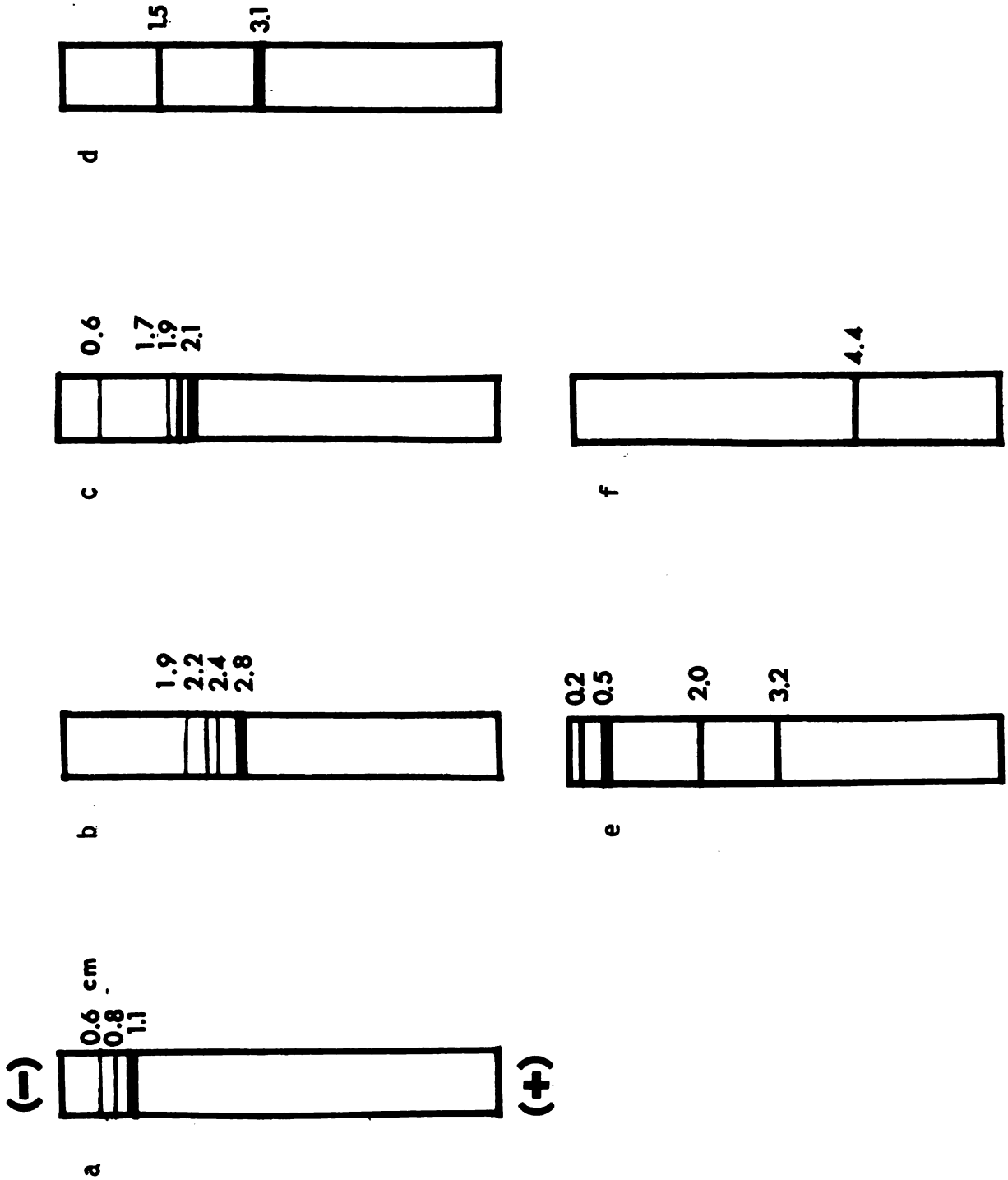

1.5
3.1
d
0.6
1.7
1.9
2.1
c
4.4
f
1.9
2.2
2.4
2.8
b
0.2
0.5
2.0
3.2
e
0.6 cm
0.8
1.1
(-)
(+)
a

TABLE 7: Thiobarbituric acid assay for sialic acid content of acetyl-CoA synthetase.

Source of acetyl-CoA synthetase		Protein/assay mg	Abs. 549 mμ	Sialic acid μg	μg Sialic acid / mg protein
Goat MG	6/8/71	0.77	0.020	0.6	0.78
Goat MG	11/12/71	0.72	0.047	1.8	2.50
Goat MG	1/19/72	1.20	0.028	1.0	0.83
Goat MG	2/25/72	0.88	0.022	0.7	0.79
Goat MG	8/1/72	1.65	0.050	1.8	1.10
Cow MG	6/28/72	0.31	0.058	2.2	7.00
Cow MG	6/13/72	0.72	0.175	6.3	8.00
Cow MG	10/29/71	1.44	0.305	11.0	7.60
Cow MG	8/30/71	3.10	0.450	16.2	5.20

chromogen absorbing at 532 mμ and interfering with the absorption due to sialic acid. The two chromogens have different alcali lability. Color due to sialic acid may be destroyed by the addition of strong alcali base while 2-deoxy ribose chromophore is stable and undergoes a spectral shift to a higher wave length (557 mμ). When the protein samples were treated with strong alcali the color faded within minutes. The amount of sialic acid present in cow enzyme is 0.7% and in goat enzyme 0.1% (Table 7).

When 0.1 N sulfuric acid was used to hydrolyze the sialic acid present, samples before and after hydrolysis gave a difference at the anodic migration (Figure 4). The gels having the hydrolyzed and the untreated protein samples were run simultaneously. BSA was used as control and no difference in the migration was detected. The hydrolyzed protein samples moved faster towards the anode and gave only one protein band. The above is good evidence that covalently linked sialic acid is present in a terminal position. The removal of sialic acid, which has a free carboxyl group, from the molecule leaves the molecule with a more positive overall charge that results in decreased anodal migration.

In another experiment protein samples were first treated with 7 M urea, known to prevent aggregation and then were hydrolyzed in 0.1 N sulfuric acid. Samples before the hydrolysis gave only one protein band as expected. Samples after the sulfuric acid hydrolysis gave again one protein band but with decreased anodal migration (Figure 5). Figure 6 shows that incubation with neuraminidase decreases also the rate of the anodal migration of acetyl-CoA

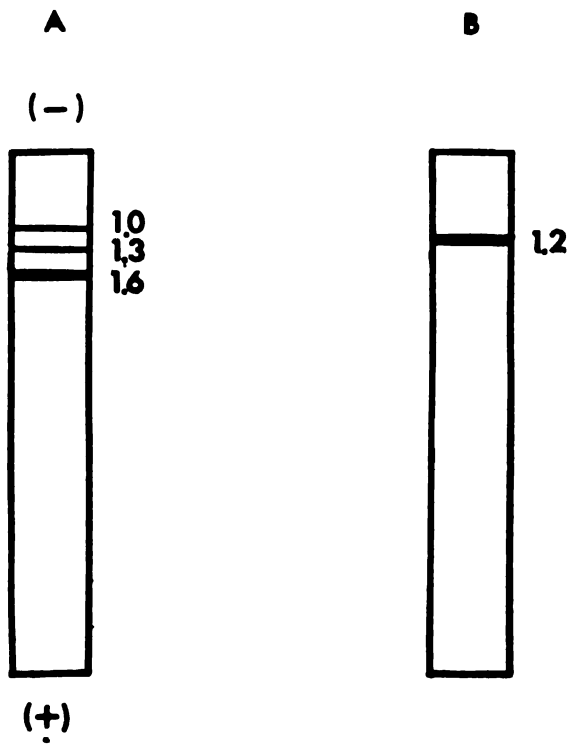


FIGURE 4: Effect of sulfuric acid hydrolysis on the electrophoretic mobility of acetyl-CoA synthetase.

A. goat enzyme before hydrolysis
B. goat enzyme after hydrolysis

BSA was used as control and no difference at the migration was detected.

FIGURE 5: Effect of 0.1 N H_2SO_4 and 7 M urea on the electrophoretic mobility of acetyl-CoA synthetase.

a: acetyl-CoA synthetase from cow MG before H_2SO_4 hydrolysis

b: acetyl-CoA synthetase from cow MG after H_2SO_4 hydrolysis

c: acetyl-CoA synthetase from goat MG before H_2SO_4 hydrolysis

d: acetyl-CoA synthetase from goat MG after H_2SO_4 hydrolysis

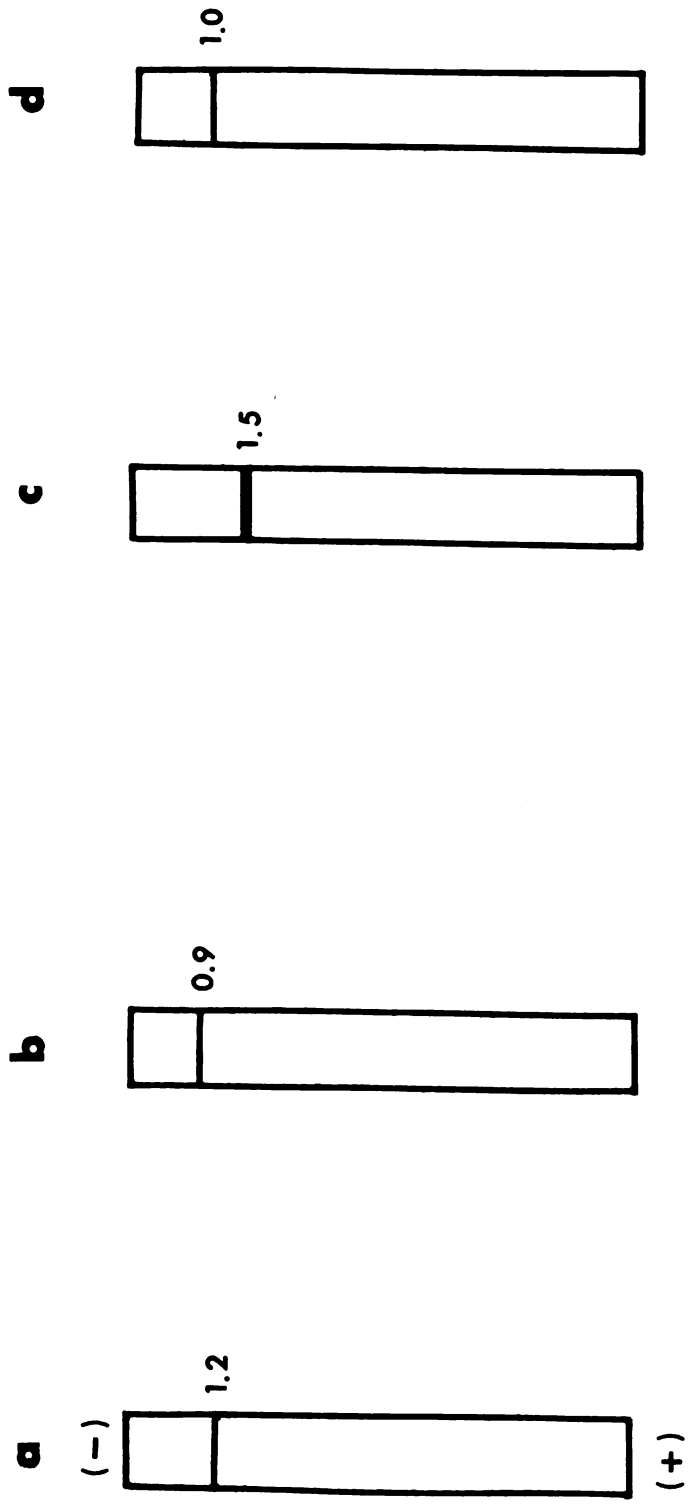
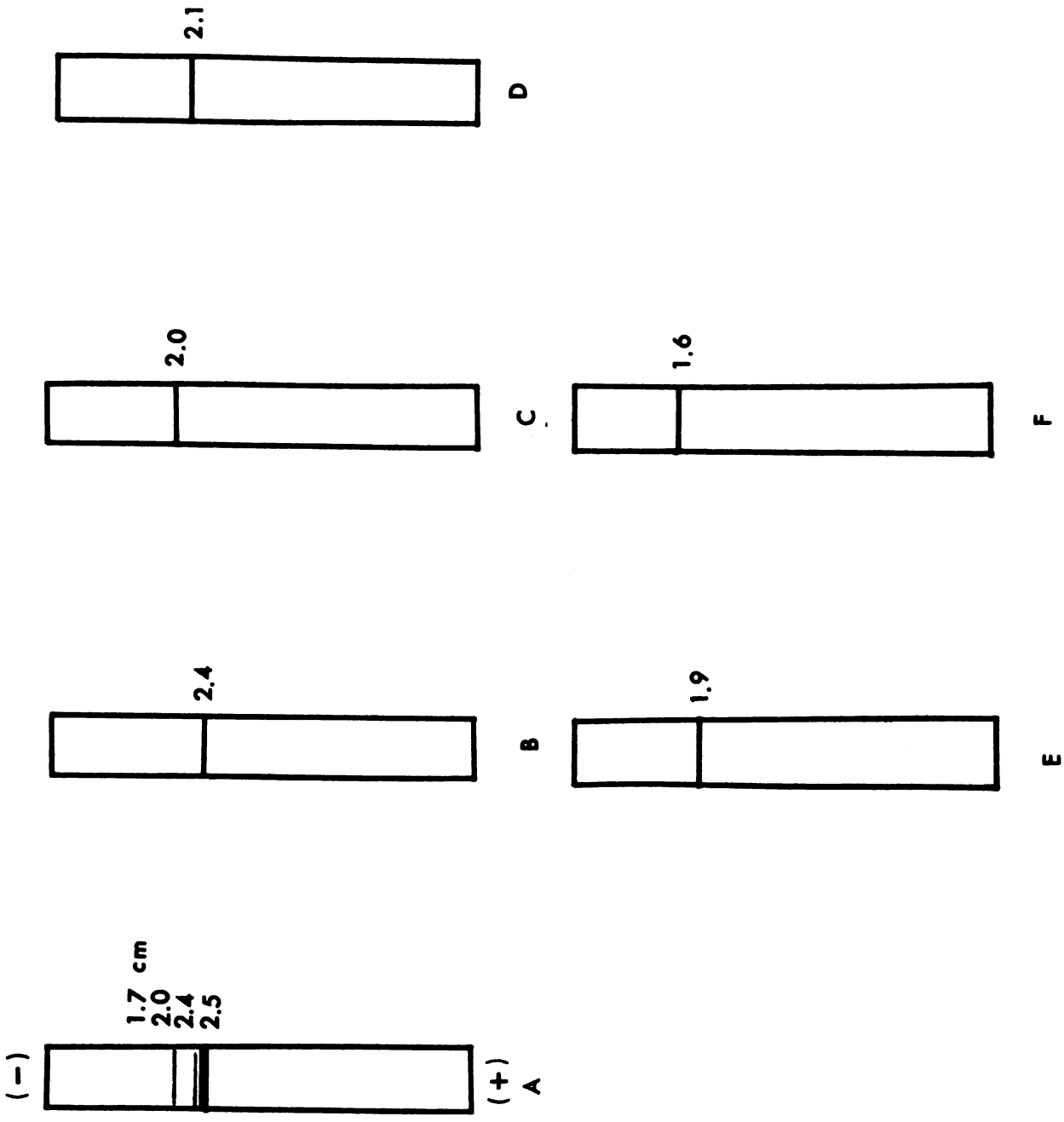

d
1.0
c
1.5
b
0.9
a
1.2
(−)
(+)

FIGURE 6: Effect of neuraminidase on the electrophoretic mobility of acetyl-CoA synthetase at pH 5.5. The enzyme was isolated from goat mammary gland.

Gel A represents a sample from the complete reaction mixture, (see details at the experimental procedures) before the addition of neuraminidase.

Gel B contains acetyl-CoA synthetase plus neuraminidase at zero time. Gels C, D, E, and F contain acetyl-CoA synthetase incubated with neuraminidase at 37° and 5.5 pH, for 30 min., 3, 5, and 11 hours respectively.


2.1
D
2.0
C
1.6
F
2.4
B
1.9
E
1.7 cm
2.0
2.4
2.5
(−)
(+)
A

synthetase. These experiments establish the presence of carbohydrates and expecially that of sialic acid.

B. Gas chromatography analysis

GLC analysis, the next step, was conducted for complete qualitative analysis. The sugars used as controls were tested for purity by measuring their optical rotation (Table 8). Tables 9 and 10 give the relative retention times (R_t) of the TMSi derivatives of methyl glycosides used as standards at 160° and 190°C respectively. Figure 9 represents the GLC trace of a control mixture of the TMSi derivatives of methyl glycosides at 160°. The cow enzyme gave the trace represented by Figure 10. Table 11 gives the relative R_t of the peaks found on the gas chromatograph of goat and cow enzyme (Figures 12 and 10 respectively). A comparison between the Figures 9, 10 and another one between the relative retention from Tables 9, 10 and 11 shows the presence of fucose, glucose and N-acetyl neuraminic acid in the cow enzyme. A peak eluting right after mannitol and having relative R_t 1.3 corresponds probably to the minor peak that the N-acetyl galactosamine standard gives. However, the major peak of N-acetyl galactosamine is not present.

Analysis of the cow enzyme (Figure 11) performed at 190° gave the same carbohydrate composition except that sialic acid was not present this time.

Figure 12 represents the GLC trace of the TMSi derivatives of methyl glycosides prepared from the goat acetyl-CoA synthetase at 190°. A comparison of the relative retention times shows the presence of fucose, galactose, glucose and N-acetyl galactosamine.

TABLE 8: Optical rotations of carbohydrates used as standards for GLC analysis.

Carbohydrate	*Initial $(\alpha)_D^{20}$	*$(\alpha)_D^{20}$ at equilibrium	$(\alpha)_D^{20}$ found
α-L-Fucose	-152.6	-75.9	77.5
α-D-galactose	+150.0	+80.0	73.7
β-D-galactose	+ 52.8	+80.2	
α-D-mannose	+ 29.3	+14.2	21.0
β-D-mannose	- 17.0	+14.2	
α-D-glucose	+112.2	+52.7	56.2
β-D-glucose	+ 18.7	+52.7	

*Values taken from the literature.

Optical rotation was measured at a concentration of 4%. The solutions had to equilibrate for 12-24 hours before final readings. For the calculations the following formula was used:

$$\text{specific rotation } (\alpha) = \frac{\alpha_{obs.} \times 100}{l \times c}$$

Where $\alpha_{obs.}$ = optical rotation observed

l = length of polariscope tube (dm)

c = concentration of the sugar as gr/100 ml of solution

TABLE 9: Relative retention times of the TMSi derivatives of methylglycosides at 160°.

TMSi derivative of the methylglycoside of:	Relative 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
2-Acetamido-2-deoxy-D-galactose	1.30
	1.50
2-Acetamido-2-deoxy-D-glucose	1.70
Methyl-N-acetylneuraminate	2.70

Time of reference peak 25 ± 2 min.

Column used was 3% OV-1

TABLE 10. Relative retention times of the TMSi derivatives of methyl-glycosides, at 190°.

TMSi derivative of the methyl-glycoside of:	Relative 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
2-Acetamido-2-deoxy- -galactose	1.10
	1.35
2-Acetamido-2-deoxy-D-glucose	1.30
	1.60
Methyl-N-acetylneuraminate	5.50

Time of reference peak 8.1 ± 2 min.

Column used was 3% OV-1

TABLE 11: GLC analysis of the TMSi derivatives of methyl glycosides prepared from bovine mammary gland acetyl-CoA synthetase. Relative retention times at 160° and 190°.

Goat enzyme Rel. Rt. of peaks found at 190°	Monosaccharide	Cow enzyme Rel. Rt. of peaks found at 160°	Monosaccharide	Cow enzyme Rel. Rt. of peaks found at 190°	Monosaccharide
0.28	L-fucose	0.16	L-fucose	0.22	L-fucose
0.33		0.19		0.25	
0.48	D-galactose	0.22		0.28	
0.57					
0.64					
0.83	D-glucose	0.66	D-glucose	0.73	D-glucose
1.00	Mannitol	0.76		0.80	
1.35	N-acetyl-galacto-samine	1.00	Mannitol	1.00	Mannitol
		1.30	N-acetyl-galacto-samine ?	1.10	N-acetyl-galacto-samine ?
2.9	Silicon ?				
		2.40	N-acetyl-neura-minic acid		

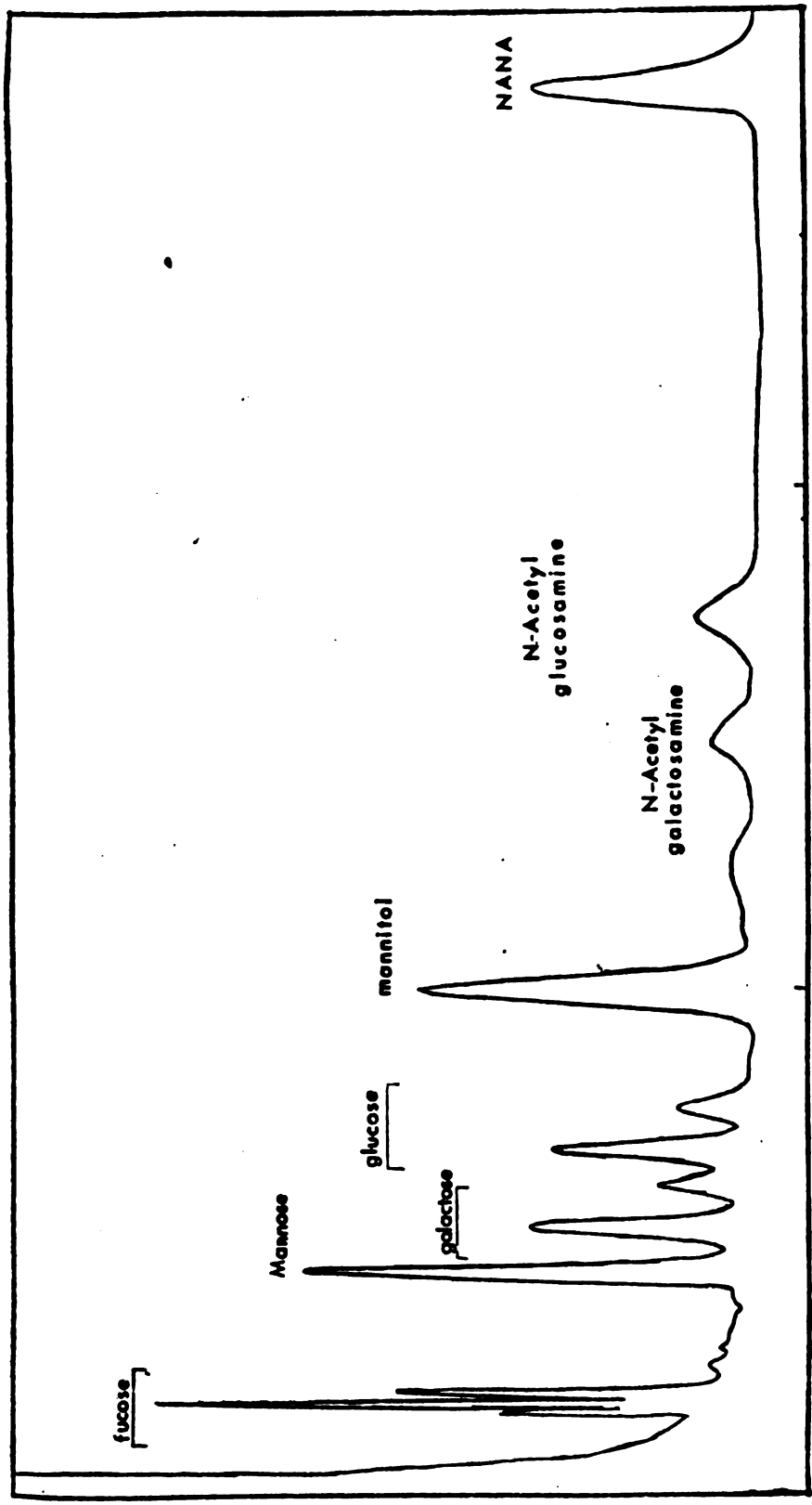


FIGURE 9: Gas chromatogram of a control mixture of TMSi derivatives of methyl glycosides at 160°C, 3% OV-I column.

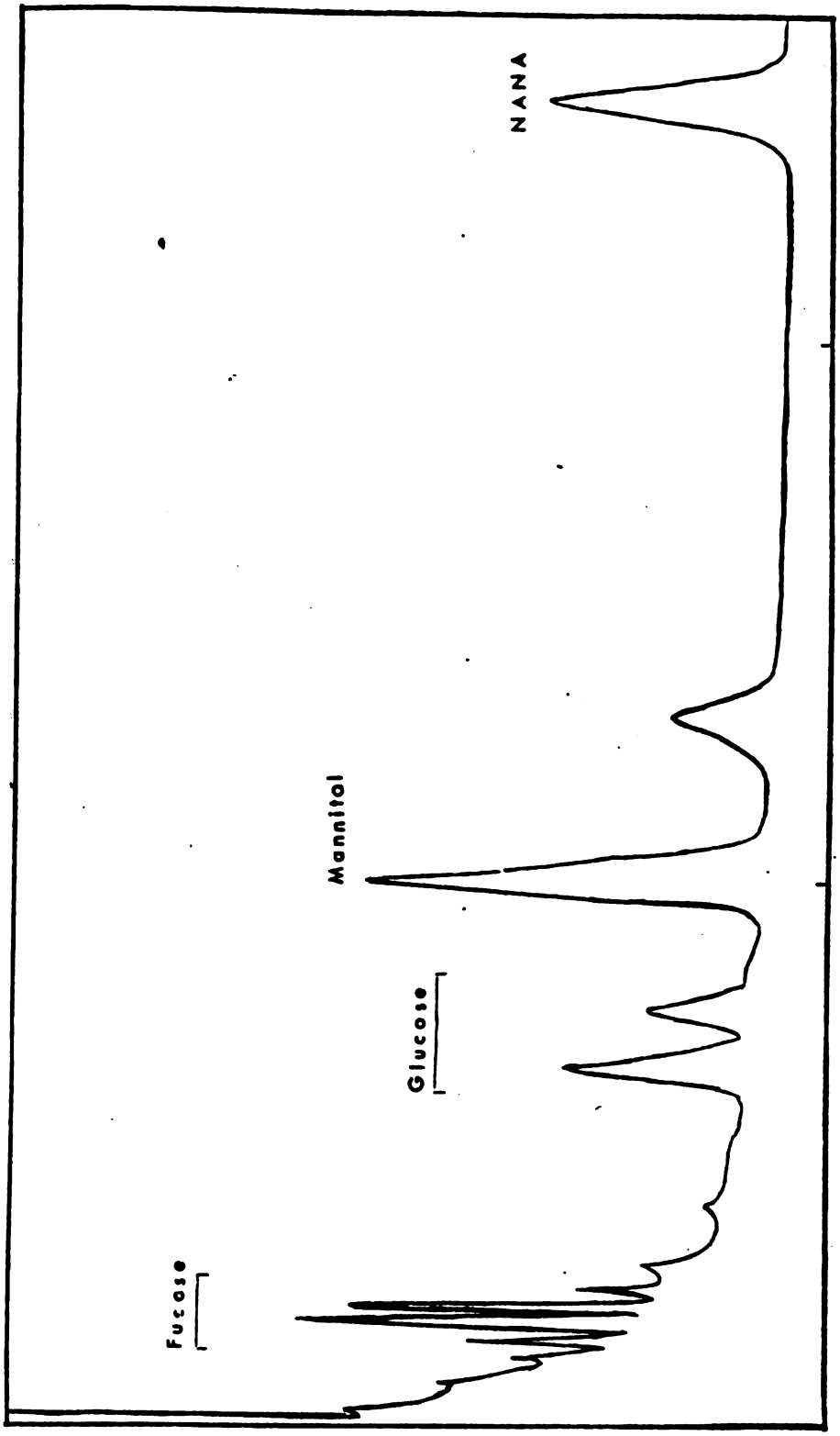


FIGURE 10: Gas chromatogram of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase, at 160°C.

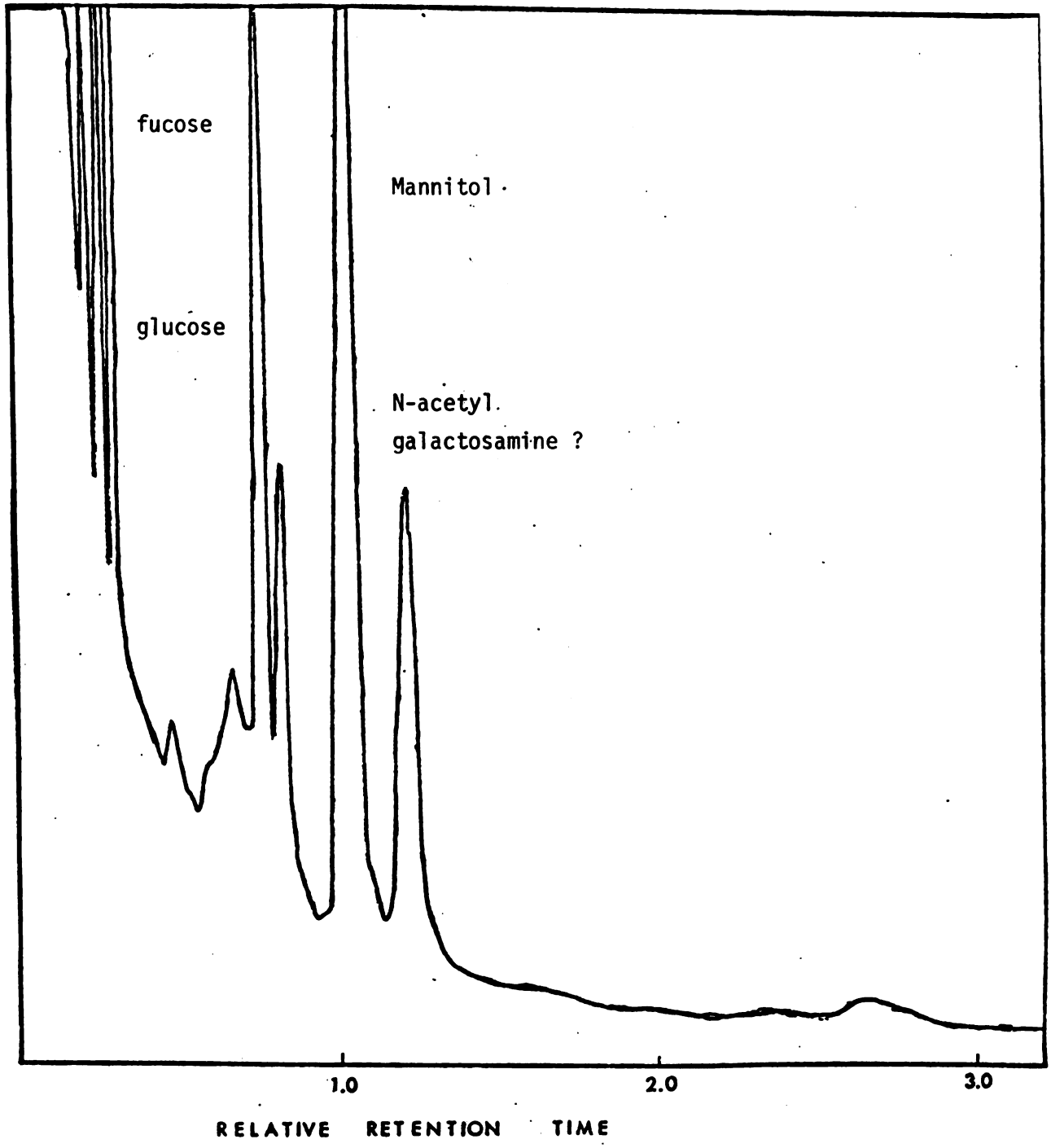


FIGURE 11: Gas chromatogram of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase, at 190°C.

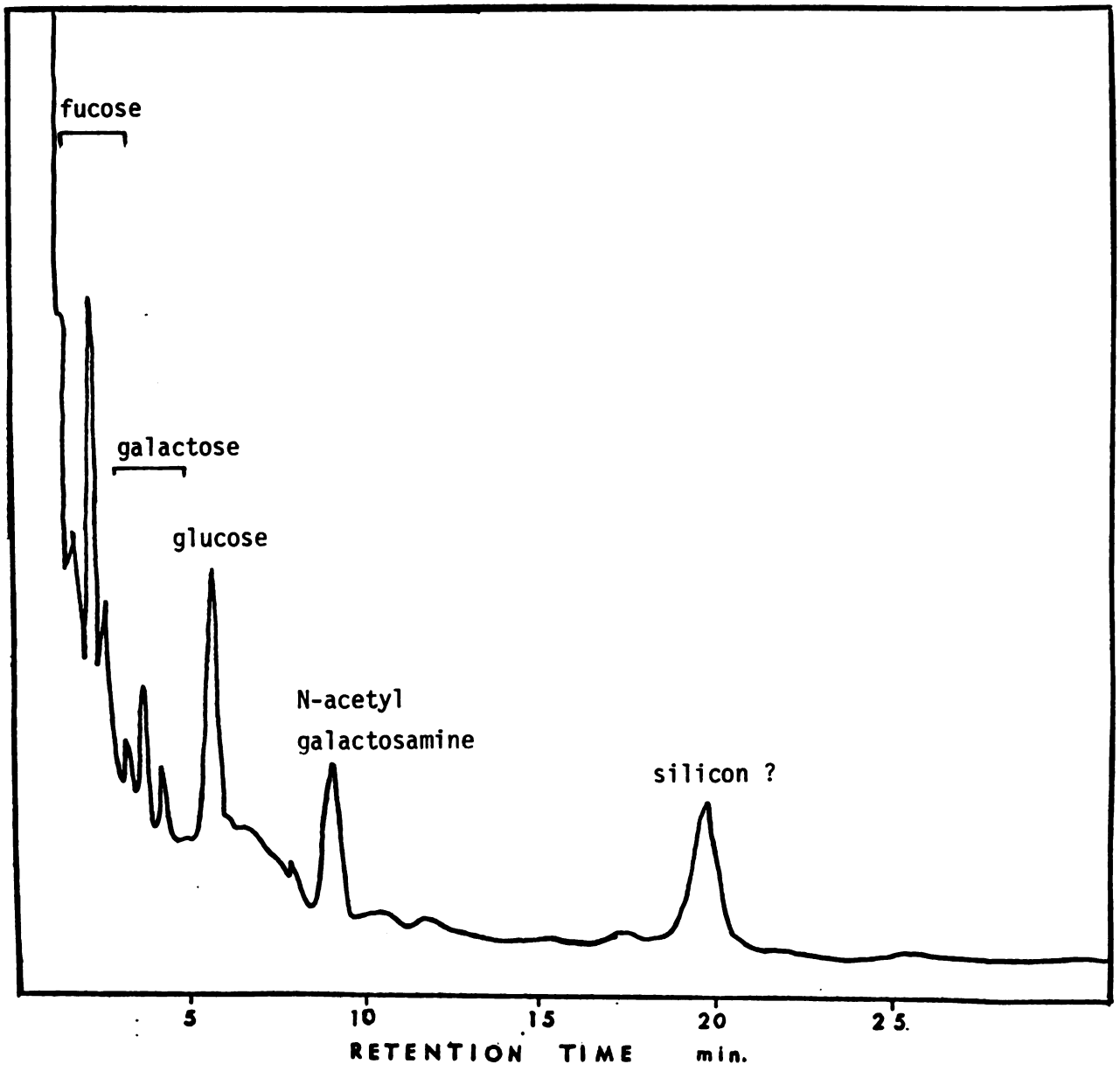


FIGURE 12: Gas chromatogram of the TMSi derivatives of methyl glycosides prepared from goat mammary gland acetyl-CoA synthetase at 190°C.

Glucose is present as the minor peak of the glucose standard and N-acetyl galactosamine as the major peak. The last peak of the chromatogram has a relative R_t of 2.9, corresponds to none of the standards and mass spectrum shows a fragmentation pattern characteristic of silicon. Silicon bleeding is very often observed from OV and SE columns.

C. Mass spectrometry analysis

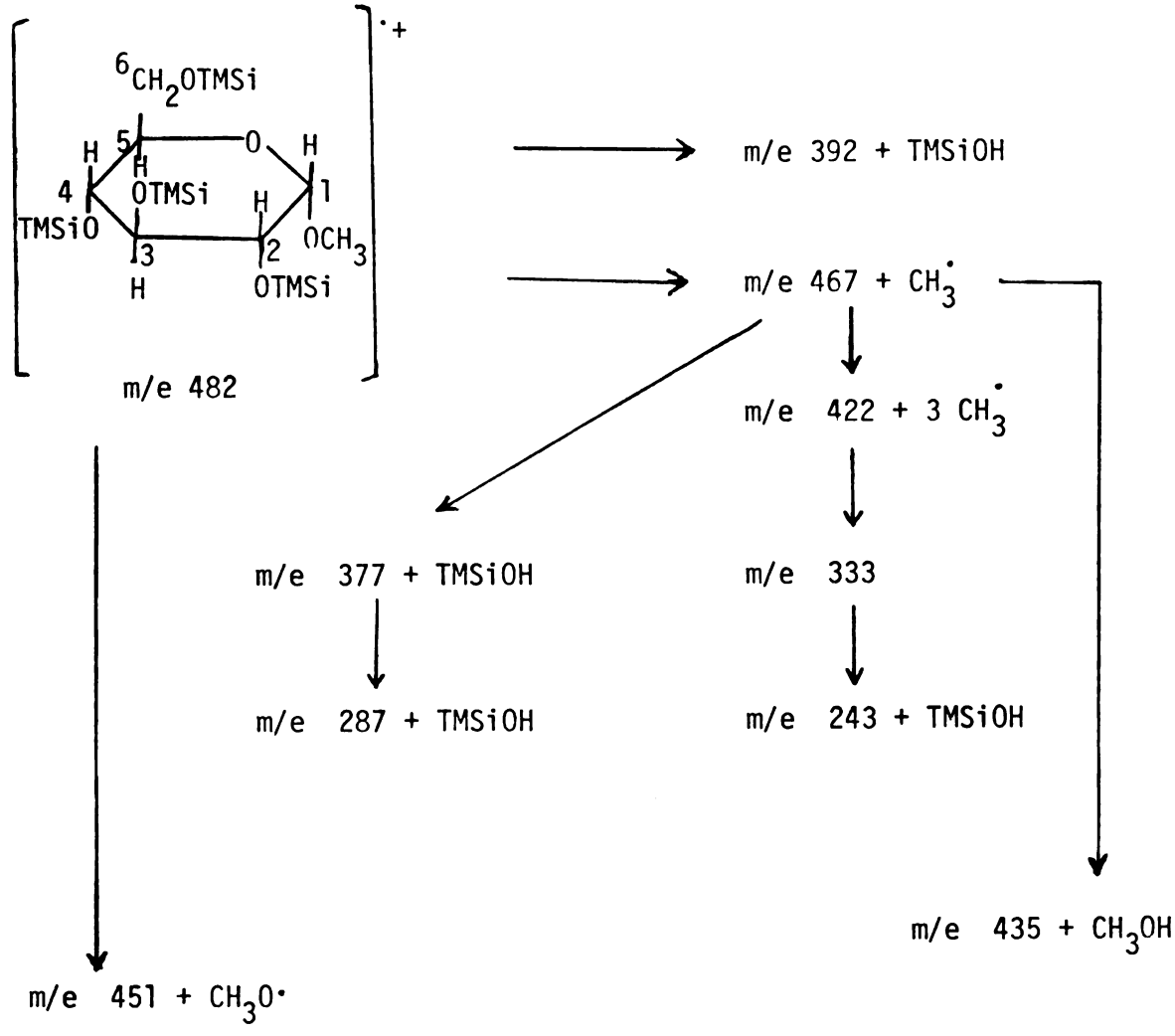
The data from the GLC analysis was confirmed using mass spectrometry. The studies were conducted with an LKB-9000 gas chromatograph-mass spectrometer using an ionizing energy of 70 EV. The GLC column, 4 feet x 1/8 inch glass was packed with chromosorb G containing 3% SE-30. The temperature was programmed from 160-240° with a 5°/min. increase. The protein samples were prepared exactly the same way as for GLC analysis.

At this point, a discussion of the mass spectra of the TMSi derivatives of methyl-glycosides is necessary. Selected peaks from the mass spectrum of methyl 2,3,4,6-tetra-O-trimethylsilyl-a-D-glucopyranoside (I) are listed in table 12.

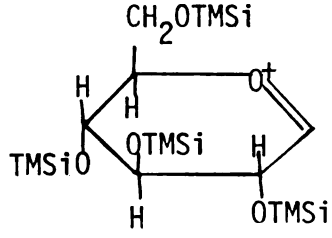
TABLE 12: Selected peaks from the mass spectrum of methyl 2,3,4,6-tetra-O-trimethylsilyl-a-D-glucopyranoside(I)

m/e	m/e	m/e	m/e	m/e
482	393	332	271	133
467	377	319	217	131
451	361	305	204	117
435	345	303	191	89
407	335	287	147	73

Molecule I fragments as follows:

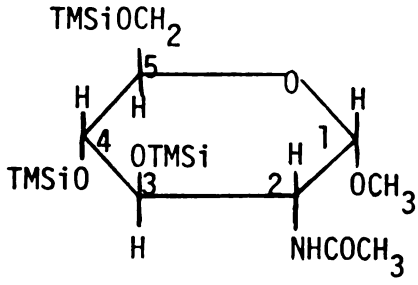


m/e 451 has the structure:

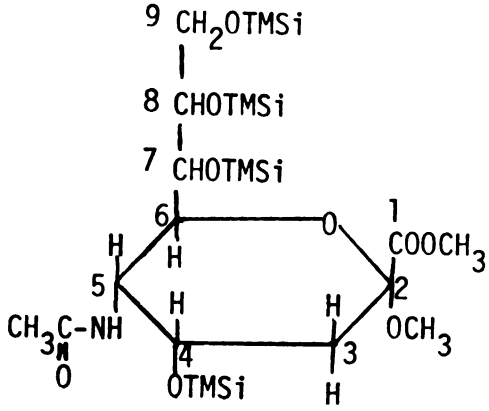


m/e 451 further fragments to give m/e 361 + TMSiOH, and 361 gives m/e 271 by elimination of TMSiOH.

The structure of some of the smaller fragments is listed in Table 13. The fragmentation of the trimethylsilyl ethers of methyl-2-acetamido-2-deoxy-a-D-glucopyranoside (II) and N-acetyl-neuraminic acid (III) will be discussed shortly. The structures of II and III are given below:



(II) m/e 451

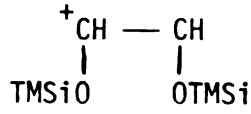
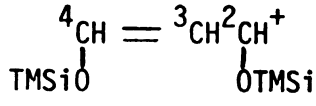
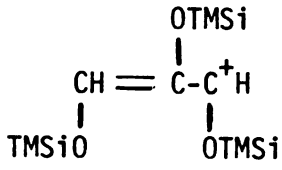
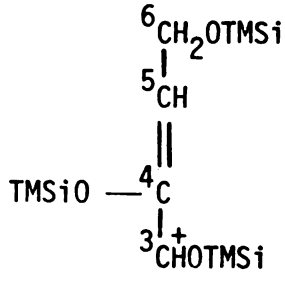


(III) m/e 625

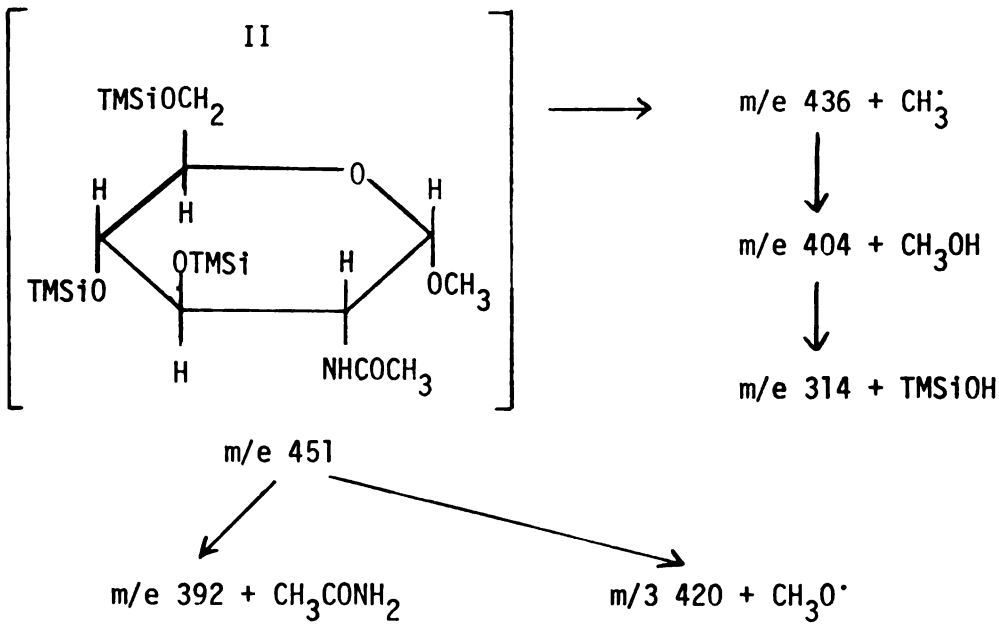
TABLE 13: Structures of some of the fragments of methyl 2,3,4,6-tetra-O-trimethylsilyl-a-D-glucopyranoside (I).

m/e	Molecular Structure
73	$^{+}Si(CH_3)_3$
103	$^{6}CH_2{=}\overset{+}{O}Si(CH_3)_3$
117	$^{6}CH_2O\overset{+}{S}i(CH_3)_2$ $^{5}CH{=}O$
129	$^{5}CH{=}^{6}CH_2$ $^{4}CHOTMSi$ $^{+}$
133	$CH_3O\overset{+}{C}HOTMSi$
147	$(CH_3)_3SiO\overset{+}{S}i(CH_3)_2$
191	$^{1}HC{=}\overset{+}{O}Si(CH_3)_3$ $OSi(CH_3)_3$

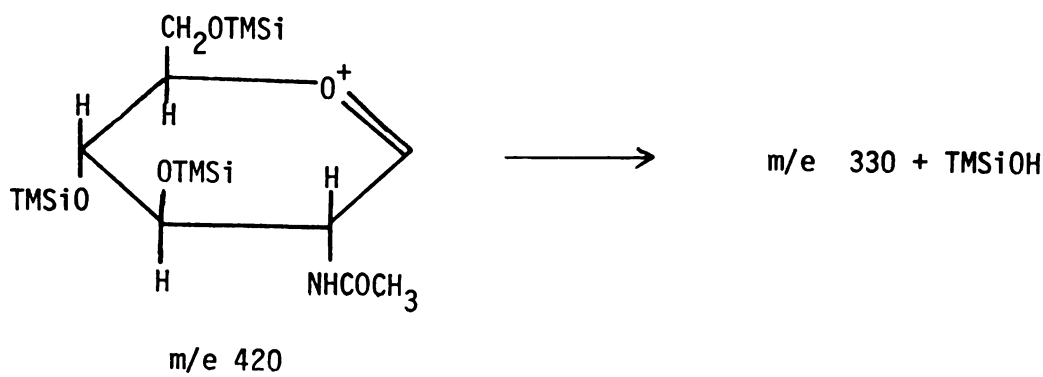
TABLE 13: continued

m/e	Molecular Structure
204	
217	
305	
319	

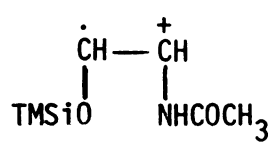
Molecule II fragments as follows:



Further fragmentation of m/e 420 which is a characteristic fragment gives:

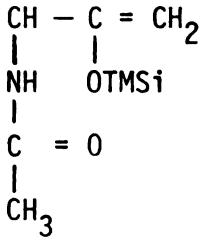


The fragment at m/e 173 is an intense ion and has the structure:



m/e 173

The same two carbon unit occurs in N-acetyl-neuraminic acid and, hence, m/e 173 can arrise either from sialic acid residues of the N-acetyl type or from the N-acetyl hexosamine residues. The related ion at m/e 186 can also be formed from either N-acetyl-neuraminic acid or N-acetyl-hexosamine.



m/e 186

Figures 13 and 14 give the GLC traces of the TMSi derivatives of methyl glycosides prepared from the cow and goat acetyl-CoA synthetase respectively. Each peak of these traces was analyzed by the mass spectrometer. Figures 15, 16 and 17 give the mass spectra of the TMSi derivatives of mannitol and the TMSi derivatives of the methyl glycosides of N-acetyl galactosamine and N-acetyl neuraminic acid respectively.

From Figure 13 (cow enzyme) scans 4, 28, 33, 48, 79, 139 (Figures 18, 19, 20, 21, 22, 23 respectively) give fragmentation patterns characteristic of the pyranoside ring. Scan 41 (Figure 24) is mannitol (Figure 15). Scan 99 (Figure 25) gives good evidence that the component is either a N-acetyl sugar or N-acetyl neuraminic acid. This peak eluted at about 220°C where N-acetyl neuraminic acid elutes under the same conditions (38).

From Figure 14 (goat enzyme) scans 3, 36, 42, 144 (Figures 26, 28,

29, 32 respectively) can be identified as sugars having the pyranoside ring. Scan 25 (Figure 27) has several of the fragments of the pyranoside ring but only the ones with lower molecular weight. Scan 90 (Figure 31) has fragments characteristic of silicon (m/e 29, 42, 56, 207, 221, 281, 355, 429, 503). Scan 47 (Figure 30) does not show fragments characteristic of sugars. The mass spectrum of the goat enzyme was obtained 8 days after the GLC analysis. Partial hydrolysis of the TMSi derivatives can occur when the samples are stored for several days. N-acetyl neuraminic acid was not found in the goat enzyme using GLC. It is known that N-acetyl neuraminic acid is unstable in storage and probably this is the reason of its absence from the gas chromatogram. However, the neuraminidase and thiobarbituric acid assay were both positive and we can conclude that N-acetyl neuraminic acid is also present in the goat acetyl-CoA synthetase. It is concluded from the above data that fucose, glucose and N-acetyl neuraminic acid are present in the cow enzyme. The goat enzyme contains fucose, galactose, glucose, N-acetyl galactosamine and N-acetyl neuraminic acid.

D. Discussion

The discovery that acetyl-CoA synthetase is a glycoprotein may explain many of the questions related to the physical and catalytic properties of the enzyme.

However, the exact role that carbohydrates play and the magnitude of the significance of this role is not clear at this time. Much of the purification data indicates that the enzyme has the tendency to aggregate. A charge is very often needed for interaction

between subunits. N-acetyl neuraminic acid present in acetyl-CoA synthetase could very well give this charge and direct this apparent aggregation and deaggregation phenomena.

Acetyl-CoA synthetase elutes in more than one protein peaks from both DE-23 cellulose column and calcium phosphate gel. All these protein peaks possessed enzymatic activity. This data suggests the existence of isozymes. Several proteins are known to exist as isoglycoenzymes or isozymes that differ only in their carbohydrate content. It is reasonable to think that acetyl-CoA synthetase is a isoglycoenzyme.

Acetyl-CoA synthetase from bovine fetal heart tissue preferentially activates propionate rather than acetate, in contrast to the adult enzyme. It would be of interest to study the carbohydrate content and molecular weight of the fetal and adult enzymes. It could be determined whether or not isozymic or aggregated forms of acetyl-CoA synthetase are responsible for this difference in substrate specificity.

There are more points of interest that should be investigated. Acetyl-CoA synthetase is not active in non-lactating mammary gland. The enzyme becomes active after parturition and its activity decreases with advancing lactation.

These phenomena described above may be explained by differences in the carbohydrate composition of the enzymes in different tissues and under different physiological states.

In summary this work has proven that acetyl-CoA synthetase is a glycoprotein and this appears to be a significant discovery that

will probably explain many of the physical and catalytic properties of the enzyme.

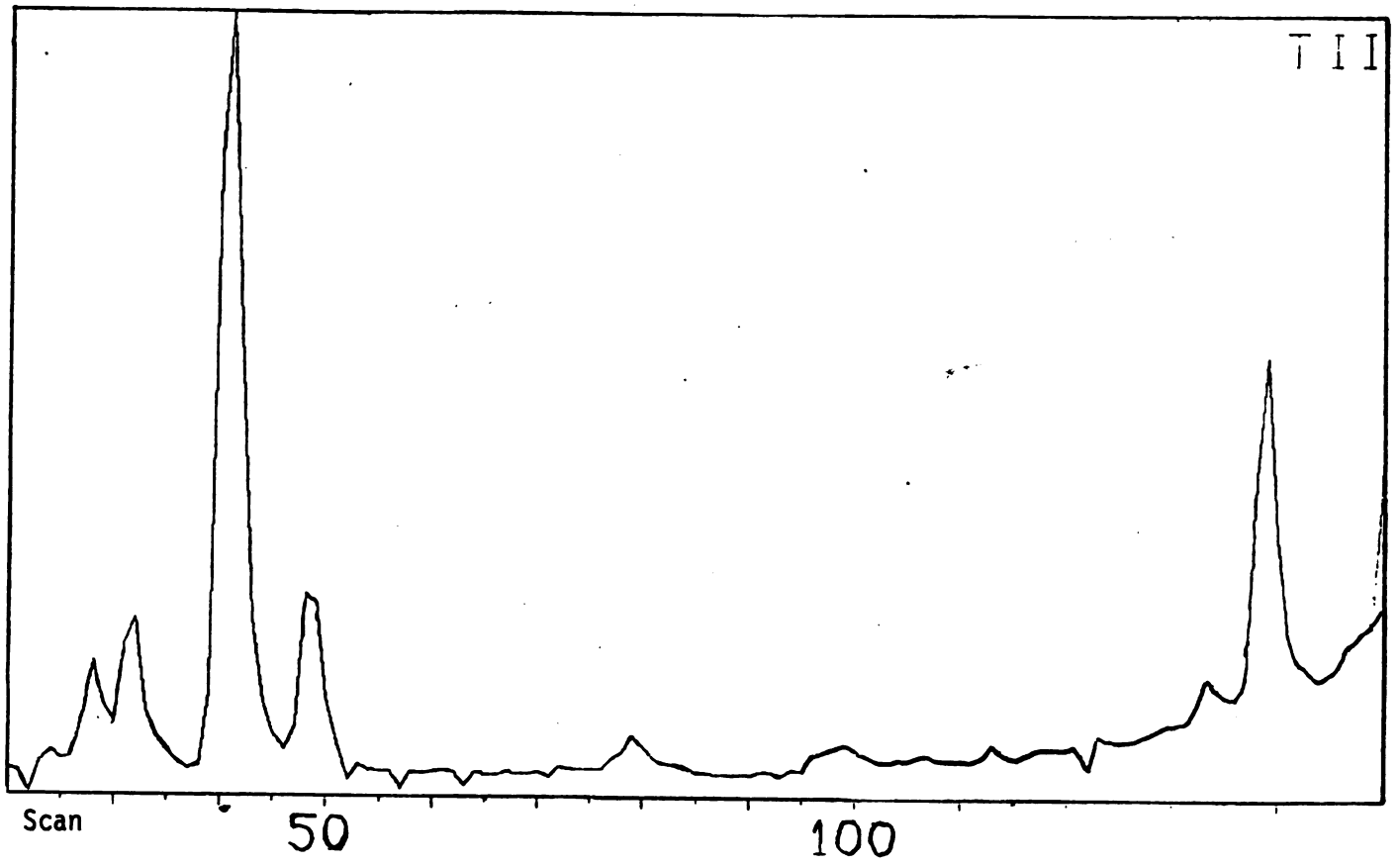


FIGURE 13: The gas chromatogram of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase that was used for the mass spectrum analysis.

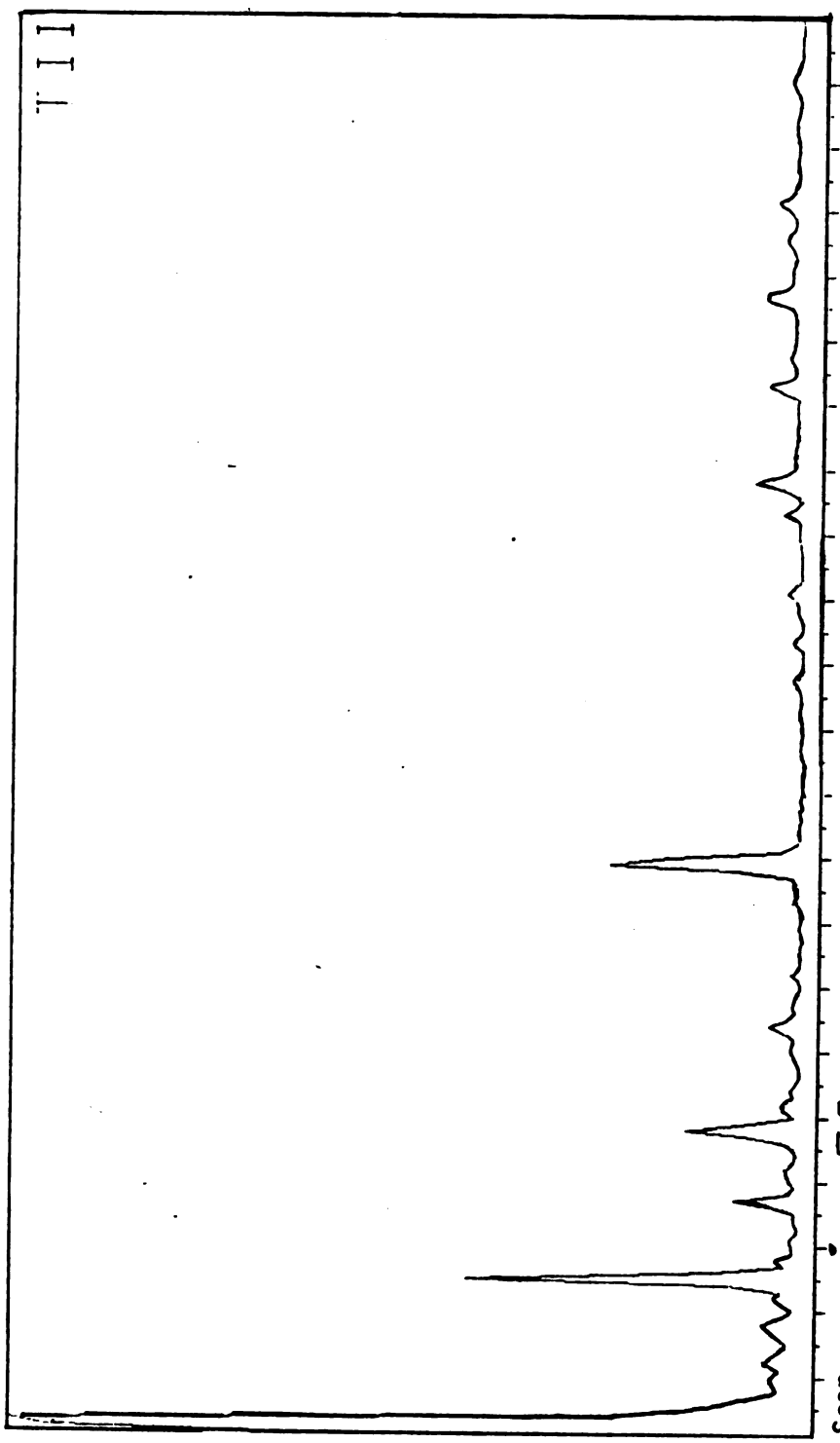


FIGURE 14: The gas chromatogram of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase that was used for the mass spectrum analysis.

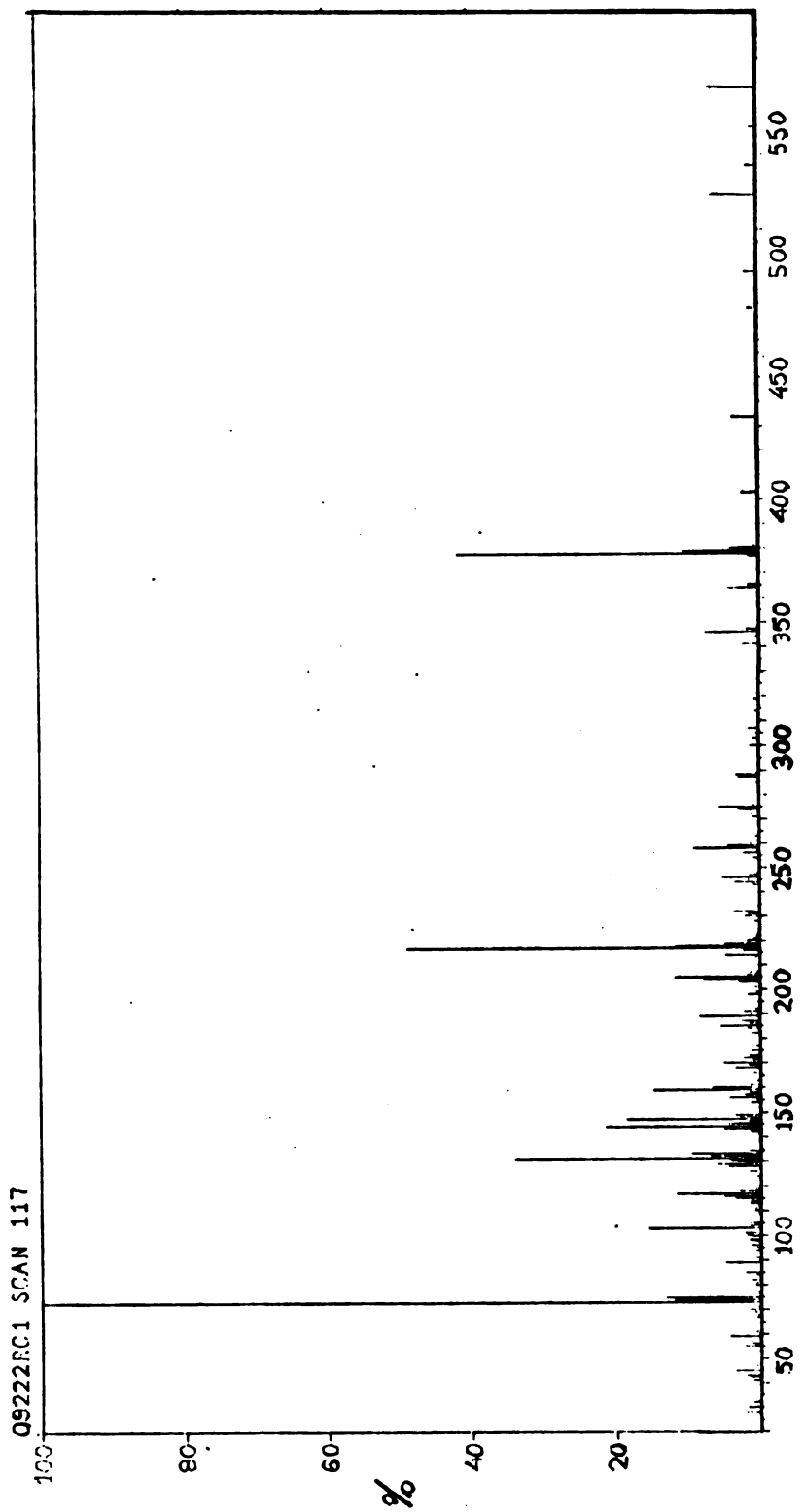


FIGURE 15: The mass spectrum of the TMSi derivative of mannitol.

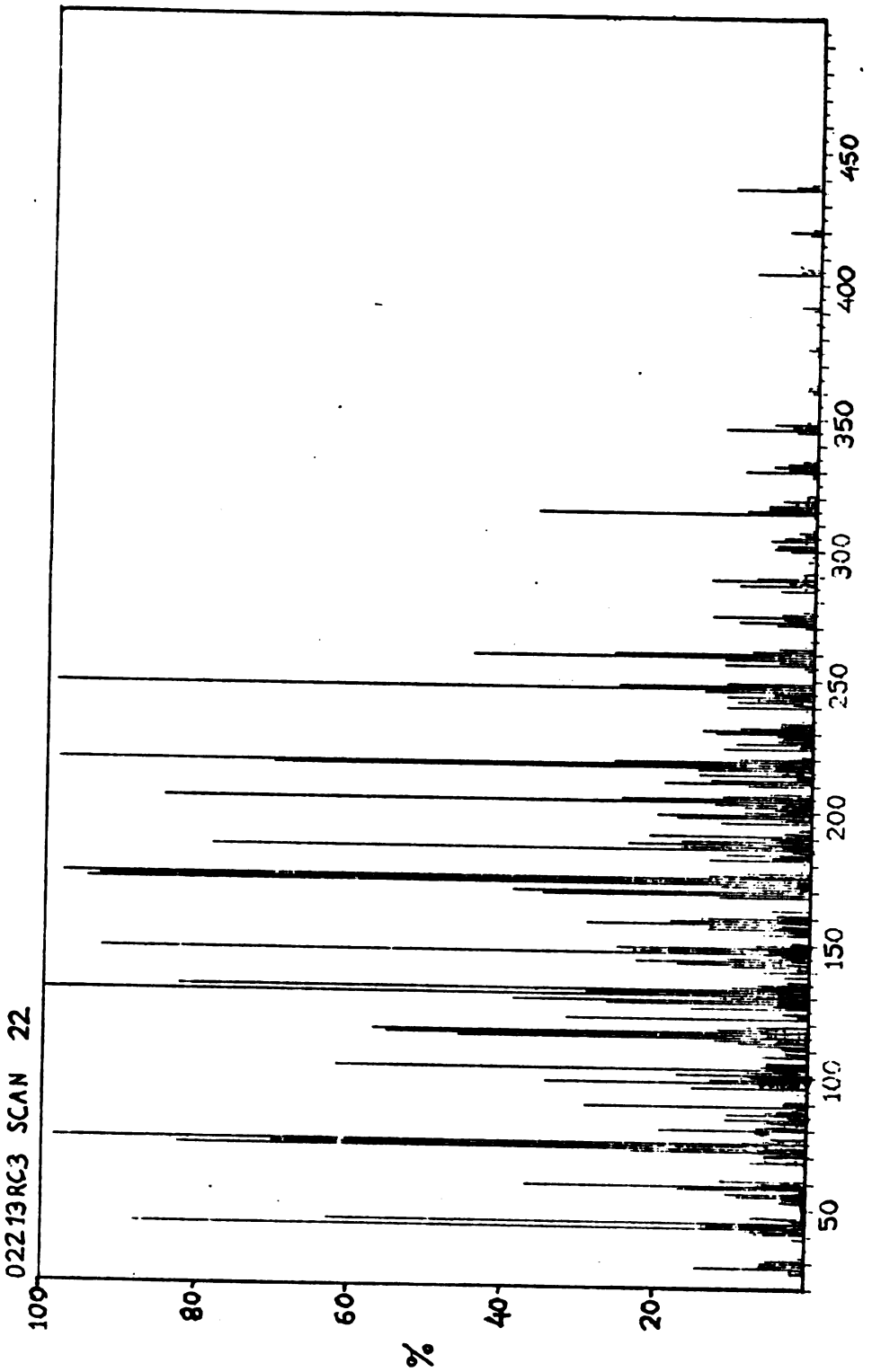


FIGURE 16: The mass spectrum of the TMSi derivative of the methyl glycoside of N-acetyl galactosamine.

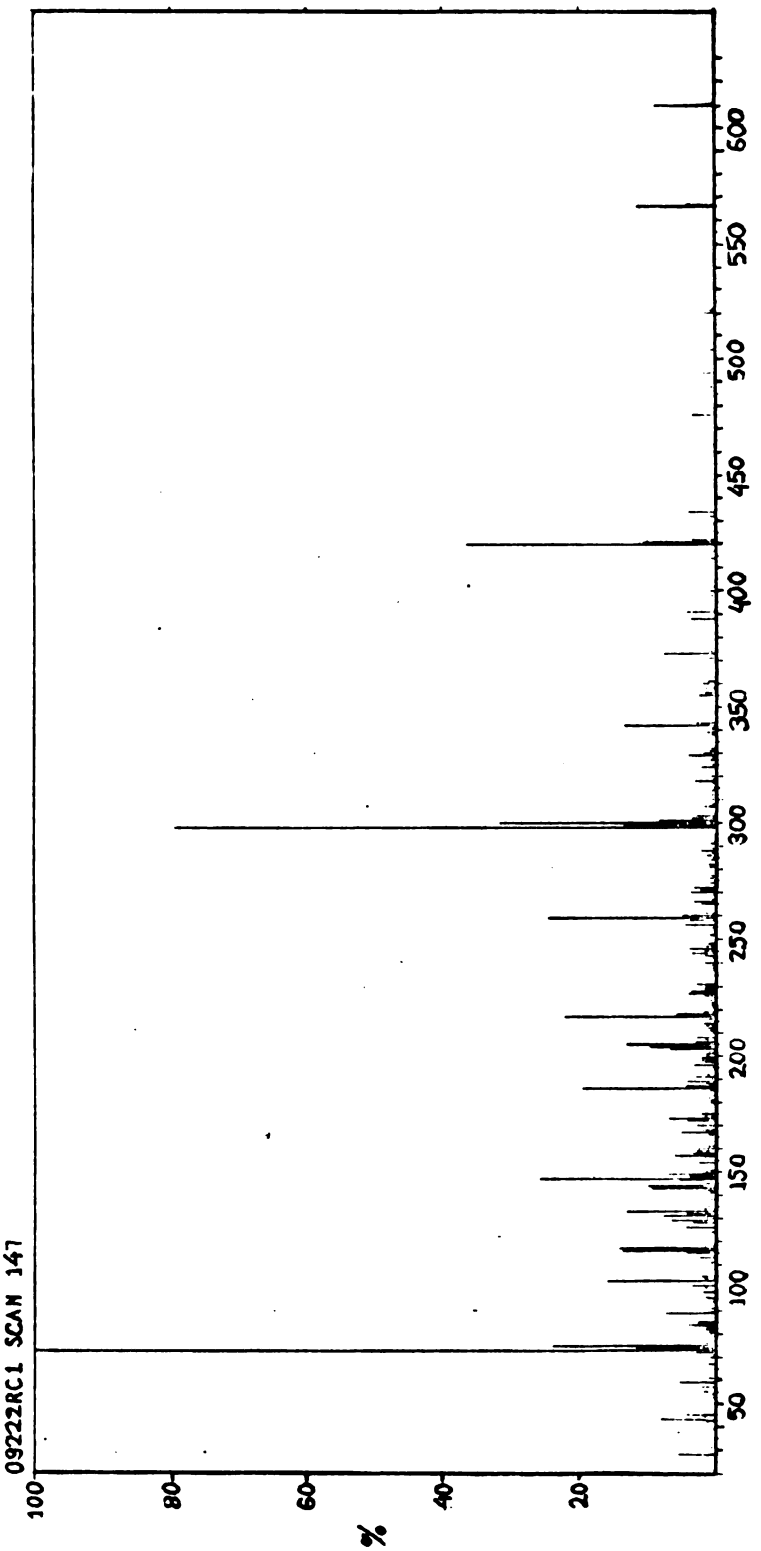


FIGURE 17: The mass spectrum of the TMSi derivative of the methyl glycoside of N-acetyl neuraminic acid.

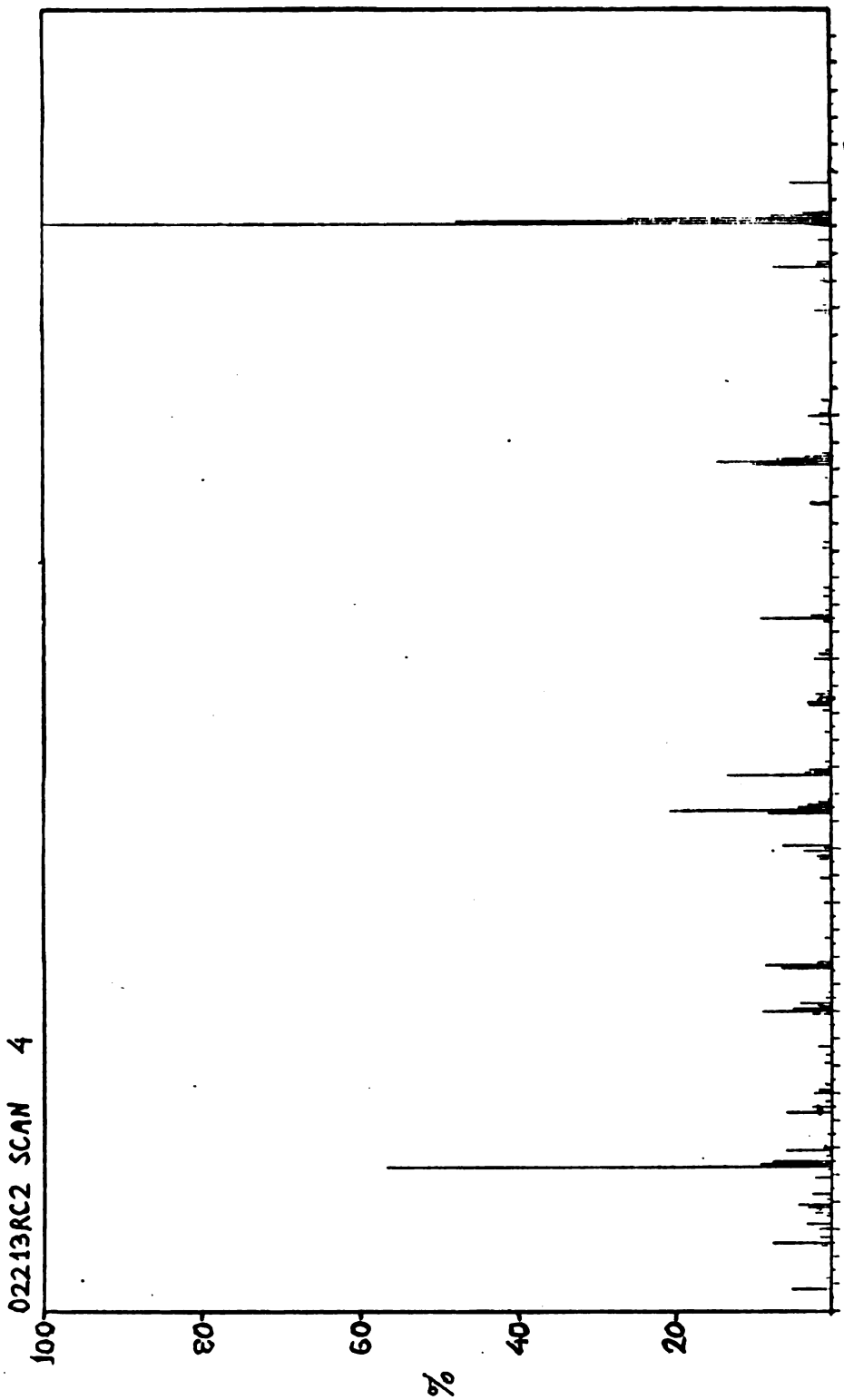


FIGURE 18: The mass spectrum from scan 4 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

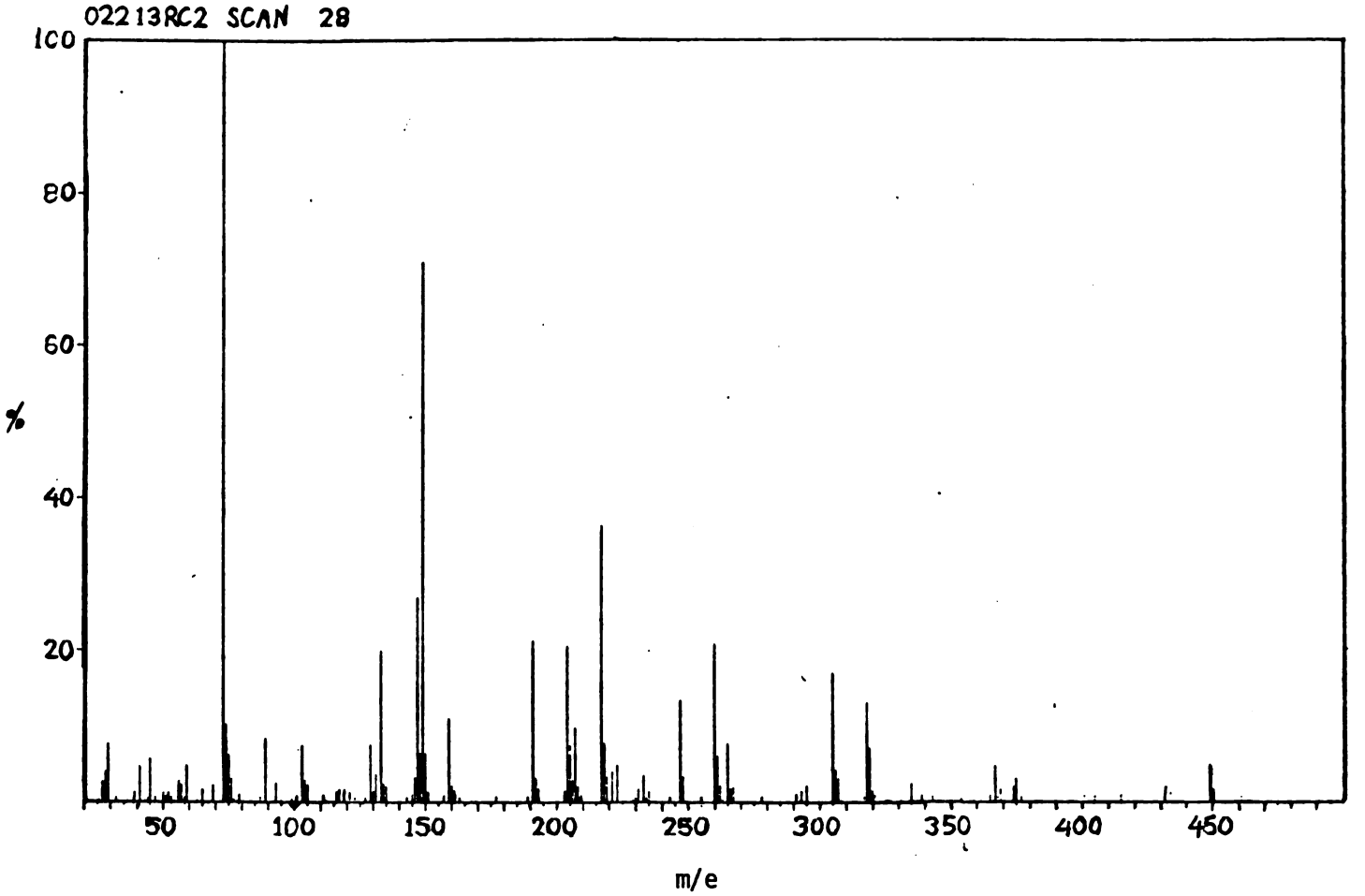


FIGURE 19: The mass spectrum from scan 28 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

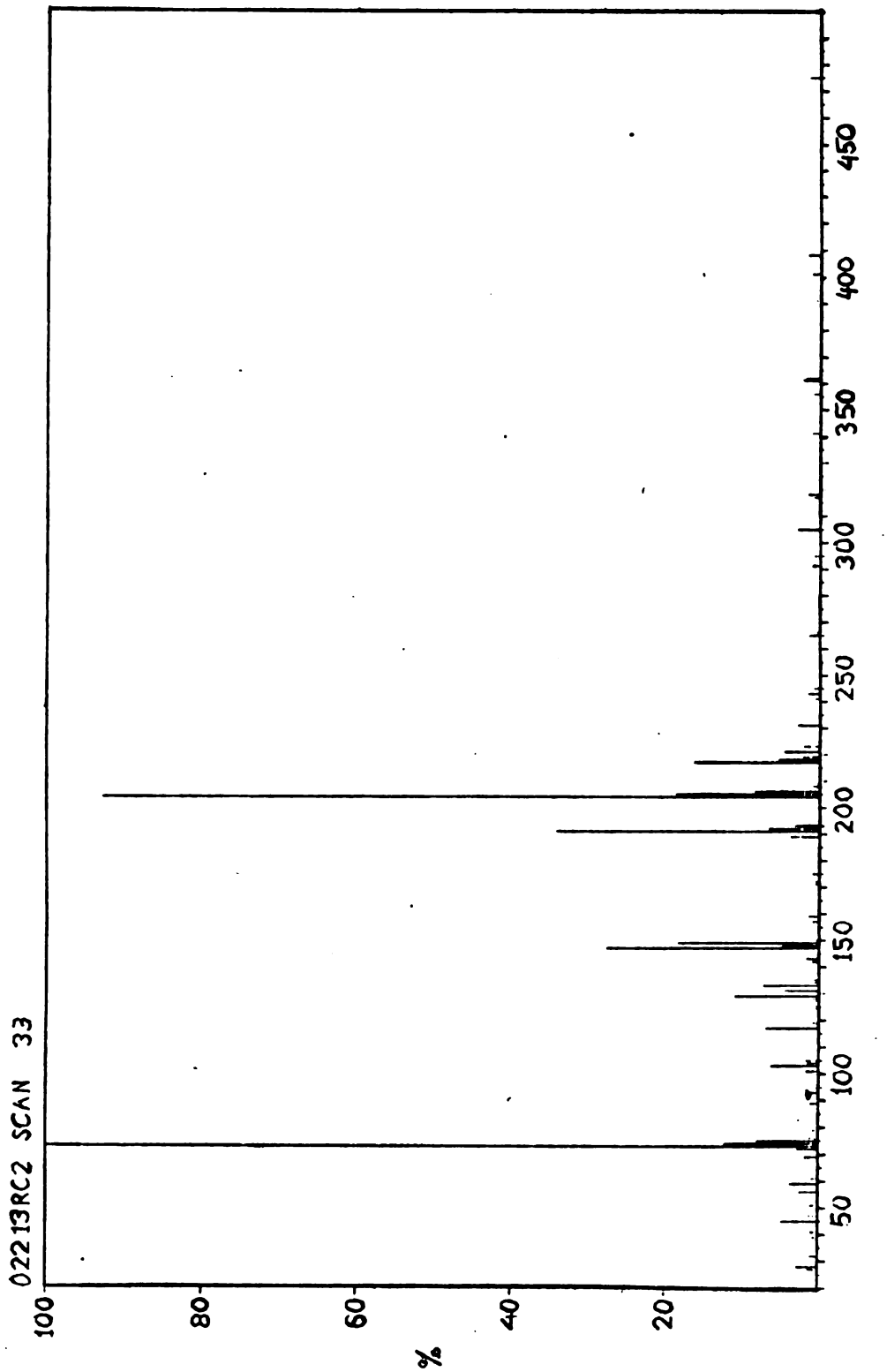


FIGURE 20: The mass spectrum from scan 33 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

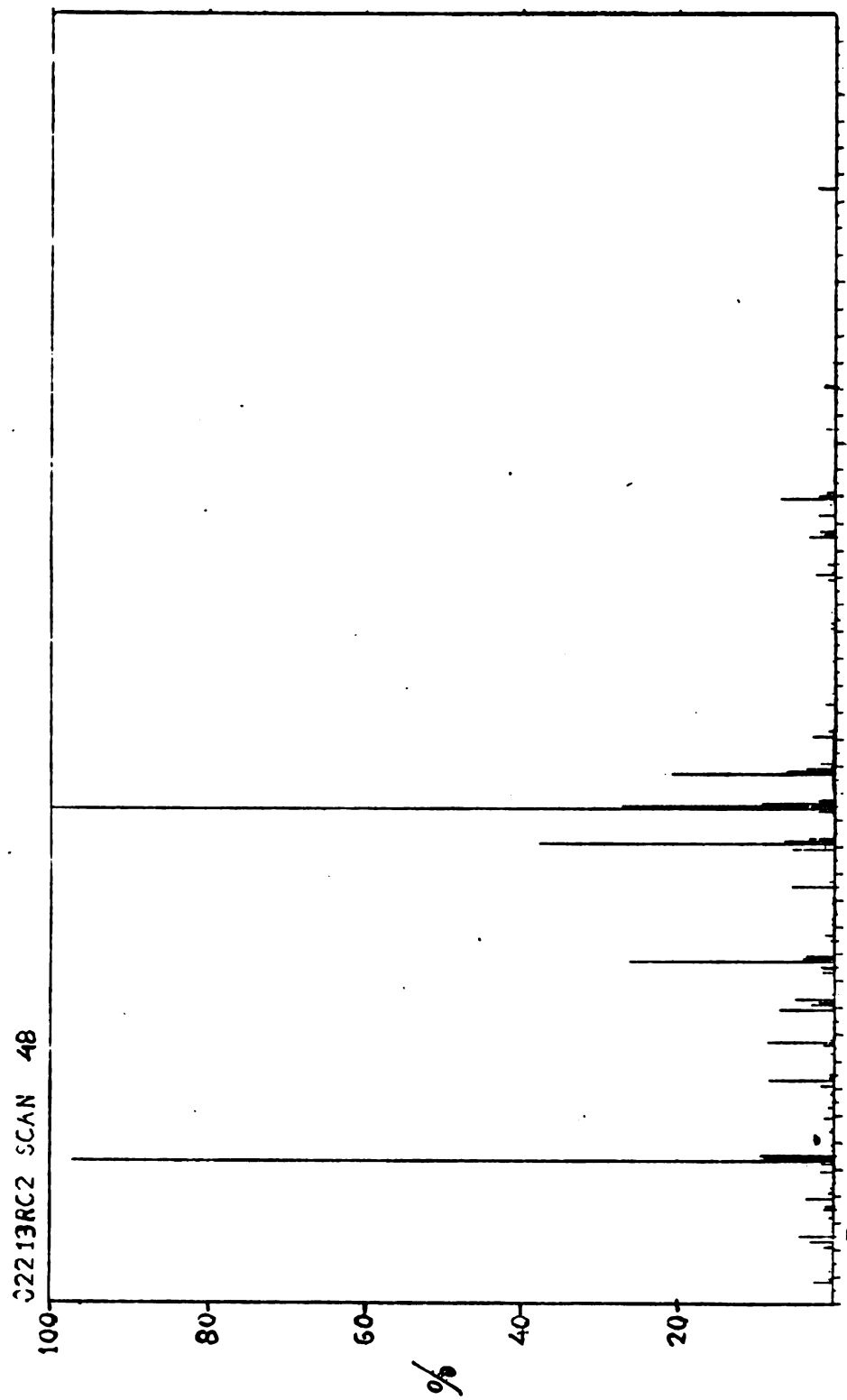


FIGURE 21: The mass spectrum from scan 48 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

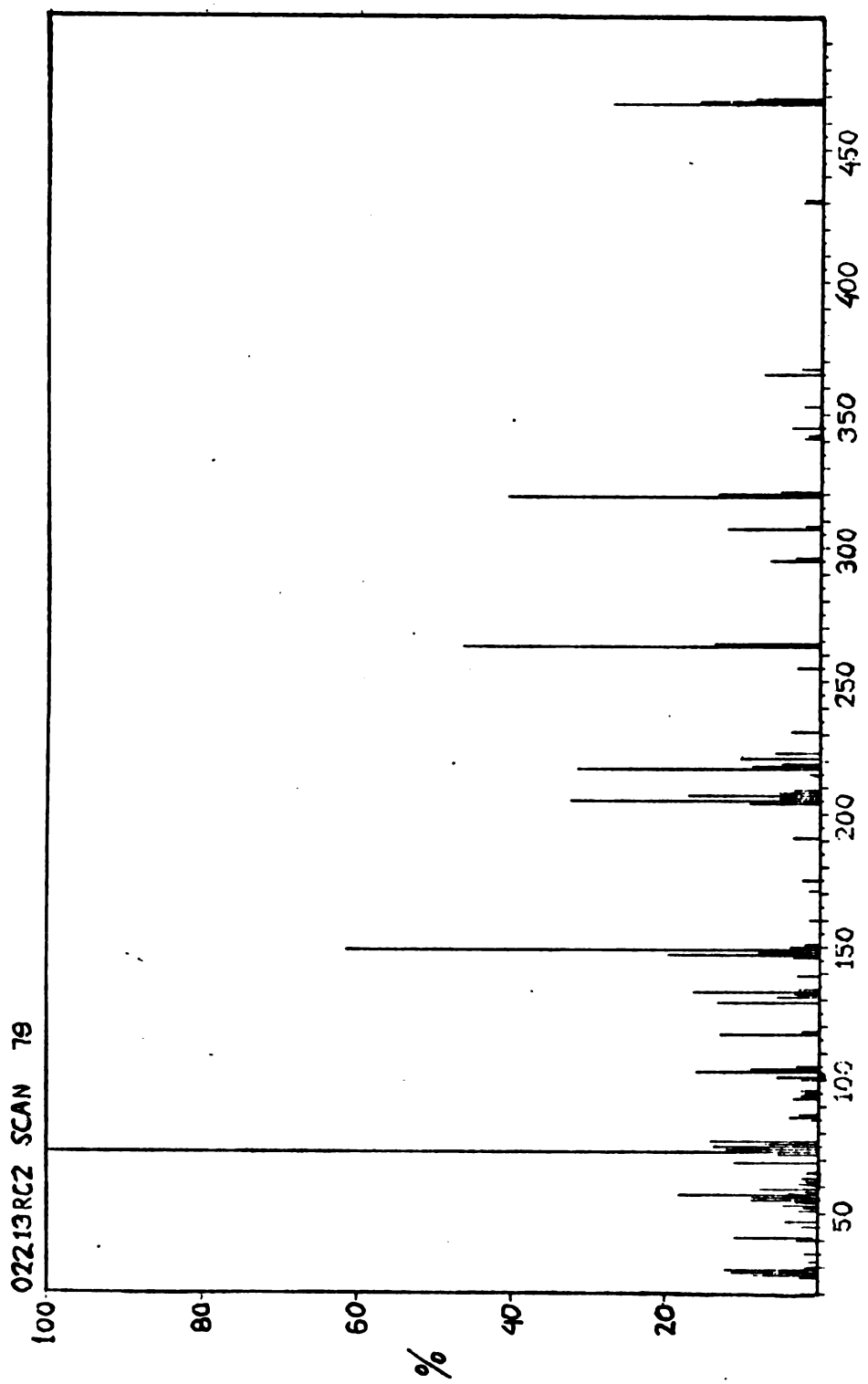


FIGURE 22: The mass spectrum from scan 79 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

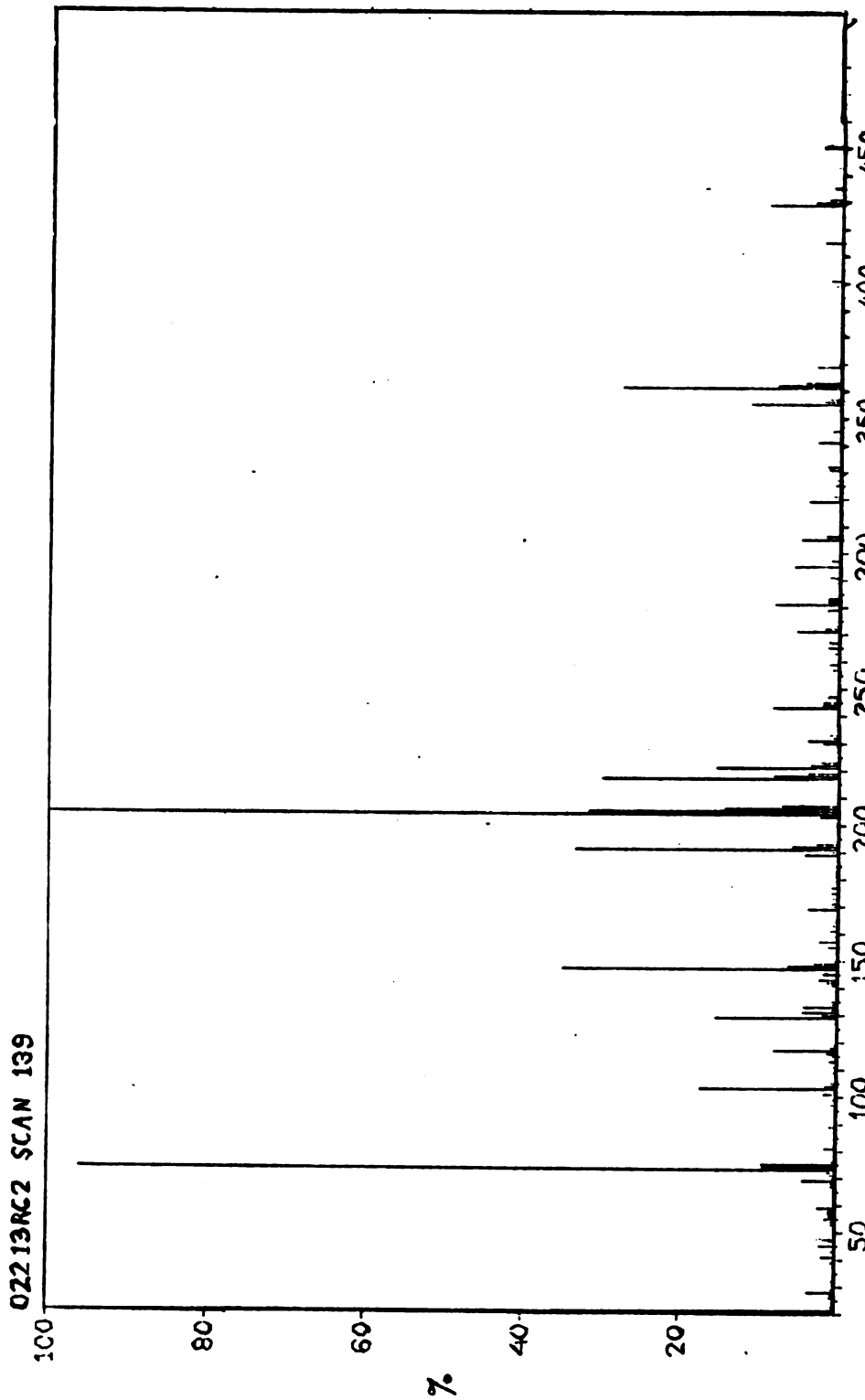


FIGURE 23: The mass spectrum from scan 139 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

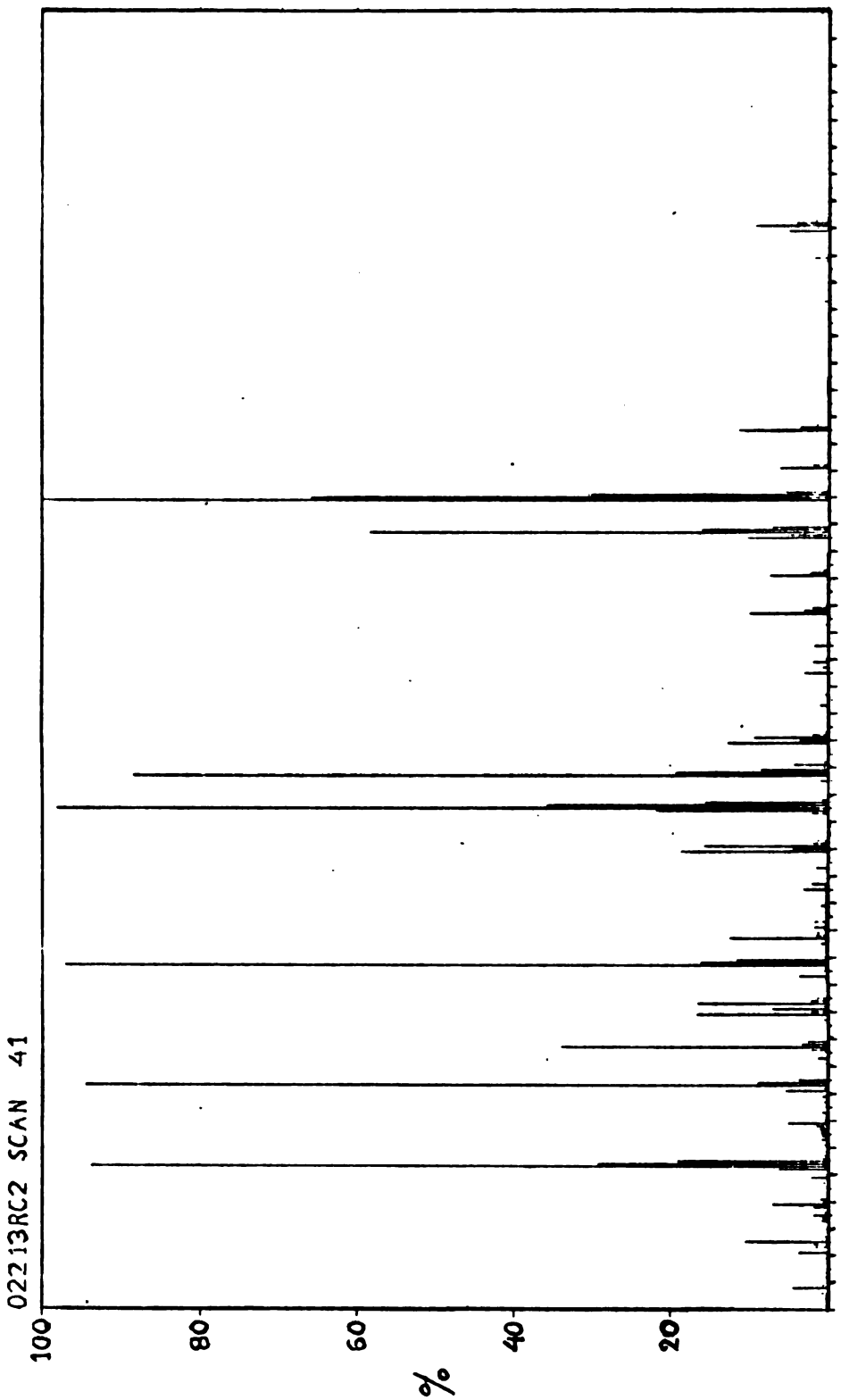


FIGURE 24: The mass spectrum from scan 41 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

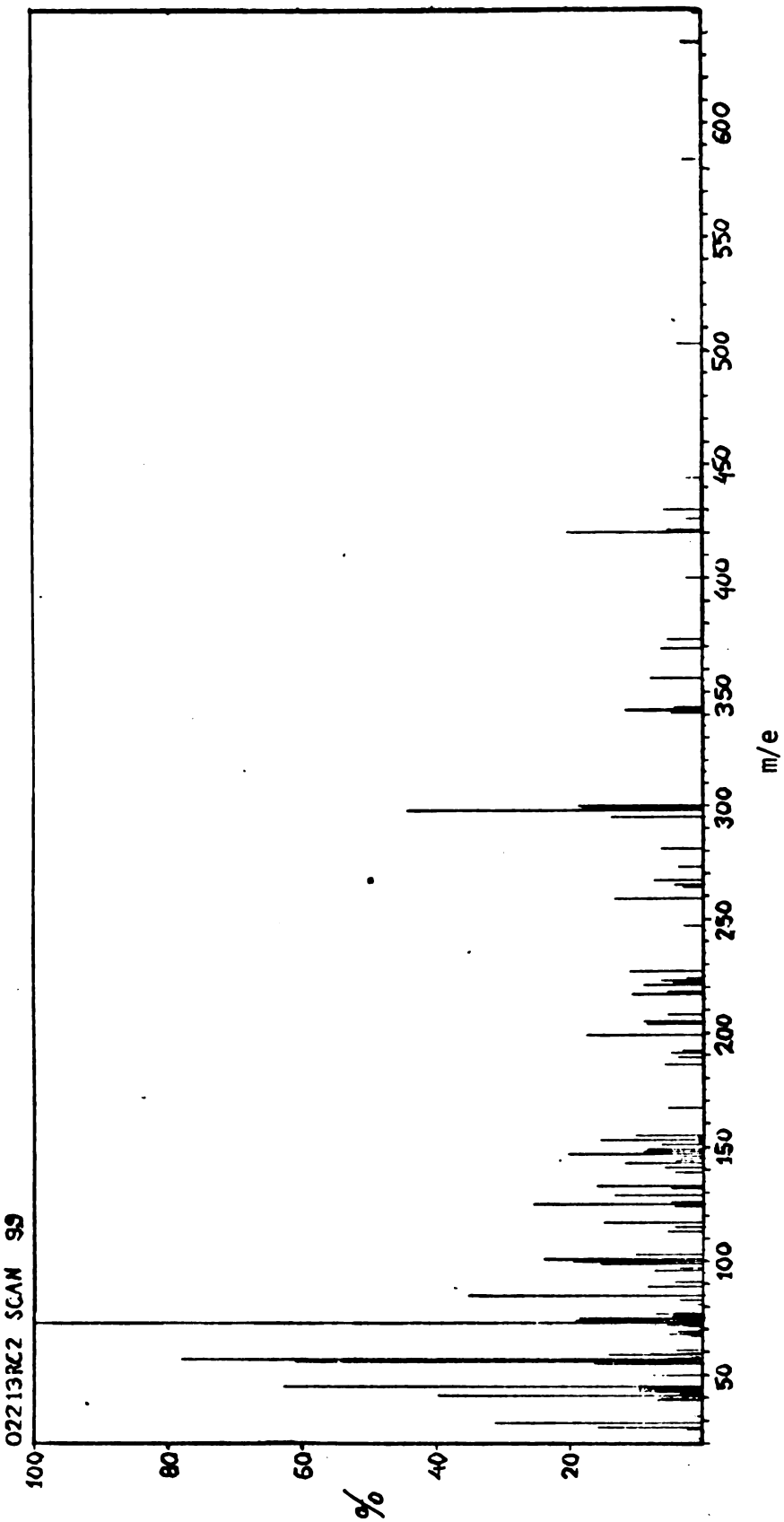


FIGURE 25: The mass spectrum from scan 99 (Figure 13) of the TMSi derivatives of methyl glycosides prepared from the bovine mammary gland acetyl-CoA synthetase.

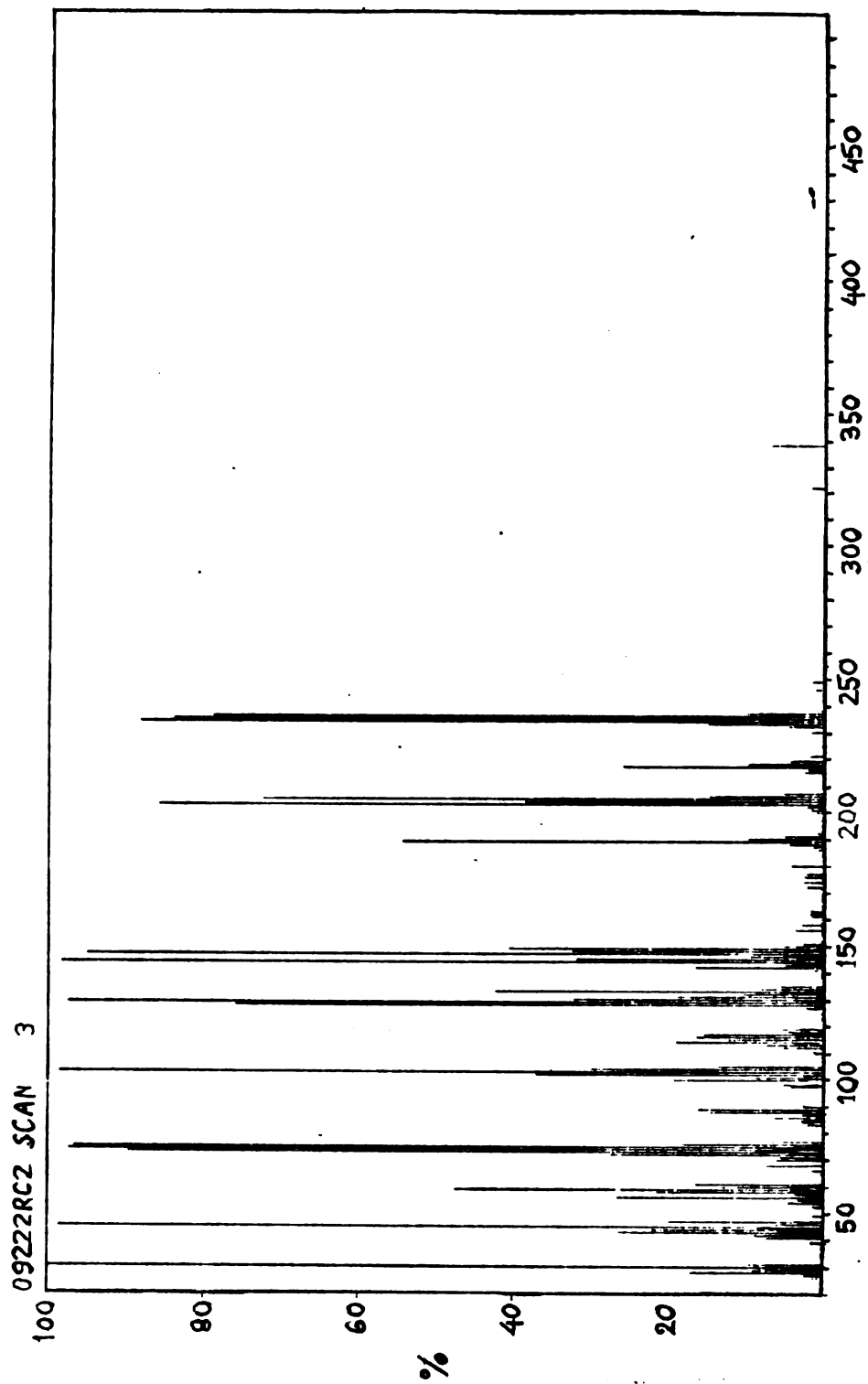


FIGURE 26: The mass spectrum from scan 3 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

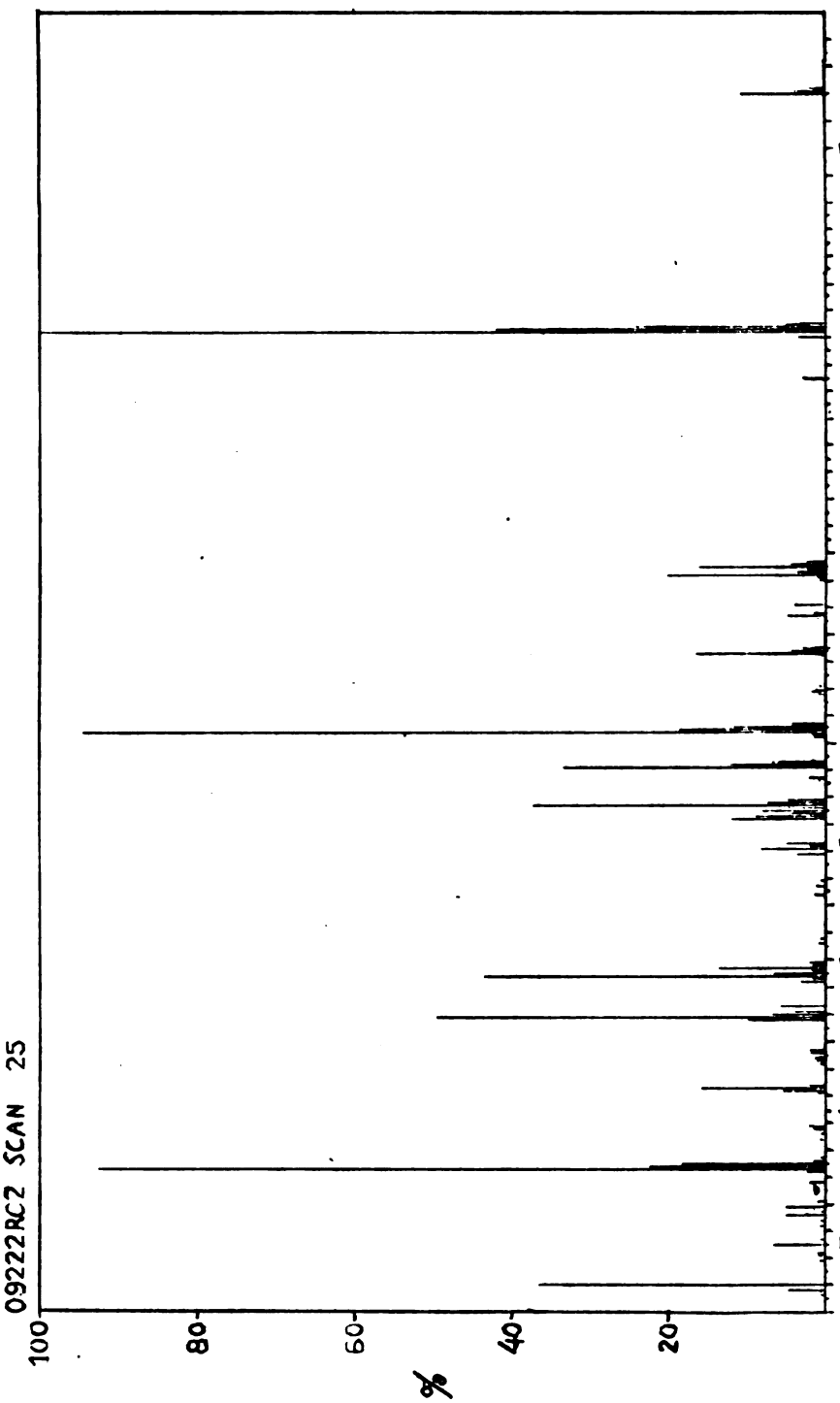


FIGURE 27: The mass spectrum from scan 25 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

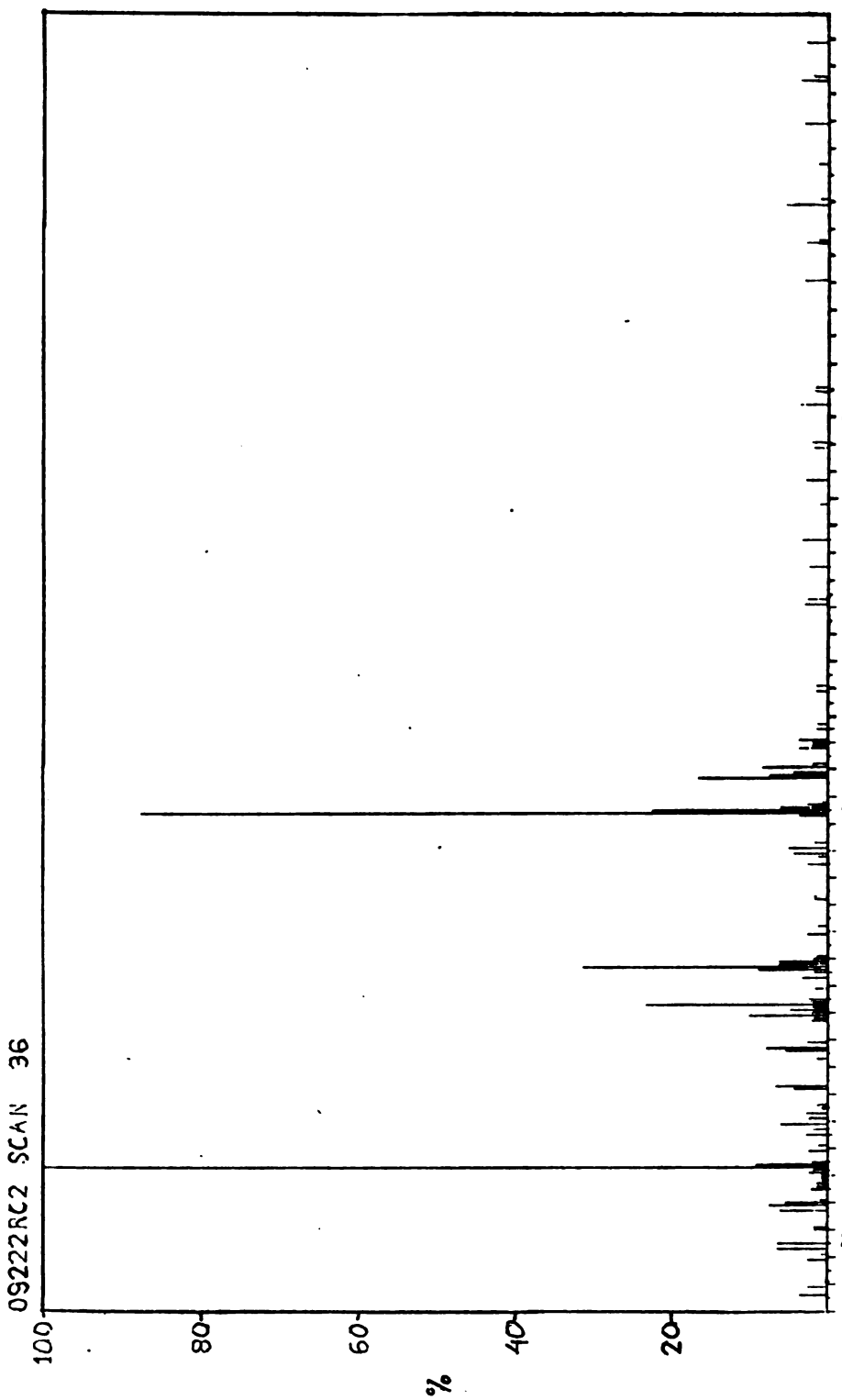


FIGURE 28: The mass spectrum from scan 36 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

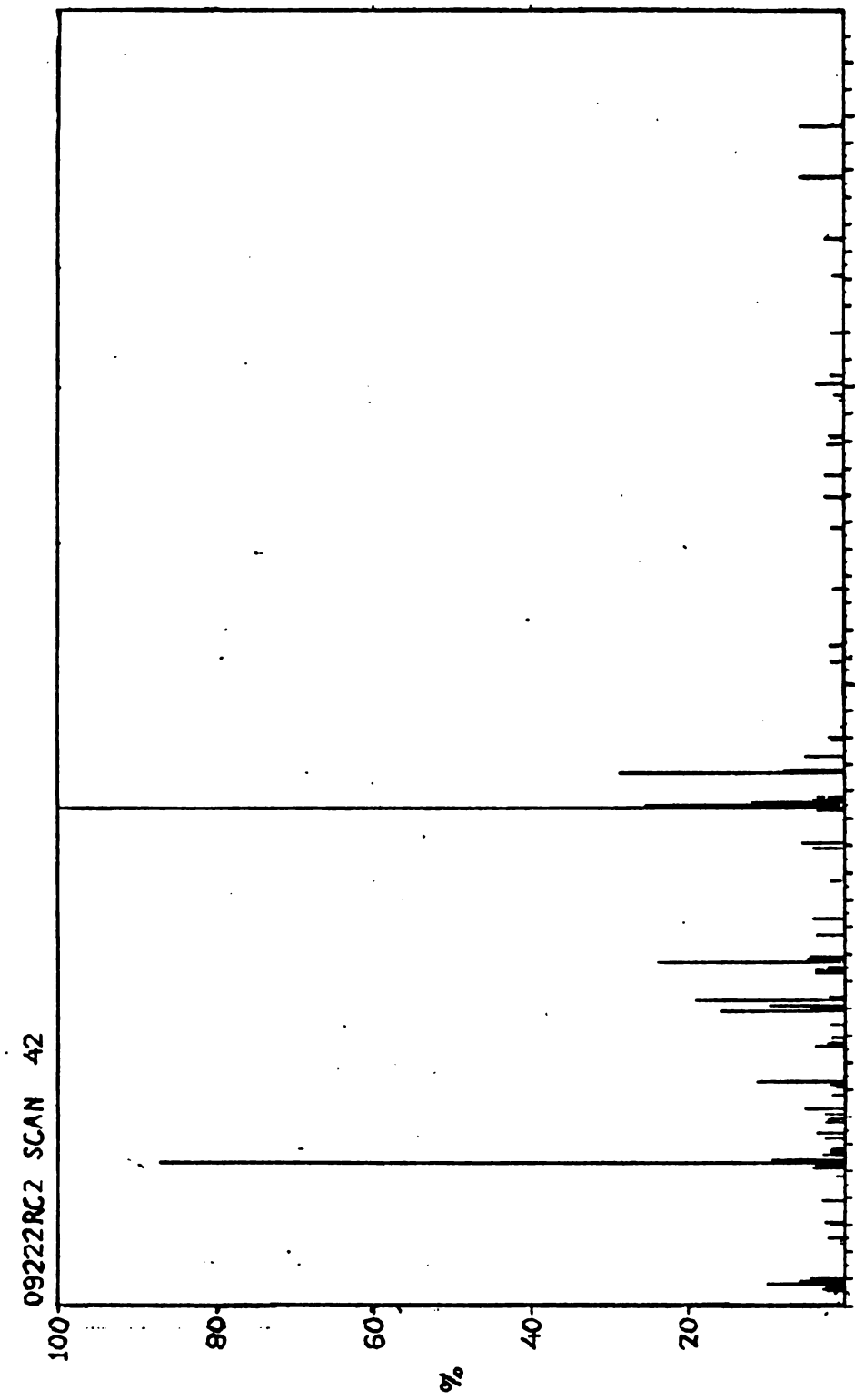


FIGURE 29: The mass spectrum from scan 42 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

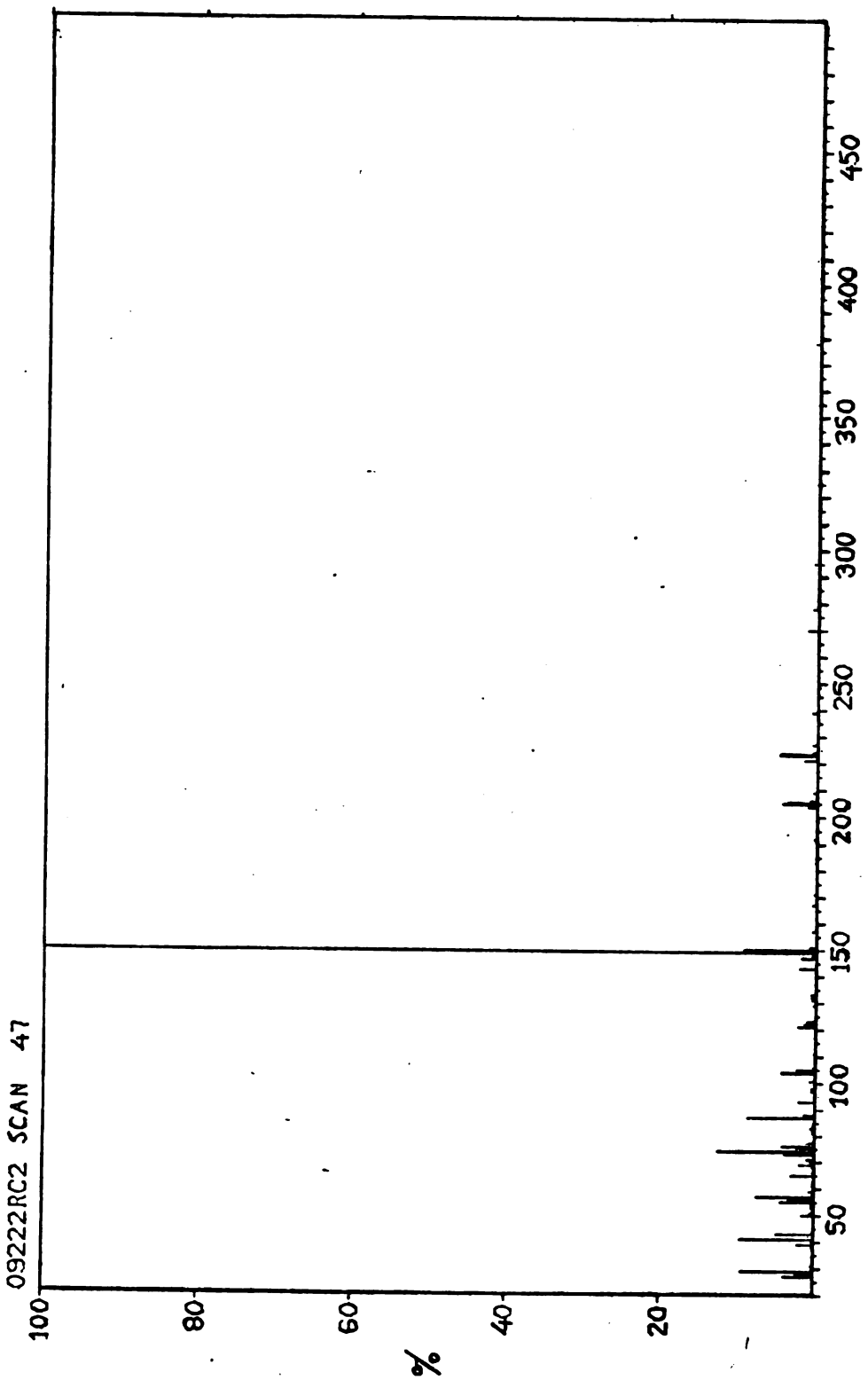


FIGURE 30: The mass spectrum from scan 47 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

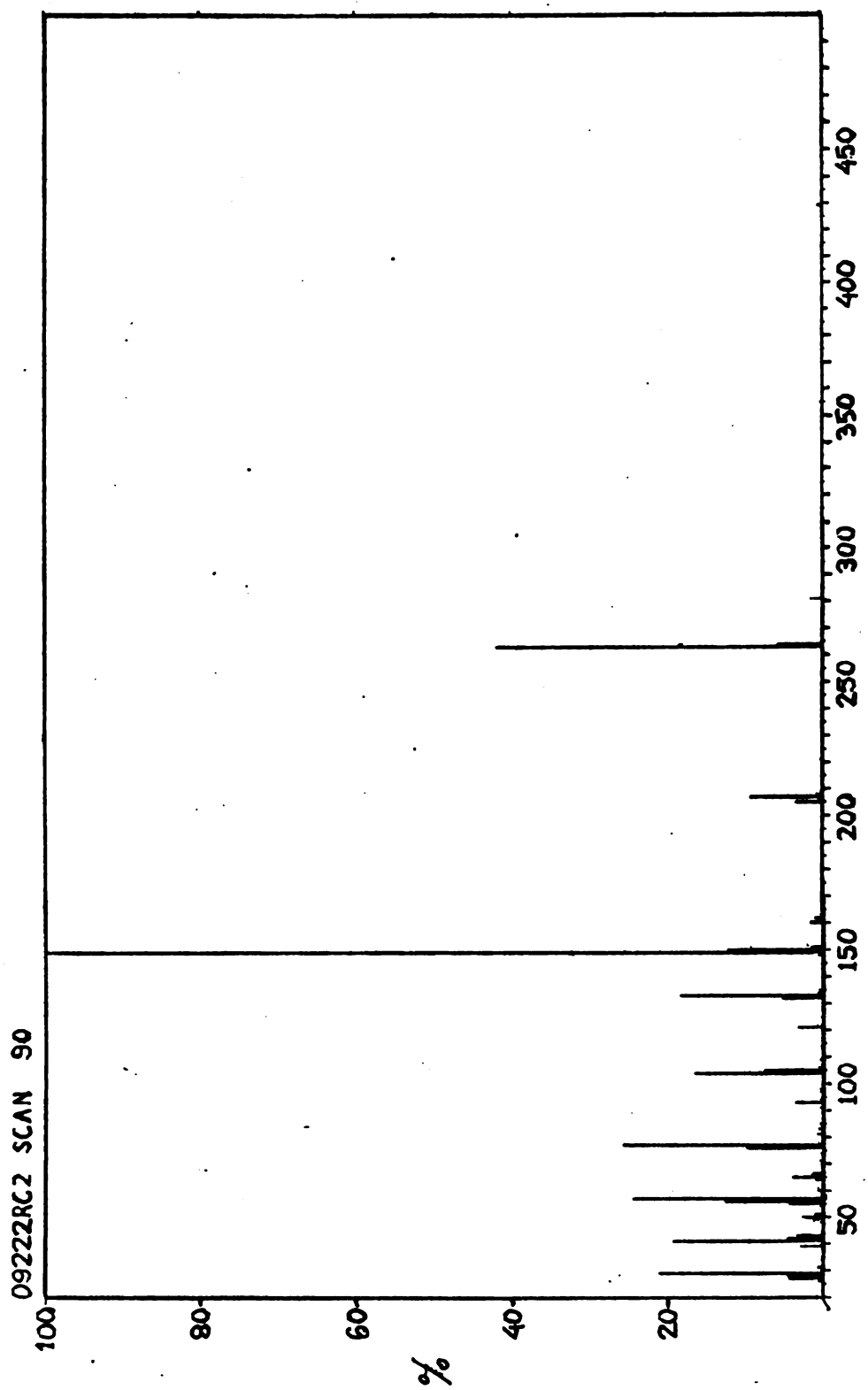


FIGURE 31: The mass spectrum from scan 90 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

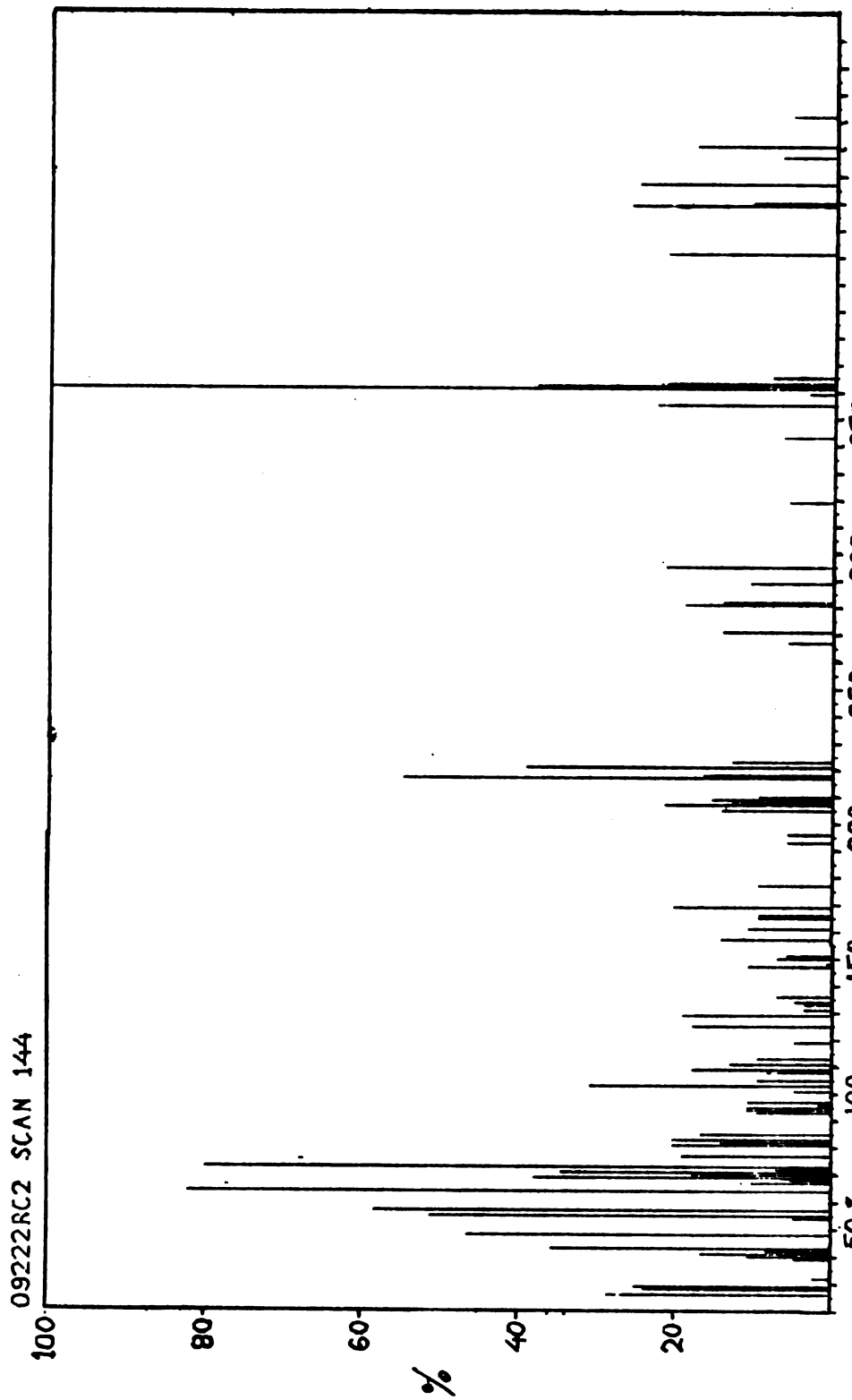


FIGURE 32: The mass spectrum from scan 144 (Figure 14) of the TMSi derivatives of methyl glycosides prepared from the goat mammary gland acetyl-CoA synthetase.

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