STUDIES ON THE GLYCOPROTEINS LEVEL IN RAT MAMMARY GLAND DURING LACTATION

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

in

DAIRYING

(DAIRY CHEMISTRY)

TO THE KURUKSHETRA UNIVERSITY,

KURUKSHETRA

BY

BENOY KRISHNA DATTA

DIVISION OF DAIRY CHEMISTRY

NATIONAL DAIRY RESEARCH INSTITUTE

(Indian Council of Agricultural Research)

K A R N A L (Haryana)

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DEDICATED TO MY PARENTS

ACKNOWLEDGEMENT

I take this unique opportunity to express my deep sense of gratitude and sincere thanks to Dr. Ajit Singh, Scientist S-1 (Radio-Tracers) Divn. of Dairy Chemistry, National Dairy Research Institute, Karnal, for his benevolent guidance, sustained encouragement, constructive criticism and valuable suggestions throughout the course of the present investigation.

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This is to certify that Shri Benoy Krishna Datta of National Dairy Research Institute, Karnal participated in the planning of this study, carried out the experimental work involved, analysed the data and prepared this report on "Studies on the glycoproteins level in rat mammary gland during lactation". He did this in partial fulfilment of the requirements for the degree of Master of Science in Dairying (Dairy Chemistry) of Kurukshetra University, under my supervision. Help and assistance given by individuals as well as Institutions in the execution of the work has been suitably acknowledged.

Ajit Singh

(AJIT SINGH)

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CHAPTER I

INTRODUCTION

The glycoproteins are important components of various cellular organelle like golgi apparatus, plasma membranes and various enzymes involved in milk synthesis. These also play an important role in mammary gland development. Milk is known to contain glycoproteins like K-casein. Since they are of mammary origin, the synthesis of glycoproteins must then be operated in the mammary gland.

The appearance of glycoproteins in milk may be either by transport from the blood or by synthesis in the mammary gland. Thus such plasma glycoproteins as transferrins, immunoglobulins and α-acid glycoproteins may be entering directly from blood. On the other hand, there is an increasing realization that a number of important proteins synthesized in the mammary gland, such as K-casein, α-lactalbumin and β-lactoglobulin, may occur both with or without carbohydrate moieties. Thus the characterization of these glycoproteins is becoming increasingly important subject. Various Glycoproteins occur in milk, of course the most predominent one is K-casein. Another group of proteins known as immunoglobulins are also present in milk and they contain carbohydrate groups. A glycoprotein designated glycoprotein-a isolated from bovine milk by

Groves and Gordon (1967) is present in both the free form and bound to immunoglobulin IgA of which it constitutes the distinctive "Secretory piece" responsible for transfer of IgA into secretions (Butler et al.,1968). Acidic Glycoproteins designated M-1 glycoproteins have been isolated from bovine colostrum and characterized by Bezkorovainy and Grohlich (1969).

The stabilizing effect is the most important function of the carbohydrate groups of glycoproteins. However, the removal of sialic acid residues from glycoprotein causes the loss of its biological activity. The higher molecular weight and high-content of sialic acid causes the lubricating effect.

The glycoproteins play an important role in infant growth. Mucin, a type of glycoprotein improves the absorption of fat and protein and increases the intestinal bifidus flora of the infant. Further the presence of glycoproteins in the diet enhances the activity of neuraminidase which helps to digest these proteins. Milk is big repository of glycoproteins, many of which are synthesized in the mammary gland. Although extensive work has been done on the changes taking place in the synthesis of proteins during proliferation of mammary gland, very little is known about the changes taking place in glycoprotein synthesis under such physiological changes. The present study is aimed to elucidate the metabolic changes

taking place in the mammary gland with particular reference to glycoprotein profile at different physiological conditions induced by lactation.

CHAPTER II

CHAPTER II

REVIEW OF LITERATURE

Mammary gland is a unique tissue which through its prodigious synthetic activity synthesizes milk proteins of which casein is of utmost importance. The mechanisms and the celluler regulation of the protein synthesis in general is one of the most fascinating research topics of the day because of the paramount importance of the protein synthesis in the ultimate manifestation of integrity of a tissue.

Isolation, characterization and glycosylation of glycoprotein:

Toshisuka Kawasake (1972) and his coworkers observed that isolated nuclei from rat liver contained carbohydrates consisting mainly of glucosamine and mannose which are normally components of glycoprotein. Rat serum proteins were labelled by injecting L-aspartic acid 2, 3, ^{3}H to see whether the amino acid lined to carbohydrate through the aspartyl-glycosylamine linkage in serum glycoproteins is derived from aspartic acid or from asparagine during biosynthesis.

The presence of 5 sugars in glycoprotein isolated from murrah buffalo's colostrum was demonstrated by Jhingran and Mehra (1980). Chromatographic analysis

showed that bound sialic acid contained N-acetyl-neuraminic-acid and N-glycolyl neuraminic acid and a total of 18 amino acids. The glycoprotein did not contain glucose.

Major Glycoproteins from Bovine milk fat globule membranes were characterized by Douglas, et al.(1976). S.D.S. acrylamide gel electrophoresis followed by periodate-Schiff staining showed a pattern of at least six glycoproteins. Alternatively the glycoproteins can be obtained by extraction of an aqueous suspension of washed cream with chloroform-methanol. Amino acid and carbohydrate composition have been determined for the purified glycoprotein. The apparent molecular weight of the Glycoprotein-2 fraction varied from 20,000 to 69,000. Serine was the most predominant amino acid.

Bezkorovainy (1967) isolated M_1-glycoprotein by subjecting milk and colostrum to C.M. cellulose chromatography followed by DEAE Sephadex chromatography at pH 8.3 and subsequently characterized this glycoprotein. The fraction isolated from colostrum M_1-Glycoprotein showed 25% carbohydrate whereas the fraction from milk contained only 7%. This fraction isolated from both the sources showed the presence of N-terminal-phenyl alanine, leucine and threonine. By repeated chromatography on Sephadex G-75 column Bezkorovainy and Grohlier (1969) resolved M_1-glycoprotein from colostrum into two separate proteins. The higher molecular weight glycoprotein (mol. wt. 72,000)

contained 28.4% carbohydrate and had an adsorption maximum at 275 nm; the lower molecular weight component (mol. wt. 12,000) contained 39% carbohydrate. Periodate oxidation experiments showed that sialic acid was linked to galactose in both the protein fractions.

Nichol's et al.(1975) isolated several glycoproteins from human colostrum whey. The molecular weight of these proteins varied from 26,000 to 35,000 and carbohydrate content from 50 to 80%. Degradation of the carbohydrate moieties of these glycoproteins indicated that each contained non-identical carbohydrate chains.

Properties of Glycoproteins

Results of ultracentrifugation, electrophoresis and N-terminal-amino acid determination indicated that a M_1-glycoprotein isolated from each of 4 individual samples of bovine colostrum and one sample of pooled milk probably consist of a series of closely related molecular species (Bozkorovainy, 1967). Their average molecular weight was 10,000 and the high β values and negative specific rotation of the colostrum glycoprotein preparations indicated highly disorganized structure. Each fraction contained, galactose, hexosamine and sialic acid, while amino acid analysis revealed high percentage of glutamic acid, proline, threonine and isoleucine and usually low amounts of basic aminoacids.

Microheterogeneity and other properties were observed in major glycoprotein by Shimizu et al (1978). On dialysis, a major glycoprotein, CB-7+8-component, was selectively precipitated. This component contained 4.16% Hexose, 2.05% hexosamine and 2.08% sialic acid could associate with PAS I to VII and CB III and form a soluble complex.

Tsuda (1976) showed that human casein separated into 2 fractions P-1 glycoprotein and P-3 phosphoprotein. The latter was purified by Ca^{+2} precipitation while P-1 prevented this precipitation thus having a stabilizing effect. From the amino acid and sugar composition of both P-1 and P-3 and further fraction of P_3 it was shown that P-1 resembles cow K-casein and P-3 cow β-casein.

The evidence of a bond between aspartic acid and glucosamine and O-threonyl glycoside bond in human Lactotransferrin was established by Spik et al (1967) by the hydrolysis of human lactotransferrin by pronase.

Later on by Basch et al (1976) processed milk fat globule membrane proteins and showed the yield and recovery of protein at several stages in this procedure. After processing, the proteins were chromatographed on Sephadex G-200 and the proteins of the soluble fraction were eluted into three major fractions (A, B & C). These fractions were dialysed extensively against water at $4^{o}C$ and then

against ion exchange resin (Amberlite MB-1) to remove the processing material such as SDS. The three fractions were then lyophillized and stored at 4°C.

Glycoproteins in milk

A component 3, a whey glycoprotein was observed by Brunner et al (1970) from Bovine milk. All components were isolated from heated and unheated skim milk and confirmed as heat stable glycoprotein native to milk.

Carbohydrates of the glycopeptides released by the action of rennin on whole milk were characterized with the help of Gas liquid chromatography by Sinkinson and Wheelock (1970).

The protein in cheese whey obtained from milk other than sialoglycoprotein are flocculated and subsequently separated by ultrafiltration and finally concentrated to obtain a glycoprotein syrup.

Thompson and Brunner (1959) observed the ratios of hexosamine: hexose : fucose : Sialic acid were 1:2.5:0.65:1.8 in soluble membrane protein, 1:3.2:0.44:3.4 in Weinstein-fraction and 1:2.5:0.61:1.7 in protease peptone. The higher hexose and sialic acid content is characteristic of similar relationship in the blood proteins, and observation which suggests the possible origin of the glycoproteins of milk.

Sinkinson & Wheelock (1970) showed that the carbohydrates are one of the main factors in determining the heterogeneity of K-casein and may possibly play a part in the action of rennin in the coagulation of milk.

Glycoproteins in other tissues:

Approximately 25% of the total glycoprotein carbohydrate recovered from rat brain by Javaid et al.(1975) is associated with mannose rich heteropolysaccharide chains. The carbohydrate content predominately consisted of mannose and N-acetyl glucosamine.

The glycopeptides can be separated from contaminating sialoglycopeptide by either column electrophoresis and gel filtration or by affinity chromatography on Concavalin A Sepharose (Eric & Javaid, 1975).

Later on Ashwell et al (1974) showed that there are a number of glycoproteins which contain galactose as the penaltimate residue terminated by N-acetyl-neuramimic acid. However, due to the variation in the number of N-acetyl-neuramimic acid residues a microheterogeneity has been observed in these glycoproteins. They also generalized the role of the terminal carbohydrate residues of glycoprotein in determining their in vivo behaviour. Indeed, with the exception of transferrin, all asialoglycoproteins so far tested show a rapid uptake by the parenchymal liver cells.

Later on Preferential synthesis of β-Lactoglobulin by the bound polyribosomes of the mammary gland was observed by Pierre Gaye and Robert Denamur (1970).

CHAPTER III

MATERIALS AND METHODS

Lactating rats, 1, 10, 20 and 28 days post partum, normal and pregnant rats were collected from the small animal house, maintained at the Institute. Mammary gland was isolated after anaesthetizing the animals with chloroform and kept in ice till use. A part of the gland was minced and homogenized in different buffer systems or distilled water as per requirement. The homogenate was filtered through muslin cloth and used for analytical studies.

I. A. Estimation of total protein

Total protein in the mammary-homogenate was estimated by the Folin's method (Lowry et al, 1951).

B. Estimation of Sialic acid

The sialic acid in the mammary gland was estimated by the thiobarbituric acid assay method of Warren (1959) with suitable modifications. One ml of mammary homogenate was mixed with 1 ml of 0.1N H_2SO_4. The mixture was hydrolysed at 80°C for 1 hr and cooled. To this 0.2 ml of N/10 NaOH was added. After thorough shaking it was centrifuged for 10 minutes. The supernatant was collected in a test tube and the residue was resuspended in 2 ml of acetate buffer pH-4.5 and centrifuged for another 10 minutes. Both the supernatants were combined and 0.1 ml of chloroform was added to clarify the solution.

0.2 ml of the above solution was taken in a centrifuge tube and 0.1 ml of sodium metaperiodate was added. The mixture was incubated at room temperature for 20 mts and subsequently 1 ml of Na-arsenite and 3 ml of the thiobarbituric acid were added. The mixture was kept in boiling water bath for 15 mts, cooled the contents and 4.3 ml cyclohexanone was added. After thorough shaking it was centrifuged in the clinical centrifuge. The upper coloured layer was collected in a test tube and the reading was taken in Klett Summerson using green filter. A blank was also run taking distilled water. The standard curve was prepared and the values were calculated taking the molecular weight of N-acetyl neuraminic-acid as 309.

Reagents

T.B.A. (Thiobarbutyric acid)

Dissolved 0.6 gm of TBA in 100 ml of warm distilled water containing 7.25 gms of sodium sulphate.

Sodium-arsenite Solution

Dissolved 10 gms of sodium arsenite in N/10 H_2SO_4 containing 7.25 gms of sodium sulphate.

Sodium meta periodate

In 3 ml of warm distilled water dissolved 1.079 gms of sodium-metaperiodate. The volume was made to 25 ml with orthophosphoric acid.

C. Hexose determination

Hexose content in the tissue was determined by the method of Dubois et al.(1956). 0.1 ml of mammary homogenate was taken in a test tube (0.1 ml water for blank) and 1 ml of 5% phenol was added which was followed by 5 ml of concentrated sulphuric acid.

After mixing, the tubes were kept in water bath at 20-30°C for 15 mts. The developed colour was read in Klett Summerson photocolorimeter using blue filter and standard curve was also prepared with glucose.

D. Estimation of Hexosamine

Hexosamine was estimated as per the method of Rimington (1940) with certain suitable modifications. To 0.2 ml Rat mammary gland homogenate was taken in a test tube to it 3 ml of 3 NHCl was added and hydrolysed in boiling water bath for 4 hours. The tube was cooled and neutralised with 3 N NaOH and diluted to 10 ml with distilled water, centrifuged for 5-10 mts. In 10 ml marked tube took one ml aliquot (1 ml of water for a blank) and added 1 ml of acetyl acetone reagent and mixed. The tubes were capped with marble and kept in boiling water bath for 15 minutes, cooled the same under tap water and added 5 ml of 95% ethanol and mixed properly. Then added 1 ml of Ehrlich's reagent, mixed and diluted to 10 ml with 95% ethanol. The reading was taken after 30 minutes in Klett-

summerson colorimeter at 530 mμ. The standard curve was drawn using glucosamine and values were calcuated.

Reagents

Acetylacetone:

One ml of acetylacetone was added in 50 ml of 0.5 N Na_2CO_3. The same was prepared freshly.

Ehrlich's reagent

To 0.8 gm of P-dimethylamino-benzaldehyde (recrystalized as the hydrochloride) dissolved in 30 ml of methanol and 30 ml of concentrated HCl. Mix methanol and concentrated hydrochloric acid in cold and then dissolved the reagent in the cold mixture.

E. Alkaline Phosphatase Estimation (Assay system)

The alkaline phosphatase was estimated using the procedure of Aschaffenburg (1953) with suitable modifications. 5 ml of buffer substrate (disodium paranitro phenyl phosphate) and kept in a water bath at 37°C. To this 3.5 ml of carbonate-bicarbonate buffer pH (10.0) was added. After 5 minutes 0.5 ml of mammary homogenate was added and the contents of the tube were mixed. The tubes were incubated at 37°C for 30 minutes. A corresponding blank was carried out by using boiled homogenate. The reaction was stopped by adding 1.0 ml of 28% T.C.A. (Trichloroacetic acid) and filtered through Whatman Filter paper No.40.

Five ml of the filtrate was taken in a centrifuge tube, 0.5 ml of 14% NaOH was added and centrifuged. The intensity of the colour developed was measured using blue filter.

Enzyme Unit:

One unit of alkaline phosphatase activity was equivalent to a preparation, liberating one µgm of P-nitrophenol in 30 minutes incubation period at 37°C.

Buffer substrate:

0.15 g of di-sodium-p-nitrophenyl phosphate was dissolved in carbonate bicarbonate buffer of pH (10.0) and the volume made to 100 ml.

F. Estimation of 5' nucleotidase

The assay system used to estimate the 5' nucleotidase was essentially the same followed by KobylKa, K & Carraway, K.L. (1973) with certain modifications. 0.6 ml of 100 mM Tris-HCL-buffer (pH 8.5) containing 10 mM 5 AMP (Adonesine Monophosphate) and 10 mM $MgCl_2$ was taken in a test tube and to it 0.4 ml of diluted enzyme solution was added and mixed. After incubation at 37°C for 20 minutes the reaction was stopped by the addition of 1 ml of 10% TCA. After filtering through Whatman paper No.40, the clear filtrate thus obtained was used for pi (Inorganic phosphorous) estimation using the procedure of Ames and Dubin (1960) as described for Thiamine pyrophosphatase determination.

G. Thiamine pyrophosphatase

The assay system used to estimate the Thiamine pyrophosphatase by the method of Cooper (1970). To a test tube containing 0.1 ml of 0.5 M Tris-Hcl buffer pH 7.8 was added 0.05 ml of $MgCl_2$, 0.1 ml of Thiamine pyrophosphatase (T.P.P.).

To this 0.2 ml of mammary homogenate was added and volume was made to 1 ml with distilled water. Zero time control was also performed by keeping the tube in ice. The contents of the experimental tube was incubated at $37^{o}C$ for 60 minutes and the reaction was terminated with the addition of 1 ml of 10% T.C.A. (Trichloroacetic acid). After centrifugation an aliquot of the supernatant solution was assayed for inorganic phosphate.

Phosphate Estimation

To 0.2 ml of filtrate was added 1.4 ml of a solution containing 1 part of 10% ascorbic acid and 6 parts of 0.42% Ammoniummolybdate in 0.5 M H_2SO_4 (mixed fresh daily). After incubation at $45^{o}C$ for 20 minutes the colour was extracted with 4 ml of isoamyl alcohol. The readings were taken at 660 mμ using Klett summerson colorimeter.

II. Gel filtration pattern of mammary gland glycoproteins using Sephadex Gel G-100

A 3x40 cm Sephadex G-100-column was prepared using phosphate buffer, pH 7.4 (Hortnagl *et al*, 1971). The column was equilibrated overnight with the same buffer. The homogenate has spun down in a clinical centrifuge and the supernatant was lyophillized. The lyophillized powder was dissolved in phosphate buffer, pH 7.4 (0.1 M) and used for gel filtration.

The eluted fractions were monitored for protein content either by the method of Lowry *et al* (1951) or were read in the Carlziss Spectrophotometer at 280 mμ.

III. SDS (Sodium Dodecyl Sulphate) polyacrylamide Gel electrophoresis:

Electrophoresis was conducted in alkaline systems using the method of Weber & Osborn (1969). A 7% polyacrylamide gel was prepared according to the method of Takajama *et al*, 1966.

(a) Gel buffer:- 0.1 M sodium phosphate buffer in 0.2% SDS.

(b) Electrophoresis buffer:- 0.1 M sodium phosphate buffer was diluted in 1:1 ratio and made to 0.1% with SDS.

(c) Acrylamide solution:- 22.2 gms of acrylamide and 0.6 gms bis-acrylamide were dissolved in 100 ml distill water. The solution was filtered and stored in cold and dark.

(d) Ammonium per sulphate solution

8 mgm of ammonium per sulphate was dissolved in 1.5 ml of gel buffer.

Acrylamide solution was prepared by mxing solution (a)-18.8 ml, soln (c) -9.7 ml, N, N, N', N'-tetramethyl ethylenediamine (TEMED)-0.045 ml and 1.5 ml of solution (d). After mixing thoroughly the solution was poured carefully into glass tubes covered at one end with parafilm. Distill water was then carefully layered over the acrylamide solution and the tubes were polymerized by incubating either at 50°C for 15 minutes or at room temperature for 1 hour.

The tissue samples which were quite insoluble in conventional aqueous buffer systems were solubilized and electrophoretically assayed accordingly to the method of Weber and Osborn(1969).

Solubilization of Protein

The proteins were incubated at 37°C for 2 hours in 0.01 M Sodium phosphate buffer pH 7.0, containing 1% SDS and 1% mercaptoethanol. The protein concentration was usually between 0.2 and 0.6 mg per ml. After incubation the protein solution was dialysed for several hours at room temperature against 500 ml of 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1% SDS and 0.1% mercaptoethanol.

The polyacrylamide gel electrophoresis was carried out using 1:1 dilution phosphate buffer. A prerun of 20 min was given before applying the sample. After giving the prerun about 50-70 µl. of protein sample was layered on the gel tops. The electrophoresis was conducted for 8 hours at a constant current of 6 MA/tube.

Detection of Glycoprotein

Carbohydrates were detected in the gel following the procedure of Fair Bank et al., 1971.

The SDS present in the gels was removed by employing the following steps. The gels were placed in slotted glass tubes and washed with (i) 25% isopropylalcohol in 10% acetic acid for 48 hours with constant stirring; (ii) 10% isopropyl alcohol in 10% acetic acid for 10 hrs (iii) 10% acetic acid for 12 hrs.

The gels were then stained for carbohydrate by treating the gels by pouring 0.5% periodic acid for 2 hours, 0.5% sodium arsenite in 5% Acetic acid for 60 minutes; 0.1% Sodium arsenite in 5% Acetic acid (repeated twice); 5% acetic acid for 20 minutes and finally with periodic acid Schiff reagent for 12 hours.

The destaining was carried out with 0.1% Sodium metabisulphite in 0.01N HCl for several hours until the ringe solution failed to turn pink with the addition of formaldehyde.

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CHAPTER IV

RESULTS AND DISCUSSION

Effect of lactation on the total protein content of rat mammary gland

Changes in the protein content of mammary tissue as affected by lactation is delineated in Table 1.

Table 1

Changes in Protein content of Mammary tissue as affected by lactation

Physiological condition	Protein content mg/gm tissue
(a) Normal	55
(b) Pregnant	70
(c) Lactating	
i) 1 day	82
ii) 10 days	91
iii) 20 days	70
iv) 28 days	60

Values are given as average of five determination.

Protein content (mg/g mammary tissue) was lowest for non-pregnant rat. Protein content was higher in pregnant rats than in non-pregnant rats. Lactation resulted in significant increase in the protein content. Analysis of mammary tissue at different stages of lactation showed that the protein content increased upto 18 days, which more or less corresponded to the peak lactation period in rat. Thereafter the protein content decreased and on 28 days of lactation it was almost similar to the level as in non-pregnant rat.

Level of carbohydrate content of rat mammary gland at different physiological conditions

Table 2 shows the changes in carbohydrate contents of rat mammary gland under normal, pregnant and lactating conditions. The levels of all constituents, namely sialic acid, hexose and hexosamine, were considerably higher in lactating mammary tissue. Since these three are important constituents of glycoproteins, the observation would suggest that lactation is accompanied by an increase in the level of glycoproteins of mammary gland. This is in consistent with the role of glycoproteins as cell receptors which increase during the proliferation of mammary cells on the onset of lactation (Keenan et al, 1970).

Table 2

Changes in carbohydrate contents of rat mammary gland as affected by Lactation

Physiological conditions	mg/g tissue		
	Sialic acid	Hexose	Hexosamine
Normal	1.23	2.14	0.02
Pregnant	1.31	2.28	0.03
Lactating	2.52	3.26	0.09

Values are given as average of five determination

Changes in carbohydrate constituents of rat mammary tissue during lactation

The level of carbohydrate constituents of mammary tissue at different stages of lactation is shown in Table 3.

Table 1

Changes in carbohydrate constituents of rat mammary tissue during lactation

Constituents (mg/gm tissue)	Stages of Lactation (days)			
	1	10	20	28
Sialic acid	1.45	2.52	1.56	1.61
Hexose	2.47	3.25	3.02	2.91
Hexosamine	0.04	0.09	0.06	0.05

Values are given as average of five determination

The sialic acid content increased from 1.45 mg/g tissue on the first day of lactation to 2.52 mg on the 10th day of lactation. Similar to sialic acid, hexose and hexosamine levels were also increased from 2.47 to 3.25 and 0.04 to 0.09 mg/g tissue, respectively. As the lactation progressed, the levels of all these constituents slowly decreased and reached to almost the same level as in normal mammary gland. This observation indicates that the level of glycoprotein content of rat mammary tissue is higher when milk synthesis activity of mammary gland is higher.

Marker enzyme levels in mammary gland during lactation

Plasma membranes and golgi vesicles take active role in regulating metabolic activity and post-synthetic modification of proteins, respectively, during the synthesis of milk in mammary gland. Hence it could be expected that levels of alkaline phosphatase and 5'-nucleotidase (marker enzymes for plasma membrane) and thiamine pyrophosphatase (marker for Golgi vesicles) in mammary gland would change as a result of lactation. Determination of activities of these enzymes in mammary glands of normal, pregnant and lactating rats showed that the levels of alkaline phosphatase and 5' nucleotidase increased as a result of pregnancy and lactation (Table 4). But the level of thiaminepyrophosphatase was higher in normal mammary gland than that of pregnant and lactating rat. The reason for the discrepancy is not clear.

Table 4

Changes in marker enzyme levels of rat mammary gland as affected by lactation

Physiological condition	Specific activity		
	Alkaline phosphatase	5' nucleotidase	Thiamine pyrophosphatase
Normal	9.61	3.86	6.52
Pregnant	13.12	4.01	5.36
Lactating	20.81	6.38	3.13

Values are given as average of five determination

Levels of some marker enzymes in rat mammary gland at different stages of lactation

The levels of alkaline phosphatase, 5'-nucleotidase and thiamine pyrophosphatase were higher at 10th day of lactation than on 1st day of lactation (Table 5).

Table 5

Changes in specific activities of marker enzymes in rat mammary gland during lactation

Enzyme	Stage of Lactation (days)			
	1	10	20	28
Alkaline Phosphatase	14.01	20.81	18.52	10.09
5'-Nucleotidase	4.40	6.38	6.01	5.17
Thiamine-pyrophosphatase	1.48	3.13	7.89	7.49

Values are given as average of five determination.

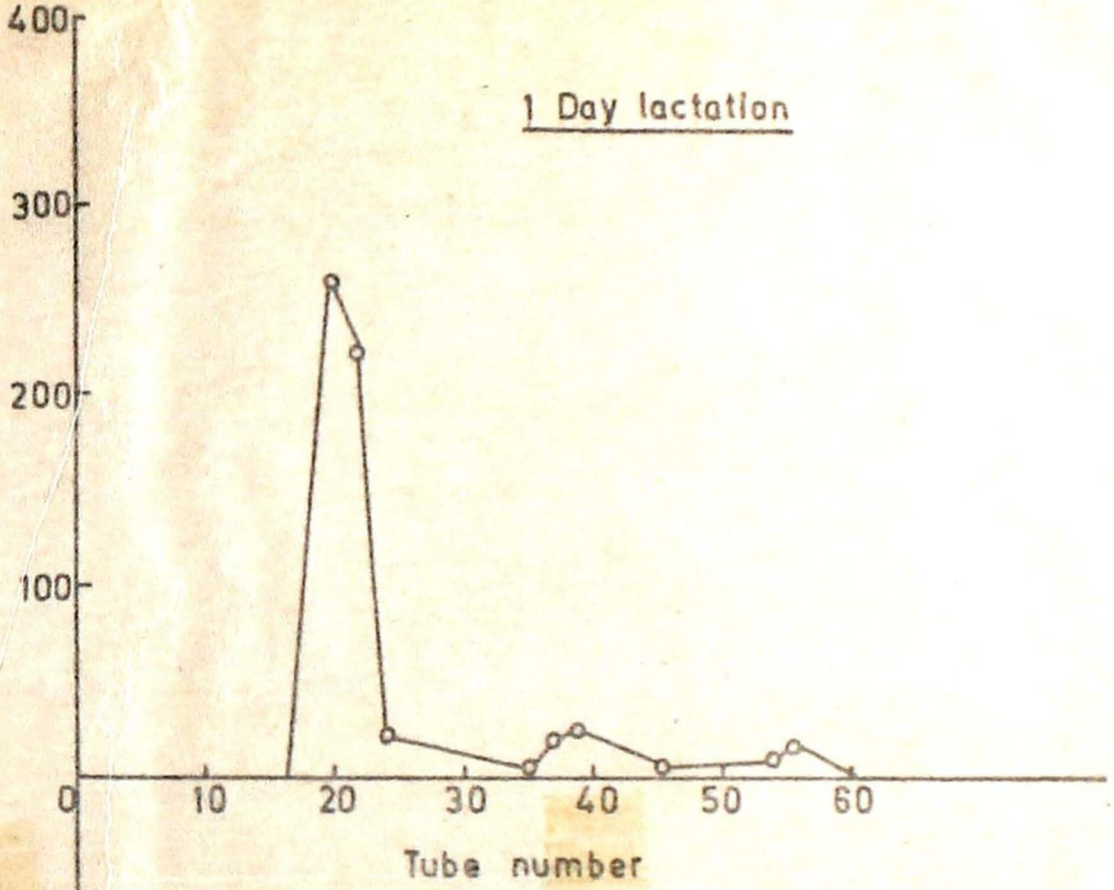
There was not much difference in the levels of alkaline phosphatase and 5'-nucleotidase between 10th and 20th day of lactation. But the levels of both these enzyme were lower on 28th day of lactation. Unlike these two enzyme levels, the level of thiamine pyrophosphatase considerably increased from 3.13 on 10th day of lactation to 7.89 on 20th day of lactation. Thereafter the level of this enzyme almost remained constant.

Sephadex G-100 gel filtration pattern of soluble proteins of mammary gland at different stages of lactation

The soluble proteins of mammary gland were prepared as given in Materials and Methods. Gel filtration of these proteins through Sephadex G-100 resulted in resolution of these proteins into three fractions from mammary gland obtained on 1st, 10th, 20th and 28th day of lactation (Fig. 1 and 2)

Amongst these three fractions, Fraction I contained highest amount of proteins at all stages of lactation. The amount of protein eluted in fraction II increased with increase in lactation. Fraction III was the minor one in all stages of lactation. Data on chemical analysis of these fractions for hexose and sialic acid are shown in Table 6. Amongst these fractions, Fraction II contained maximum amounts of hexose and sialic acid. It contained nearly thrice the amount of hexose and sialic acid then that of Fraction I and Fraction III. Moreover, the hexose and sialic acid contents of Fraction II increased substantially from 1st day of lactation to 10th day of lactation and thereafter a gradual decrease was noticed with advancement of lactation. This observation further supports the earlier observation made in this study that glycoprotein content in mammary gland is higher at the peak lactation stage. Hexose and sialic acid contents/mg protein in Fraction I & III did not vary much at different stages of lactation.

Fig-1


400
300
200
100
0
10
20
30
40
50
60
1 Day lactation
Tube number

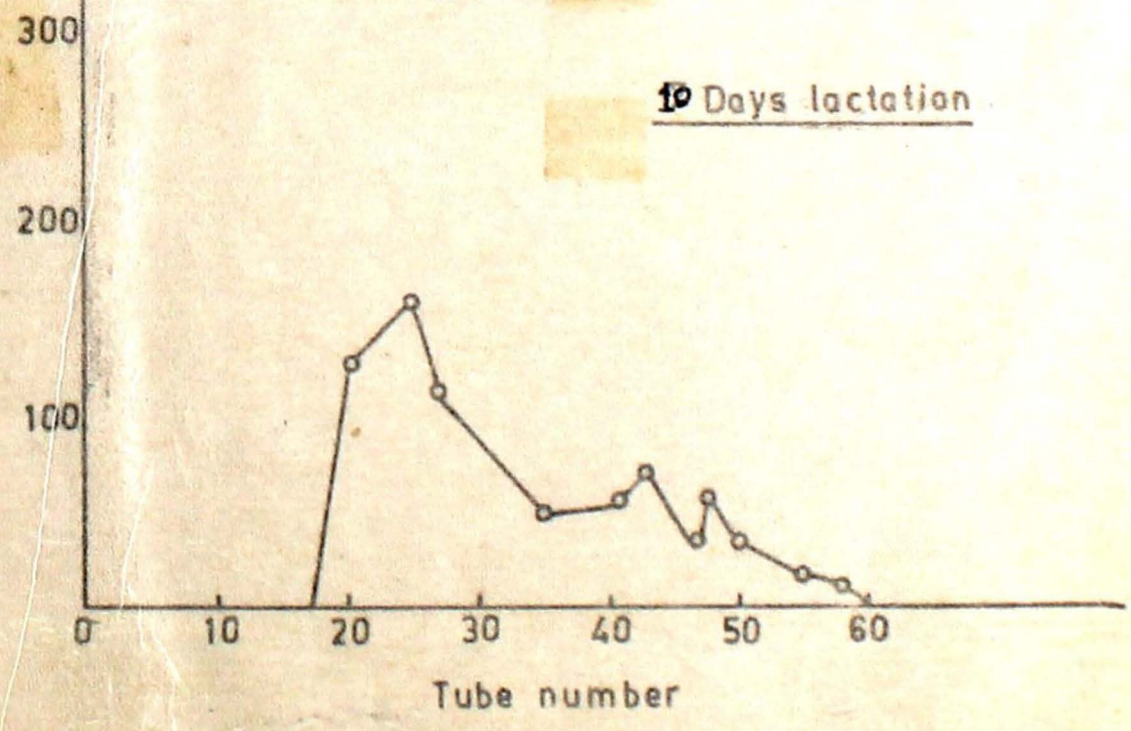

300
200
100
0
10
20
30
40
50
60
10 Days lactation
Tube number

Fig-1

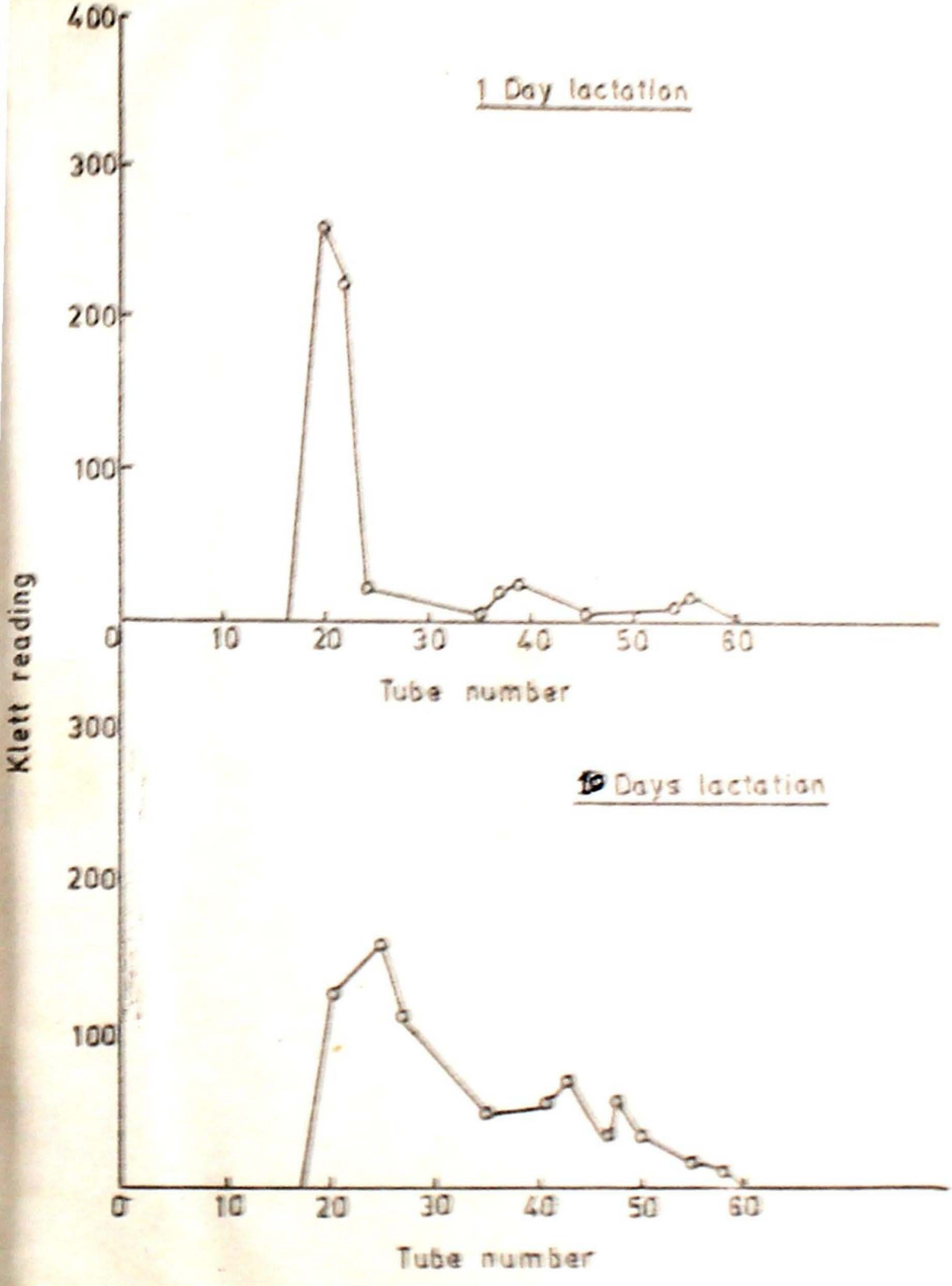

1 Day lactation
400
300
200
100
0
10
20
30
40
50
60
Tube number
Klett reading
10 Days lactation
300
200
100
0
10
20
30
40
50
60
Tube number

Fig-2

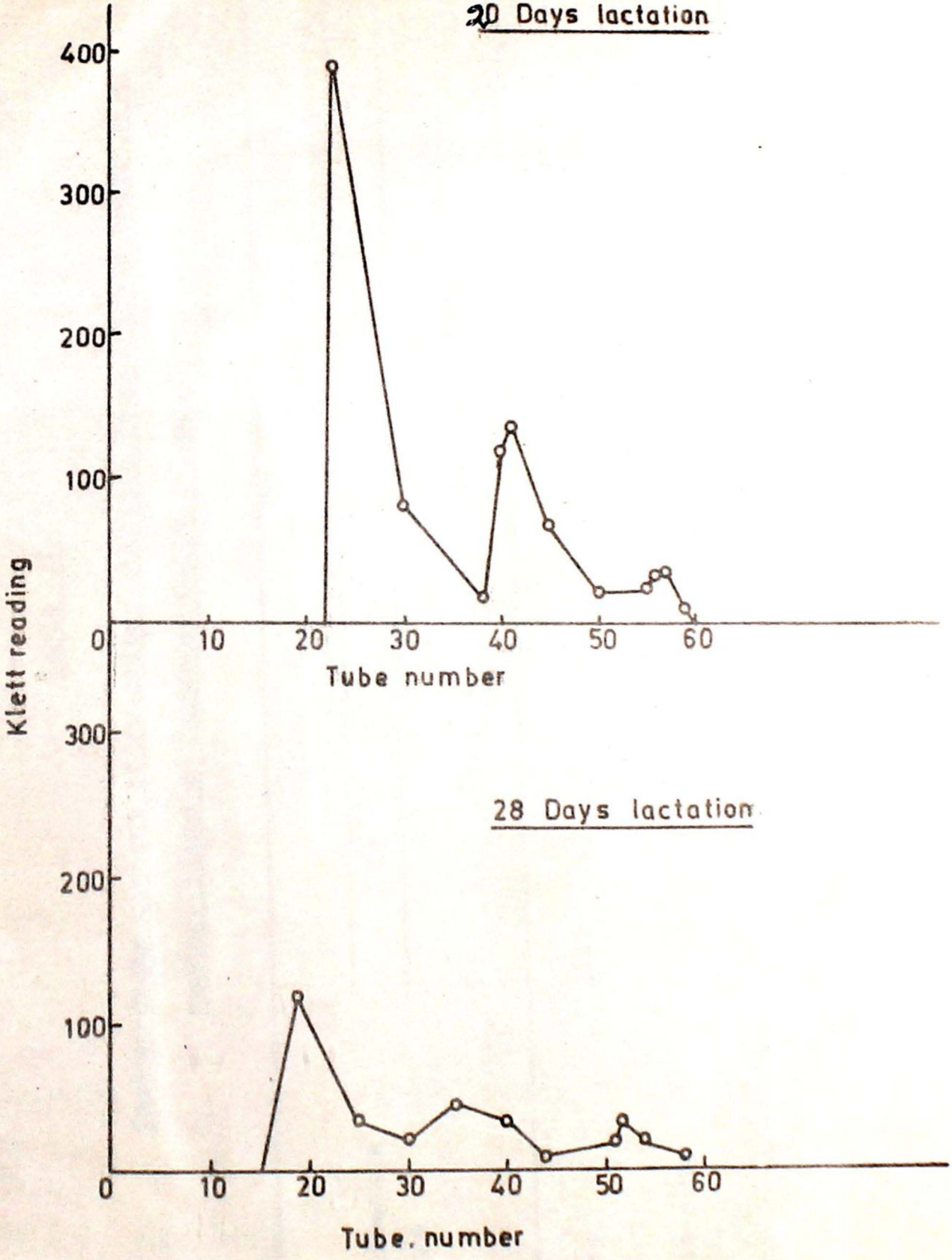

20 Days lactation
400
300
200
100
0
10
20
30
40
50
60
Tube number
Klett reading
300
28 Days lactation
200
100
0
10
20
30
40
50
60
Tube. number

Table 6

Carbohydrate content of fractions obtained from soluble proteins of mammary gland on Sephadex G-100 Column.

Constituents mg/g	Days of Lactation											
	1			10			20			28		
	Fractions											
	I	II	III	I	II	III	I	II	III	I	II	III
Hexose	21.2	45.1	18.6	26.4	72.1	28.1	24.2	52.6	21.2	22.4	50.2	19.6
Sialic acid	18.3	31.2	16.4	20.9	45.6	18.8	21.5	30.3	18.7	19.5	28.6	20.4

Electrophoretic pattern of glycoproteins of different fractions obtained on Sephadex gel column

Fig.3 reveals the electrophoretic pattern of glycoproteins of Fractions I, II and III obtained by molecular sieve chromatography. There were three common glycoprotein components in Fraction I isolated from mammary gland at Ist, 10th, 20th and 28th day of lactation. But samples from 10th day of lactation contained an additional low molecular weight glycoprotein component.

When Fraction II obtained at 1st, 10th, 20th and 28th days of lactation was subjected to electrophoresis, it was resolved into 4, 5, 4 and 4 glycoprotein components. The major component in all these patterns was a slow moving high molecular weight protein. This band was more thick on the 10th day of lactation than 1st day of lactation. The band became thinner as the lactation advanced. An additional fast moving glycoprotein band observed on 10th day of lactation was absent at early and late stages of lactation. The last fraction showed only a single glycoprotein band of which pattern remained almost constant at all stages of lactation.

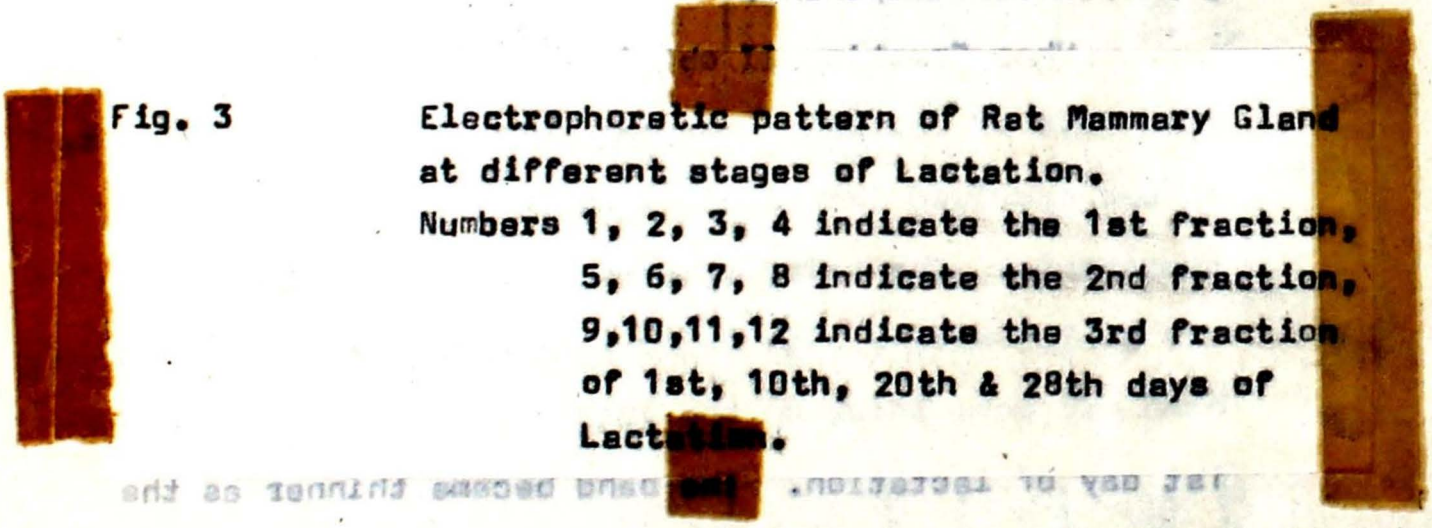


Fig. 3 **Electrophoretic pattern of Rat Mammary Gland at different stages of Lactation.**
Numbers 1, 2, 3, 4 indicate the 1st fraction,
5, 6, 7, 8 indicate the 2nd fraction,
9,10,11,12 indicate the 3rd fraction
of 1st, 10th, 20th & 28th days of Lactation.

Fig-3

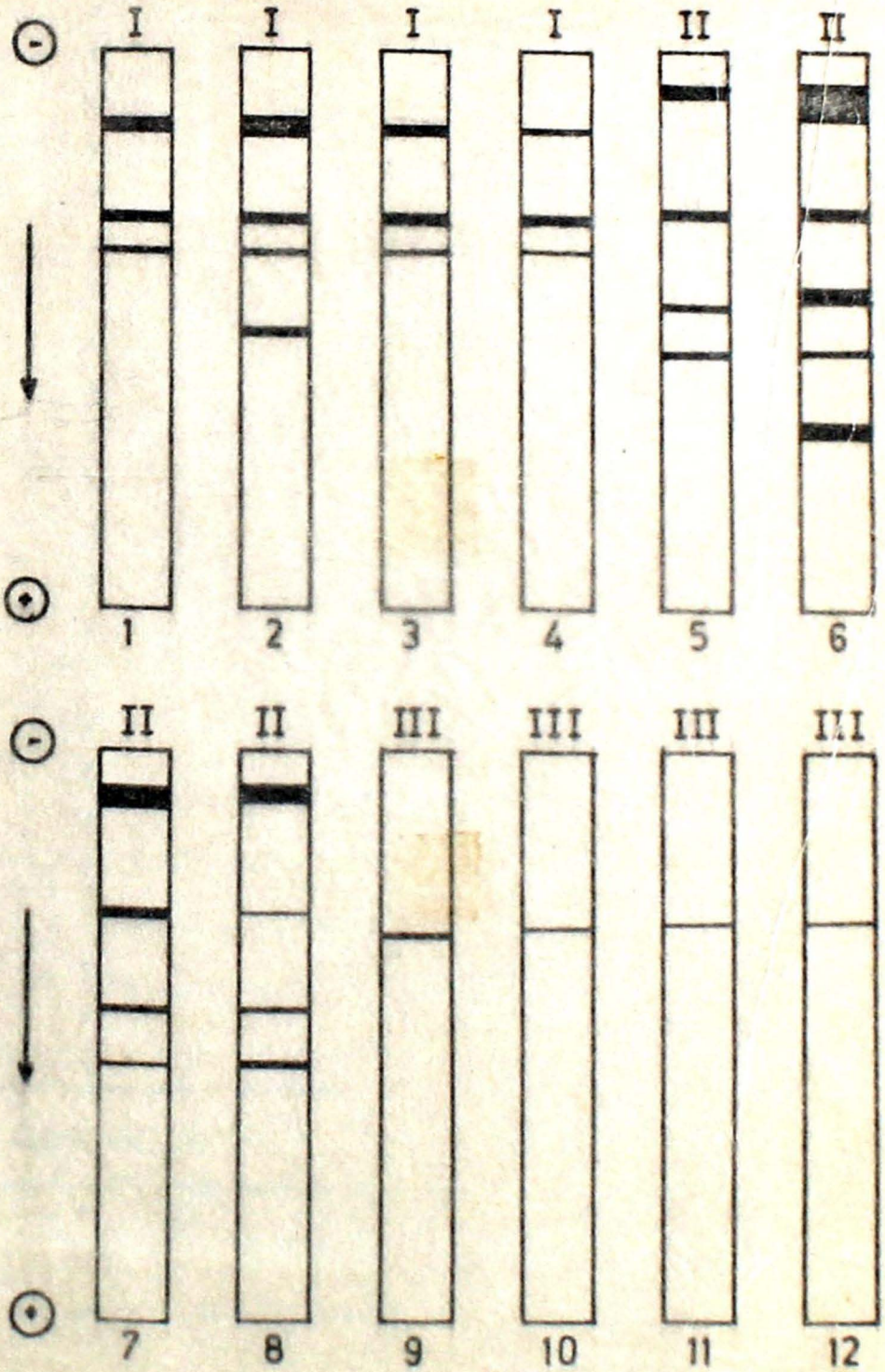
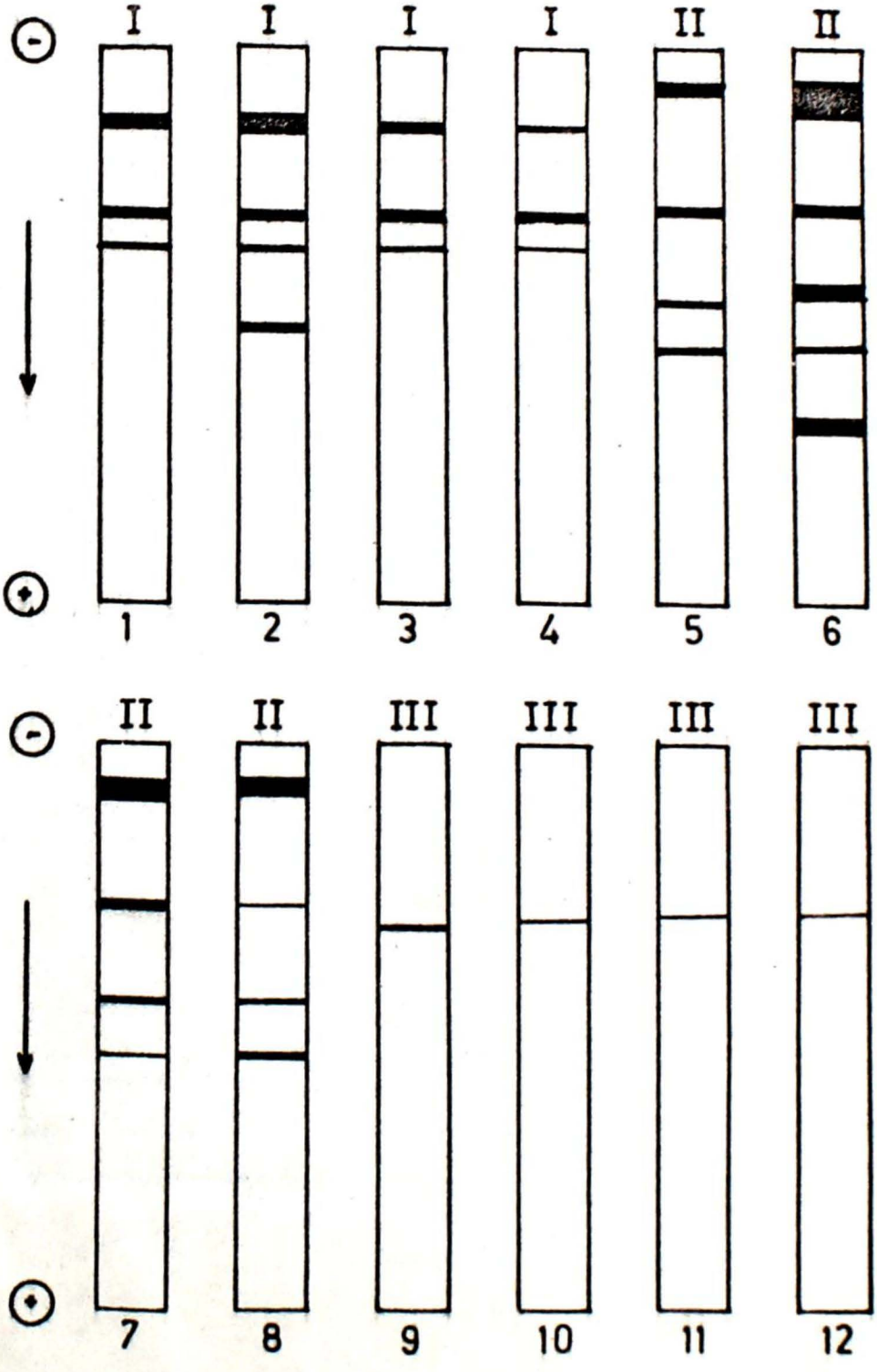


Fig-3



SUMMARY

In the present study, investigation has been made on the changes in glycoprotein content of rat mammary tissue as affected by lactation. Salient features of the study are delineated below:

1. The protein content/g of rat mammary tissue was affected by the stage of lactation. The protein content increased upto peak lactation, decreased gradually as lactation advanced and towards the end of lactation it was similar to the level as in normal female rat.

2. The level of all carbohydrates analysed (sialic acid, hexose and hexosamine/g of mammary tissue) were considerably higher in lactating mammary tissue.

3. The sialic acid, hexose and hexosamine content increased from first day of lactation to the 10th day of lactation, decreased as the lactation advanced, and towards the end of lactation, the level reached to almost the same as in normal mammary tissue.

4. The level of some marker enzymes namely alkaline phosphatase and 5 nucleotidase increased as a result of pregnancy and lactation. But the level of thiamine pyrophosphatase was higher in normal mammary tissue than in pregnant/lactating rat.

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4. The level of some marker enzymes namely alkaline phosphatase and 5 nucleotidase increased as a result of pregnancy and lactation. But the level of thiamine-pyrophosphatase was higher in normal mammary tissue than in pregnant/lactating rat.

5. The levels of alkaline phosphatase 5′ nucleotidase were higher at 10th day of lactation than at 1st day of lactation and remained constant upto 20th day of lactation. The thiamine pyrophosphatase activity increased considerably from 1st day to 20th day of lactation.

6. Sephadex G-100 filtration of soluble proteins from mammary gland resolved into three fractions irrespective of the stage of lactation. However, the proportion of these fractions varied with the stage of lactation.

7. Amongst the three fractions obtained on molecular sieve chromatography, Fraction I contained at least three glycoprotein components as revealed by electrophoresis at all stages of lactation.

8. Fraction II contained maximum amounts of hexose and sialic acid. On electrophoresis, it was resolved into 4, 5, 4 and 4 glycoprotein components at Ist, 10th, 20th and 28th day of lactation, respectively. The major component in all these electrophoretic patterns was a low moving high molecular weight protein. The concentration of this component was highest at 10th day of lactation.

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