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FINAL TECHNICAL REPORT

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Structural studies on phospho-
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information applicable to ex-
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DETAILED REPORT:

STRUCTURAL STUDIES ON PHOSPHOPROTEINS OF MILK

Under the tenure of the present programme, investigation was carried out only with respect to the main phosphoprotein of milk, namely casein. When this programme was started in 1963, the exact number of components in casein and their chemical nature was not well understood, although there were many reports on the physico-chemical characterisation of caseins. The investigation was initiated with a view to ascertain the number of components in casein and to study their chemical nature. Since the buffalo milk is as easily available in India, as the cow's milk and used extensively, it was thought worthwhile to extend the investigation on cow caseins to buffalo caseins as well. Such a study may provide data for comparison of the results as obtained by several investigators in the case of haemoglobins(1).

Further, a change in the composition of casein in the milk of these two species naturally results in a change in its physico-chemical properties. A study of this would afford a better understanding of the changes taking place in milk proteins during various processing operations.

The present programme of work concerning the structural studies on casein can be classified under three headings:

- (A) Attempts to obtain a simple and rapid method for the preparation of pure components of cow as well as buffalo caseins.
- (B) Comparative studies on the physico-chemical properties of the casein from cow and buffalo milk.
- (C) Polymorphism in buffalo casein.

(A) ATTEMPTS TO FRACTIONATE COW AND BUFFALO CASEINS

The complete fractionation of casein into distinct homogeneous fractions by a single step has not yet been achieved. In the initial stages fractionation of casein was attempted using Sephadex-G-200 gel filtration technique. However, fractionation of casein could not be achieved by this method. When it was attempted to fractionate casein on Sephadex G-200 column in presence of guanidine hydrochloride, the Sephadex was found to lose its compactness and gel characteristics.

Since aqueous guanidine is known to dissociate proteins at a lower concentration than urea, attempts have been made to fractionate cow casein by the combined aqueous guanidine hydrochloride and ammonium sulphate fractionation followed by DEAE cellulose chromatography. This method also did not achieve any significant separation. Finally the method of Dumas *et al*(2) was used with slight modifications. With this method beta-casein comes out as a single pure band and alpha-casein is slightly contaminated with K casein. This could be removed successfully with a single precipitation in presence of calcium chloride. The method is essentially as follows.

The whole casein was dissolved in imidazole buffer (0.05M) at pH 7.0 containing 3.3M urea and passed through a column of DEAE cellulose (2 x 40 cms) previously equilibrated with the same buffer. The elution was performed with a linear gradient of sodium chloride (0.1M to 0.6M). Two major peaks and several minor peaks were obtained. The same method with modification in the linear gradient of sodium chloride could be used for the separation of individual components of alpha- and beta-caseins. The fractions were pooled and precipitated using ammonium sulphate. This was dispersed in water, dialysed and lyophilised.

The whole purification steps to prepare alpha-casein and beta-casein was followed using acrylamide gel disc electrophoresis in presence of 4.5M urea. In this method only 7.5% cross-linked, pH 9.5 standard gel (stacks at pH 8.3 and runs at pH 9.5) was used. Cylindrical columns of gel matrices in three sections i.e. small pore lower gel, large pore spacer gel and large pore sample gel were prepared in glass tubes 11 cm. long and 0.6 cm inner diameter. The procedure for the preparation of these gels was essentially similar to that of Davis and Ornstein(3) except that urea was incorporated into the gel at a concentration of 4.5M. The gel monomer solution, buffer concentrates and catalysts were mixed in required proportions(4) and stored in the

A weighed amount of urea was added to the premixed small pore and large pore gel solution to make them 4.5M with respect to urea and the solutions were poured into the tubes for polymerization. The change in buffer concentration resulting from the volume change due to the addition of urea was ignored. The buffer used for electrophoresis are tris-glycine buffer at pH 8.5 or tris-borate-EDTA buffer of pH 9.2, both containing 4.5M urea.

After purification of alpha- and beta-casein the electrophoretic pattern was not different from that obtained with whole casein. Cow casein generally contained 2 alpha-casein and 3 beta-casein components whereas buffalo casein had 3 alpha-casein and one or two beta-casein components. The polymorphism of these casein components will be discussed later.

The purification of casein also was followed using acrylamide gel electrophoresis. Before the electrophoresis the casein solution was reduced using mercaptoethanol.

Preparation of K-casein:

The preparation of K-casein from buffalo milk was tried according to the method of Hill(5). In this method urea need not be used. Thus any change in native structure of K-casein introduced by urea could be avoided.

In this method, 20 gms. of acid precipitated casein was taken in 300 ml water and the pH adjusted to 8. This was cooled to 3°C and 25 ml of 4M CaCl₂ are added and the pH was maintained at 6.7 to 7.2. This was kept cool for 1 hr. and then warmed to 35°C. The precipitate formed was removed by centrifuging for 15 minutes at 500 rpm. Supernatant was removed and dialysed against water overnight at 3°C. The dialysed solution was concentrated to 50 ml and the pH was adjusted to 4.7. The precipitate of crude K-casein obtained in this way was redissolved in 50 ml water by the slow addition of 1N NaOH to bring the pH to 7.5. The solution was cooled and 4 ml of 4M calcium chloride was added and the pH was kept at 6.7-7.2 during the addition and then it was warmed to 35°C. The precipitate formed was spun at 30000 g for 90 minutes. The supernatant was dialysed for 3 to 4 hr. against 5 litres of water with stirring and then overnight against 16 litres of 0.05N acetic acid buffer at pH 6.25 at 6°C. This was subjected to DEAE cellulose chromatography essentially according to the method of Hill(5).

In the first run 3 peaks were obtained. The first one was pure beta-casein. Second and third peaks had K-casein in combination with different proportions of beta-casein. When further subjected to DEAE-cellulose chromatography they moved as single bands in their respective positions. This shows that buffalo K-casein forms con-

siderably stable complex with beta-casein. In this no trace of alpha-casein was present. Later we attempted to separate K-casein in presence of urea by the method essentially according to Zittle and Custer(6). Here complete purification of K-casein was achieved in two purification steps.

The contamination of K-casein in the alpha-casein fraction was removed as follows. The lyophilised powder was dissolved at pH 7.0 cooled to 2°C and made 0.13M with Ca⁺⁺ and warmed to 35°C. The precipitate was removed and again dissolved at pH 8. The calcium was removed using Dowex-50(H⁺). The solution was further dialysed and lyophilised.

alpha-, beta- and gamma-casein of buffalo milk were also prepared by the method of Hipp et al(10).

(B) PHYSICO-CHEMICAL CHARACTERISTICS OF WHOLE CASEIN AND ITS MAJOR COMPONENTS

The amino acid composition of cow casein has been determined by a number of workers(7). However, no accurate data about buffalo caseins are available. Hence amino acid composition of whole casein, pure preparations of alpha, beta- and K-caseins of cow and buffalo milk was determined.

In view of the differences observed in the amino acid composition, rate of proteolysis of whole casein as well as alpha-casein of cow and buffalo milk was studied using pepsin, trypsin and alpha-chymotrypsin.

Further the "finger prints" of tryptic digests obtained with whole casein and with alpha-casein were determined to find any obvious differences.

Extensive studies have been made earlier on the phosphorus containing peptides isolated from the proteinase digests of casein. The digestion of casein by pepsin results in the formation of an insoluble product with a higher phosphorus content than casein. However, digestion of dephosphorylated casein with pepsin does not result in the separation of similar product from the digests. In view of the difference in the composition of some amino acids of the whole casein of cow and buffalo milk it was felt worthwhile to compare the properties of peptides obtained by hydrolysis with pepsin.

Experimental methods:

The alpha-, beta- and gamma-casein were prepared by adopting both urea and alcohol fractionation techniques of Hipp et al(10). The nitrogen content of the isolated casein was determined by the microkjeldahl method as described by

Digestion of casein with pepsin and isolation of peptide from the digests:

Casein was dissolved in water and the solution was adjusted to pH 1.3 with 0.2N HCl. It was digested with crystalline pepsin (keeping ratio of enzyme to protein being 1:70) for 2 hours at 37°C. The insoluble product (gel) that had separated was isolated by centrifugation. It was washed successively with water, alcohol, acetone and finally with ether and dried. The supernatant was neutralized with dilute alkali and the peptides were precipitated by treatment with 50 ml of 10% neutral lead acetate solution. The precipitate was isolated by centrifugation, washed and suspended in water. It was decomposed by passing H_2S and filtered. The filtrate was further stirred with 5 g of Dowex-50 (H^+ form) to ensure complete removal of lead and other metal ions and then lyophilized to obtain in the form of dry powder.

The amino acid composition of the gel and soluble peptides was determined according to the method described earlier.

Soluble peptides were also obtained from dephosphorylated casein. Here there was no separation of insoluble gel like product during digestion.

The separation of these peptides was achieved using a column of Dowex-1-Cl. The peptides were dissolved in distilled water at pH 7.8 and passed through a column of Dowex-1-Cl (0.9 x 25 cm). This was first washed with two column lengths of water and then developed with 0.001, 0.005, 0.01, 0.05 and 0.1N HCl. Fractions of 5 ml. were collected. The fractions were analysed by the ninhydrin method of Rosen(18).

The nitrogen and phosphorus content of gel and soluble peptides are represented in Table 3. It is clear that the soluble peptides of cow as well as buffalo casein have higher proportion of phosphorus compared to gel peptides fraction.

Amino acid composition of gel peptides of cow and buffalo caseins was determined and represented in Table 4. Significant differences were encountered in the composition of some amino acids between these fractions. However, the peptide elution pattern obtained on chromatography did not show any difference. Five major peaks were obtained from both cow and buffalo casein "gel peptides" fraction and 4 major and one minor peak was obtained from "soluble peptides" fraction.

was estimated by the method of Fiske and Subbarow(12). The paper and agar electrophoretic mobilities of these fractions were determined according to the method of Giri(13) with 0.05M veronal buffer, pH 8.6 at 200 volts for 4 hours.

For the amino acid analysis the improved method described before was used for the preparation of alpha-, beta- and K-caseins. The amino acid analysis was carried out using the standard automatic recording equipment described by Spackman, Stein and Moore(8).

Dry samples of casein were hydrolysed separately with 6N HCl for 24 hrs. at 110°C in sealed tubes under vacuum. The HCl was then evaporated in a vacuum desiccator and the residues were taken up separately in citrate buffer at pH 2.2. The separation of neutral and acidic amino acids were performed on a 60 x 0.9 cm chromatographic column at pH 3.25 to 4.25 (0.2M sodium citrate buffer). The basic amino acids and ammonia were analysed on a 10 x 0.5 cm column at pH 5.28 (0.35M sodium citrate buffer).

Cysteine was determined as cysteic acid after performic acid oxidation according to the method of Hirs(14).

Proteolysis of cow and buffalo caseins:

1% solutions of caseins in 0.1M sodium phosphate buffer, pH 7.8 were incubated at 37°C with crystalline enzymes. The enzyme substrate ratio was maintained at 1:30. At regular intervals, definite aliquots were withdrawn from the incubation medium and immediately treated with a known volume of 10% trichloroacetic acid (TCA) such that its final concentration remained 5%. The protein precipitates were recovered by centrifugation, washed with 5% TCA and their nitrogen content were determined by digestion with concentrated H_2SO_4 and estimating the ammonia by the Nestler reaction as described by Koch and McMeekin (1924) (15).

"Finger Printing" of tryptic peptide:

The aliquots removed from the incubation mixture at the end of 24 hrs. were also treated with TCA as described above, soluble portion was recovered and TCA was removed with ether. The aqueous portions free from TCA were evaporated to dryness and the peptides present in these samples were separated in two dimensions by paper electrophoresis and paper chromatography as described by Ingram (1958) (16).

The dephosphorylated casein was prepared according to the method of Sundararajan et al.(17).

TABLE 1: THE NITROGEN AND PHOSPHORUS CONTENTS AND THE AGAR ELECTROPHORETIC MOBILITIES OF WHOLE CASEIN, alpha-, beta- AND gamma- CASEIN ISOLATED FROM BUFFALO MILK.

Fraction	% Nitrogen	% Phosphorus	Agar electrophoretic mobility cm/hr
Whole casein	14.89	0.70	<u>alpha</u> -casein-1.10 <u>beta</u> -casein -0.62 <u>gamma</u> -casein -0.04
<u>alpha</u> -casein	15.37	0.71	1.10
<u>beta</u> -casein	14.25	0.70	0.62
<u>gamma</u> -casein	15.18	0.55	0.04

TABLE 2: AMINO ACID COMPOSITION OF WHOLE CASEIN AND DIFFERENT FRACTIONS OF CASEIN FROM COW AND BUFFALO MILK.

Amino acid	%(gm/100 gm)							
	Whole cow casein	Whole buffalo casein	Cow <u>alpha</u> -casein	Buffalo <u>alpha</u> -casein	Cow <u>beta</u> -casein	Buffalo <u>beta</u> -casein	Cow K-casein	Buffalo K-casein
Lysine	7.0	6.3	6.4	5.9	4.7	5.3	4.3	4.8
Histidine	2.4	2.2	2.2	1.9	1.9	1.8	2.3	1.2
Ammonia	2.6	2.6	2.7	3.8	1.2	2.0	8.7	2.8
Arginine	3.8	2.7	3.5	3.7	1.9	1.3	3.3	2.6
Aspartic Acid	6.8	6.2	6.5	8.1	5.6	4.5	8.5	7.5
Threonine	3.6	4.0	4.2	5.0	4.5	4.0	6.0	9.2
Serine	4.3	4.2	5.9	5.3	6.5	6.5	5.1	5.5
Glutamic Acid	19.9	21.8	22.2	22.6	21.9	21.0	19.5	19.1
Proline	10.5	12.8	5.2	5.7	11.9	13.2	9.8	6.3
Glycine	1.5	1.3	1.8	1.7	1.6	1.3	1.25	0.6
Alanine	2.6	2.6	2.4	4.1	2.5	2.0	4.3	9.4
Valine	5.8	5.6	4.5	4.8	5.4	5.5	4.1	5.6
Methionine	2.5	1.0	2.5	2.0	2.5	3.4	1.6	8.5
Isoleucine	4.8	4.5	4.6	5.4	3.8	3.7	4.5	6.0
Leucine	8.9	8.6	6.5	5.9	11.1	10.8	4.6	4.9
Tyrosine	5.9	6.4	5.5	8.1	4.0	3.8	6.0	5.5
Phenylalanine	5.4	6.0	4.6	5.8	6.7	6.2	5.5	5.0
Cysteic acid *	0.5	1.12	Nil	Nil	Nil	Nil	1.2	2.8

* In these values no account has been made for the possible presence of small amounts of ...

TABLE III. NITROGEN AND PHOSPHORUS CONTENTS OF THE PEPTIDES OBTAINED FROM THE PEPTIC DIGESTS OF CASEINS.

	Nitrogen (%)	Phosphorus(%)
Casein (cow) ..	16.37	0.77
Gel peptides (cow) ..	16.14	2.64
Soluble peptides (cow) ..	14.23	4.59
Gel peptides (Buffalo) ..	16.48	2.63
Soluble peptides ..	15.61	4.06

TABLE IV. AMINO ACID COMPOSITION OF GEL PEPTIDES ISOLATED FROM THE PEPTIC DIGEST OF COW AND BUFFALO CASEINS.

Amino Acid	% g/100gm.	
	Cow	Buffalo
Lysine ..	7.8	6.8
Histidine ..	1.0	2.8
Ammonia ..	3.1	3.0
Arginine ..	3.4	1.5
Aspartic acid ..	8.8	8.0
Threonine ..	4.9	3.9
Serine ..	8.8	7.8
Glutamic acid ..	31.2	32.5
Proline	2.9
Glycine ..	1.1	1.5
Alanine ..	1.5	3.6
Half cystine ..	3.2	..
Valine ..	4.4	4.5
Methionine ..	1.0	2.0
Isoleucine ..	8.9	8.2
Leucine ..	5.1	5.5
Tyrosine ..	2.5	2.1
Phenylalanine ..	2.8	3.3

RESULTS:

In Table-1 are given the percentage of nitrogen, phosphorus and electrophoretic mobility of alpha-, beta-, and gamma-caseins of buffalo milk. Although the percentage of nitrogen content was same in all the three fractions, the phosphorus content of gamma-casein was considerably lower than the other two fractions.

In Table-2 are represented the amino acid composition of alpha-, beta- and K-caseins of cow and buffalo milk obtained by the improved method described earlier. This indicates that in the whole casein of cow and buffalo milk there are differences particularly with respect to cystine (determined as cysteic acid) and methionine content. When the amino acid content of individual fractions were compared, no significant difference was observed in the case of alpha- and beta-caseins of cow and buffalo milk. However, in the case of K-casein, significant difference in the composition of many amino acids was observed. Cystine was not present in alpha- as well as beta-casein of cow and buffalo milk. Therefore the difference observed in the cystine content in the whole casein must be mainly due to difference in the cystine content of K-casein. In fact, it was found that cystine content of buffalo K-casein is almost double the value of cow K-casein as it was found in the case of cystine content of whole casein.

The rate of proteolysis of whole casein and alpha-casein of cow and buffalo milk was studied using trypsin, pepsin, alpha-chymotrypsin and the results are presented in the Tables 5 to 10.

TABLE-5: PROTEOLYSIS OF BUFFALO AND COW WHOLE CASEINS WITH TRYPSIN.

Time	Percentage Proteolysis	
	Buffalo Casein	Cow Casein
Zero	Nil	Nil
1 min.	11.77	15.33
15 min.	30.98	28.08
30 min.	41.52	43.74
45 min.	47.95	44.88
1 hr.	53.20	50.69
1.5 hr.	54.98	56.50
2.0 hr.	59.70	58.24
3.0 hr.	62.58	62.88
24 hr.	79.52	81.45

TABLE 6: PROTEOLYSIS OF BUFFALO AND COW WHOLE CASEINS WITH PEPSIN.

Time	Percentage Proteolysis	
	Buffalo casein	Cow casein
Zero ..	Nil	Nil
15 min. ..	27.4	27.9
30 min. ..	40.5	44.2
45 min. ..	49.3	48.8
1.0 hr. ..	50.9	67.4
1.5 hr. ..	53.9	72.1
2.0 hr. ..	56.8	77.7
3.0 hr. ..	65.8	83.3
24 hr. ..	91.0	96.3

TABLE 7: PROTEOLYSIS OF BUFFALO AND COW WHOLE CASEINS WITH alpha-CHYMOTRYPSIN.

Time	Percentage Proteolysis	
	Buffalo casein	Cow casein
Zero ..	Nil	Nil
15 min. ..	57.4	57.8
30 min. ..	66.9	58.8
45 min. ..	71.5	75.0
1.0 hr. ..	83.4	77.1
1.5 hr. ..	84.9	78.3
2.0 hr. ..	86.4	85.0
3.0 hr. ..	87.6	87.5
24 hr. ..	100	100

TABLE 8: PROTEOLYSIS OF BUFFALO AND COW alpha-CASEINS WITH TRYPSIN.

Time	Percentage Proteolysis	
	Buffalo <u>alpha</u> -casein	Cow <u>alpha</u> -casein
Zero ..	Nil	Nil
1 min. ..	4.68	-
15 min. ..	18.81	8.70
30 min. ..	30.58	13.95
45 min. ..	36.46	23.83
1 hr. ..	45.87	27.32
1.5 hr. ..	48.83	34.30
2.0 hr. ..	51.76	33.12
3.0 hr. ..	52.93	37.78
24 hr. ..	75.30	68.60

TABLE 9: PROTEOLYSIS OF BUFFALO AND COW alpha-CASEINS WITH PEPSIN.

Time	Percentage Proteolysis	
	Buffalo <u>alpha</u> -casein	Cow <u>alpha</u> -casein
Zero ..	Nil	Nil
15 min. ..	4.5	7.3
30 min. ..	31.8	16.4
45 min. ..	38.6	29.1
1.0 hr. ..	43.3	33.8
1.5 hr. ..	54.5	36.3
2.0 hr. ..	71.0	50.9
3.0 hr. ..	79.5	69.1
24 hr. ..	82.5	85.4

TABLE 10: PROTEOLYSIS OF BUFFALO AND COW CASEINS WITH
alpha-CHYMOTRYPSIN.

Time	Percentage Proteolysis	
	Buffalo <u>alpha</u> -casein	Cow <u>alpha</u> -casein
Zero	Nil	Nil
15 min.	59.9	12.4
30 min.	65.4	21.7
45 min.	73.8	37.1
1.0 hr.	82.0	45.9
1.5 hr.	89.2	52.0
2.0 hr.	97.5	76.0
3.0 hr.	100	90.4
24 hr.	100	100

The rate of proteolysis of whole cow casein with trypsin, pepsin and alpha-chymotrypsin was almost similar to that of buffalo casein. However the rate of proteolysis of cow alpha-casein with trypsin, pepsin as well as alpha-chymotrypsin was slower than buffalo alpha-casein.

The "finger prints" of tryptic digests obtained with whole casein and with alpha-casein isolated from cow and buffalo milk were almost similar.

Several investigators have studied the insoluble phosphopeptones formed during the proteolysis of casein by pepsin. Degradation of dephosphorylated casein with pepsin however does not result in the formation of an insoluble product. In furthering the comparative studies on casein from cow and buffalo milk the soluble and gel peptide elution pattern on Dowex-1-Cl was determined. It was found that both cow and buffalo caseins gave five major peaks in gel peptide fraction and four major peaks and one minor peak in the case of soluble peptide fraction. In the case of dephosphorylated casein three major peaks and four minor peaks were obtained.

The elution profile of the soluble and gel peptides and dephosphorylated and hydrolysed casein of cow and buffalo casein was very similar.

Discussion:

The data presented here about the physicochemical properties shows considerable similarities between cow and buffalo casein. Buffalo casein has all the four major components namely alpha-, beta-, gamma- and K-casein corresponding to the respective components in cow casein. Recently it has been shown that human casein does not possess the component corresponding to the beta-casein(19). The electrophoretic mobilities of alpha-, beta-, and gamma-casein are almost same as that of cow casein in Agar gel and acrylamide gel electrophoresis. The mobility of K-casein in acrylamide gel electrophoresis after reduction by mercaptoethanol is same as that of cow K-casein. Other similarities were found in amino acid composition. The main difference appear to be in the cases of sulphur containing amino acid namely cystine. It is interesting to note that significant difference in the amino acid composition of alpha- and beta-casein of cow and buffalo milk were not encountered. Cystine is absent in buffalo alpha- and beta-casein just as in the case of cow alpha- and beta-caseins. The difference in cystine content found in whole casein appears to be mainly due to difference in the cystine content of K-casein. The higher cystine content of buffalo K-casein may be one of the reasons for the slow release of sialic acid from buffalo casein compared to cow casein by the action of rennet(20).

The significant differences found in the amino acid composition of cow and buffalo K-casein may explain the difficulties encountered in the preparation of cheese from buffalo milk(21,22). Other interesting differences between K-casein of cow and buffalo milk have been shown by Gupta and Ganguli(20,23).

K-casein is very important in studying casein micelles. Action of rennet on the K-casein in the preparation of cheese is very well known. In view of the significant differences encountered in the amino acid composition of buffalo K-casein as compared to cow K-casein, detailed study of the nature of buffalo K-casein should be undertaken. Further the greater stabilizing properties of buffalo K-casein was probably responsible for obtaining stable complexes of K-casein with beta-casein observed when preparation of K-casein was followed according to the method of Hill(5). This difference in the property of buffalo K-casein may be utilized for the preparation of milk concentrates, where it is beneficiary.

The difference in the initial rate of proteolysis of alpha -casein of cow and buffalo milk could not be explained on the basis of their amino acid composition. However, this may depend on the primary structure of the protein and may not be revealed in the gross composition of amino acids.

(C) POLYMORPHISM IN BUFFALO CASEIN

The genetic polymorphism of the various casein components is well established. At the moment there are three alpha₁ and several beta -casein varieties are known. These were found to differ from each other by substitution of a single amino acid, usually a basic amino acid for an acidic one and vice versa. Since there were considerable differences in amino acid composition in different major components of casein, it was of interest to study the polymorphism present in buffalo casein. Acrylamide gel disc electrophoresis was used for this purpose. The method has been described earlier. Two buffers namely tris-glycine at pH 8.5 containing 4.5M urea and tris-EDTA-boric acid buffer at pH 9.2 containing urea were used. When K-casein was to be separated, mercaptoethanol was added to the casein solution and incubated for 1 hr. before the electrophoresis. Sample gel will not gel in presence of mercaptoethanol. Therefore, the samples were applied in concentrated sucrose solution whenever mercaptoethanol was used.

There were three alpha -casein bands in all the 52 samples of buffalo milk tested with the centre band always in a higher proportion than the other two. In the case of beta -casein only four samples had two bands and the rest had single band. Among these four, three samples are from "Murrah" buffalo and one from "Surti" buffalo. K-casein band differs considerably. There are only two bands in all the samples tested. There was no difference in the pattern of casein components from local buffaloes and Murrah buffaloes. In this analysis samples of milk from 40 individual Murrah buffaloes and 10 individual local buffaloes were analysed.

Our earlier results showing five components in beta -casein was probably due to faulty preparation, as this could not be found in the 52 samples of milk tested above.

Recently Ganguli and Majumdar(24) have shown the presence of two components in "Murrah" buffalo K-casein using starch gel electrophoresis.

These results indicate that considerable variation in casein components found in different breeds of cow are not present in buffalo casein. However, analysis of

more number of samples from other breeds of buffaloes are necessary to come to any conclusion about the polymorphism in buffalo casein.

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Extensive work has been carried out in recent years on the synthesis and structure of proteins generally and in particular of conjugated proteins. Among conjugated proteins, phosphoproteins form a very important group because of their role in various metabolic reactions such as enzyme catalysis (1), phosphorylation of membrane bound proteins, metal ion transfer (2,3), electron transfer (4), energy storage (5) and in cellular regulation (6).

For a very long time studies on phosphoproteins have been confined to storage proteins such as casein of milk and phosphovitin of egg yolk. In all these phosphoproteins, phosphate is mostly esterified to the hydroxyl group of serine. So far only traces of phosphothreonine have been reported in casein (7) and in phosphovitin (8).

The mechanism by which phosphate is incorporated into the casein has attracted considerable attention in the past. Though much work has been carried out on the structure and biosynthesis of casein, the mode of incorporation of inorganic phosphate (Pi) into the protein is not well understood. This could take place in either of the following two ways (i) incorporation of Pi into casein as phosphoserine or (ii) incorporation of Pi into casein at a peptide or protein level.

A critical study of the available literature indicates that the incorporation of Pi into casein takes place at a peptide or protein level. This conclusion has been reached mostly based on the evidence obtained from the in vitro experiments: (i) mitochondrial preparations of mammary gland and liver could incorporate P_i^{32} into casein and other proteins (9); (ii) under similar conditions phosphoserine could not be incorporated into the phosphoproteins; and (iii) serine could not be phosphorylated by mammary gland extracts. Further, partially dephosphorylated casein is a better precursor for the enzyme phosphoprotein kinase (10) and not the completely dephosphorylated casein (5). The mitochondrial preparation used earlier could phosphorylate not only partially dephosphorylated casein and casein but also many other proteins, casein and phosphovitin being the best substrates. Unlike the in vivo phosphorylation of some specific serine residues of casein, any available serine moiety of the protein can be phosphorylated in an in vitro system. Phosphoserine, obviously could not be incorporated into casein because the enzyme system used was not amino acid incorporating system. Phosphoserine need not be formed from serine but can be formed from phosphohydroxy pyruvate

as well (10). All the phosphoprotein kinases irrespective of the sources from which they were prepared utilize casein and phosphovitin as their best substrates and not their respective tissue proteins. Therefore, this problem was reinvestigated. At first ethionine, an inhibitor of protein synthesis was taken to study the incorporation of Pi^{32} as well as of radioactive amino acids.

If Pi^{32} gets in to casein as phosphoserine, then ethionine should inhibit this process. If the phosphorylation is taking place at the protein level, ethionine should not inhibit such a process. With this idea in view, experiments were undertaken to study the incorporation of radioactive amino acids and P^{32} into casein, proteins of mammary gland, liver, kidney and blood by injecting ethionine into intact rats. Since ethionine is not a specific inhibitor of protein synthesis, the effect of puromycin and cycloheximide was also studied on the synthesis of protein.

Experimental Methods

In the in vivo experiments rats in lactation for a period of 20 days were used. DL-ethionine was injected intraperitoneally at a level of 1 mg/gm body weight. Half the dose was given at zero time. After 2 hrs. the second half of the dose was administered. Inorganic phosphate P^{32} and C^{14} labelled amino acids were injected intraperitoneally 1 hr. after the first injection of ethionine. Control rats received equal volume of distilled water. After 5 hrs. of the first injection, the animals were sacrificed, liver, blood and kidneys were removed as quickly as possible and chilled in 0.9% saline. Residual milk was extracted from the mammary gland by repeated extraction with 0.9% cold saline(11). Phosphoproteins from the above organs as well as from milk were prepared by the method described by Freidkin and Lehninger(12). In one experiment, these powders were dissolved in minimum amount of dilute ammonia and dialysed exhaustively against 0.05M phosphate buffer of pH 7.0 and then against distilled water and reprecipitated by adjusting the pH of the solution in a dialysis bag to pH 4.6. No appreciable radioactivity was lost. Radioactivity of these powders was determined by the use of a Geiger counter attached to a "Panex" scalar. Dephosphorylation was carried out at 34°C for different periods of time using 0.05M $Ba(OH)_2$ solution.

IN vitro experiments using tissue slices

Rats lactating for a period of 5 to 10 days were used. The rats were killed by decapitation, the liver and mammary glands were removed and excised in cold saline. Tissue slices were prepared using the "Stadie Riggs" tissue slicer. A known weight of slices (about 200 mg) of liver as well as of mammary gland were taken in each of the 50 ml flasks containing 5 ml of the Krebs-Ringer bicarbonate buffer pH 7.5. One set of flasks were used as control. To another set of flasks was added inhibitors of protein synthesis. These flasks were saturated with oxygen, closed tightly with corks and incubated in a "Gallenkamp" metabolic shaker at 37°C for 1/2 hr. Equal volume of 20% TCA was added and homogenized. Isolation of phosphoprotein was accomplished by the method described earlier and the radioactivity determined.

P^{32} phosphoserine was prepared according to the method of Murray and Spinks(13). Preparation of phosphoserinol was carried out essentially according to the method of Manning and Meister(14). The isolation of final product was carried out by chromatography on Dowex x 50cm (H^+ 200-400 mesh, 1.5 x 20 cm) and elution with 0.5N HCl.

Preparation of P^{32} -phosphate labelled amino acyl-S-RNA

Lactating rats were stunned and decapitated, the mammary glands were removed and immediately washed in ice cold 0.9% saline and minced. This was incubated in the presence of "Keller and Zamecnick" medium A(15) pH 7.0) containing P^{32} phosphate for two hours at 37° in an atmosphere of oxygen. After incubation, it was chilled immediately and homogenized using "Virtis" homogenizer. Water saturated phenol containing 1% sodium dodecyl sulphate was added to this homogenized medium and shaken vigorously for one hour. This was spun at 15,000 g for 10 minutes in a "Servall" refrigerated centrifuge and the top layer was carefully removed. The bottom layer was mixed with an equal volume of cold medium A and centrifuged again and the top aqueous layer was collected. To the combined aqueous portion was added three volumes of 95% ethanol. The resultant precipitate was dissolved in 100 ml of cold 0.02M acetate buffer, pH 5.8, containing 0.1M sodium chloride, and added dropwise to a column of DEAE cellulose (2 x 10cm) previously equilibrated with the same buffer.

Elution was performed by a linear gradient of sodium chloride from 0.1M to 2M in a total volume of 500 ml. 5 ml fractions were collected every 15 minutes. RNA was eluted between the tube numbers 45 to 55. These portions which were completely devoid of contaminating protein were pooled and then extracted twice with an equal volume of cold phenol saturated with water and with ether respectively. To the aqueous solution containing RNA, three volumes of 95% ethanol were added and was allowed to stand at -14°C overnight. The precipitate formed was spun down and washed with 80% alcohol, dissolved in a few ml of cold water and dialysed against cold water overnight and lyophilized.

Results

The results obtained on the effect of ethionine in vivo on the incorporation of radioactive phenylalanine and tryptophane into the protein of various organs are presented in Table 1. The results indicated that ethionine inhibited the incorporation of both the amino acids into the proteins of liver, kidney, blood and casein.

Table 1: EFFECT OF INJECTED ETHIONINE ON THE INCORPORATION OF PHENYLALANINE U-C¹⁴, TRYPTOPHANE-7-C¹⁴ AND PHOSPHATE-P³² INTO THE PROTEINS OF VARIOUS ORGANS AND CASEIN OF LACTATING RAT.

% decrease in the incorporation of labelled compounds over the control

	Phenylalanine-U-C ¹⁴	Tryptophane-7-C ¹⁴	Phosphate-P ³²
Liver	33	18	2
Kidney	—	—	1
Blood	52	85	0
Mammary gland	65	30	67
Casein	64	33	74

The results presented in Table 1 clearly revealed that ethionine did not inhibit the incorporation of P_i^{32} into the proteins of liver, kidney and blood. Surprisingly it inhibited the incorporation of P_i^{32} into casein and the phosphoproteins of mammary gland. This experiment was repeated five times and similar pattern of results were obtained. Out of the five sets of experimental results only twice a decrease of 2 to 3% in the liver and a decrease of 1% in the kidney were obtained.

The results presented in Table 2 revealed that 70% of the P_i^{32} from liver and 80 to 100% from mammary gland and casein respectively can be dephosphorylated. This is in accordance with the results reported in the literature. The results presented in Table 3 clearly showed that on paper electrophorogram, the radioactive peak more or less coincided with the protein peak as detected by bromophenol reagent.

Table 2. P_i^{32} -RELEASE FROM PROTEIN OF LIVER MAMMARY GLAND AND CASEIN BY BARIUM HYDROXIDE.

	Percentage release of P_i^{32} at-	
	2 hrs.	24 hrs.
Liver	70	70
Mammary gland	84	84
Casein	85	100

Table 3. ELECTROPHORETIC PATTERN OF CASEIN AND MAMMARY GLAND PHOSPHOPROTEIN.

(Electrophoresis was carried out for 3 hours and 30 minutes using Whatman No.1 filter paper strips and 0.05M veronal buffer of pH 8.6 at a voltage gradient of 8.8 V/cm.)

	Distance moved from the origin of radioactive peak moved	Distance moved by protein peak from the origin (B.P.B. test)
Casein	+2 to 4 cm.	+1.5 to 3 cm.
Mammary gland phosphoprotein	+3.5 to 5 cm.	+2 to 4 cm.

The results of the effect of ethionine, puromycin and cycloheximide on the incorporation of valine-C¹⁴ and phosphate-P³² are represented in Table 4. It could be seen that all the inhibitors of protein synthesis inhibited the incorporation of valine-C¹⁴ into liver as well as mammary gland proteins. Ethionine is markedly less effective than puromycin and cycloheximide in inhibiting the incorporation of amino acid. The incorporation of phosphate-P³² into liver proteins was not inhibited by any of the inhibitors of protein synthesis whereas that into mammary gland proteins was inhibited. However, the extent of inhibition of phosphate-P³² into mammary gland protein is almost 1/3 of inhibition of valine-C¹⁴ by all these inhibitors of protein synthesis.

Table 4. EFFECT OF INHIBITORS OF PROTEIN SYNTHESIS ON THE INCORPORATION OF C¹⁴-VALINE AND P³²-PHOSPHATE INTO THE PROTEIN OF LIVER AND MAMMARY GLAND OF THE R.T.

(Tissue slices were incubated for 2 hours in the case of experiments with ethionine and 30 minutes in the case of puromycin and cycloheximide).

Inhibitors of protein synthesis used	Labelled compounds used	%decrease in the incorporation of labelled compounds over the control	
		Liver	Mammary gland
Ethionine (10 mg/ml)	C ¹⁴ -valine	69.0	29.4
	P ³² -phosphate	5.2	10.4
Puromycin (200 µg/ml)	C ¹⁴ -valine	92.0	88.0
	P ³² -phosphate	1.3	19.9
Cycloheximide (200 µg/ml)	C ¹⁴ -valine	90.0	83.0
	P ³² -phosphate	2.8	28.0

Attempts to demonstrate the presence of phosphoserine in rat mammary gland:

One day old lactating rats were used in these experiments. Mammary gland was obtained from normal lactating rats as well as from those given ethionine

intraperitoneally in the same manner as described in experiment (a). The animals were sacrificed, a 10% homogenate of the washed mammary glands was prepared in cold 10% TCA. TCA was removed by ether extraction and the pH was adjusted to 7.5 to 8.0. The sample was loaded on a Dowex-1-Cl (200-400 mesh) column (14 X 1.5 cm). The column was washed with 200 ml water followed by 200 ml of 0.001N HCl. Elution was continued with 0.01N HCl and 5 ml fractions were collected. 80 fractions were collected and analysed for radioactivity. Of the six radioactive peaks obtained only two of them answered the ninhydrin test. Tubes 23 to 31 constitute the first peak and 32 to 41 form the second peak. First peak gave intense colour with ninhydrin but the second peak gave only a faint colour. These two peaks were concentrated and a part of the concentrates were chromatographed on Whatman No.1 filter paper for 24 hrs. using 75 parts of ethanol and 50 parts of 0.1N acetate buffer of pH 4.6 as the solvent system. On chromatography of the second peak, ninhydrin colour did not coincide with the radioactivity. The radioactivity was associated with U.V. quenching spots.

Attempts to demonstrate incorporation of phosphoserine into S-RNA of rat mammary gland

Ten day old lactating rat mammary glands were homogenized and spun at 100,000 g for one hour. The supernatant was adjusted to pH 5 with 1N HCl. The precipitate was centrifuged and after one wash with 0.14M KCl, it was dissolved in the medium of pH 7.5 containing 0.35M sucrose, 0.035M KHCO₃, 0.025M KCl and 0.01M MgCl₂. This was used as the enzyme source and contained enough S-RNA to accept amino acids. This enzyme preparation did not release phosphate from either phosphoserine or casein. The incubation mixture to study the transfer of serine to S-RNA consisted of enzyme, ATP (10 μ moles), Mg⁺⁺ (10 μ moles) and labelled serine together with unlabelled serine (20 μ moles). After one hour incubation at 37°C, the reaction was arrested by the addition of 10% TCA. The protein nucleoprotein complex was washed thrice with cold 5% TCA containing unlabelled serine followed by alcohol ether mixture and finally by ether. Amino acyl S-RNA was extracted from this with 1.0M sodium chloride at boiling water temperature and precipitated from sodium chloride solution with ethanol on a filter and after drying the counts were determined.

This experiment was repeated with P³²-phosphoserine. The results presented in Table 5 indicate that both C¹⁴ labelled serine and P³² labelled phosphoserine could be transferred to S-RNA.

Table 5. IN VITRO INCORPORATION OF C¹⁴ -SERINE AND P³² PHOSPHOSERINE INTO S-RNA OF LACTATING RAT MAMMARY GLAND.

(0.5 μ c of serine-3-C¹⁴ and 0.5 μ c of P³²-phosphoserine are added in the incubation mixture).

	cpm/mg incorporated over the blank
Serine-3-C ¹⁴	120
P ³² -Phosphoserine	160

The incorporation of amino acids was found to be very low. This is probably due to the low efficiency (about 4%) of the radioactive counter used.

Experiments to demonstrate phosphoserine in the SRNA fraction:

A. A 10% homogenate of the lactating mammary glands was prepared in 0.25M sucrose. Amino acyl S-RNA was prepared from the 1,00,000 g supernatant by the conventional phenolization method. The preparation was dialysed against water for 18 hours at 5°C. Amino acids were released from the amino acyl S-RNA by incubating in a medium of pH 10.1 for 30 minutes at 37°C. The contents were dialyzed, the dialysate was concentrated and passed through Dowex-1-Cl column. Basic and neutral amino acids were removed from the column by washing with 100 ml of water followed by 100 ml 0.001N HCl. Elution was continued with 100 ml of 0.01N HCl. 0.01N HCl eluate was concentrated and analysed by two dimensional paper chromatography using phenol: water (75:25) and butanol: acetic acid:water (4:1:1) as the two solvent systems. Ninhydrin positive spots corresponding to aspartic acid, glutamic acid and phosphoserine were detected. Recently Carlsen et al(20) reported similar results from phosphoserine synthesising chick liver system.

This experiment was repeated using P³² as tracer. P³²-phosphate (100 μ c/100gm body weight of rat) was injected to rats intraperitoneally. The animals were sacrificed after two hours. A 10% homogenate of the washed mammary glands was prepared in 0.25M sucrose

and phosphoserine was isolated as described above. The spot corresponding to phosphoserine had considerable radioactivity. The 0.01N HCl eluate from the column was mixed with 10 mg cold phosphoserine and crystallized from tissues with ethanol and ether. The crystals were taken in 2 ml of water and chromatographed with ethanol:acetate buffer at pH 4.6. The ninhydrin spot contained all the radioactivity.

Although phosphoserine could be demonstrated in S-RNA fraction, there could be doubt regarding the specific attachment of phosphoserine to the -CCA terminal of S-RNA molecule as in the case of other amino acids(16). The following two procedures were used to prove conclusively the presence of phosphoseryl-S-RNA in rat mammary gland. The first method is followed essentially according to Preise et al(17). and the second method according to Soda et al(18).

1. The amino acyl-S-RNA isolated from rat mammary gland can be digested with ribonuclease and the amino acyl adenosine isolated. The presence of phosphoserine in this fraction will prove the presence of phosphoseryl-S-RNA in rat mammary gland. If P^{32} -phosphate was incorporated into S-RNA, then the amino acyl adenosine fraction will not have nucleotide phosphate. Phosphoserine is the only amino acid which contains phosphate under these conditions. The presence of radioactivity in this fraction would prove the presence of phosphoseryl S-RNA.

2. The amino acyl S-RNA isolated from rat mammary gland can be reduced using lithium borohydride to amino alcohol. The presence of phosphoserinol in this fraction would prove the presence of phosphoseryl-S-RNA in rat mammary gland.

P^{32} -phosphate incorporation into amino acyl-S-RNA fraction was followed by using rat mammary gland minces. The resulting labelled, amino acyl-S-RNA was isolated, purified and lyophilized according to the procedure described under methods. A 10 mg. of the lyophilized powder was dissolved in 0.5 ml of acetate buffer at pH 5.8 and treated with 100 μ g of ribonuclease for 15 minutes at 30°C. An aliquot was placed on Whatman No.1 filter paper and chromatographed at 4°C with secondary butanol:formic acid:water (70:10:20) as solvent system. The R_f values of the various mononucleotides, serine, phosphoserine and adenosine are represented in Table 6. In comparison with other amino acyl adenosines, phosphoseryl adenosine was expected to

to have an R_f value between phosphoserine and adenosine(17). Following chromatography, the strip was cut into 1 cm section, eluted with 0.05M NH_4OH and the eluate counted. Almost all the radioactivity was found at the origin and no radioactivity could be located in the region where phosphoseryl S-RNA could be detected.

Table 6. R_f VALUES OF THE VARIOUS MONONUCLEOTIDES, SERINE, PHOSPHOSERINE AND ADENOSINE.

	R_f value
Phosphoserine	0.12
Serine	0.33
Adenosine	0.28
AMP	0.04
CMP	
GMP	
UMP	

2. The P^{32} -labelled amino acyl-S-RNA was prepared as described earlier. 10 μ g of the lyophilized amino acyl S-RNA was suspended in 10 ml of 0.15M lithium borohydride in tetrahydrofuran (distilled over lithium aluminium hydride) and the mixture was shaken at 26°C for one hour. 2 ml of water was added and the solution was heated on a steam bath until almost all of the tetrahydrofuran had evaporated and the residual solution was neutralized by the addition of 0.1M NaOH. The solution was desalted by passing through a column of Dowex-50 (H^+) followed by elution of the radioactive material with 6N HCl. The desalted material was concentrated in vacuo to dryness over sodium hydroxide until all the HCl was removed. To this residue was added phosphoserinol dissolved in a minimal amount of water and chromatographed on strips of Watman No.3 paper using solvent consisting of tert butanol:methyl ethyl ketone:water:concentrated NH_4OH in the proportion 50:50:25:15. After chromatography, the paper strips were cut into 1 cm sections and their radioactivity determined in a Geiger-Muller counter attached to a "Panex" scalar. No radioactivity was detected in the region corresponding to phosphoserinol. Other phosphate

protein biosynthesis, phosphate could be introduced into phosphoproteins at four stages (i) incorporation of free phosphoserine into the growing polypeptide chain after activation and esterification to a S-RNA; (ii) phosphorylation of seryl-SRNA; (iii) phosphorylation of the polypeptide chain before its release from the ribosomes and (iv) phosphorylation of a pool of nascent protein after its release from the ribosomes.

If the phosphorylation is taking place after the complete protein is formed, the inhibitors of protein synthesis should not inhibit the incorporation of phosphate- P^{32} . Thus the phosphorylation of proteins of liver, kidney and blood takes place after the complete protein is formed. Mirsky and his colleagues(6) have also reported that protein phosphorylation by isolated lymphocyte nuclei proceeds independently of protein synthesis.

However, ethionine inhibited the incorporation of Pi^{32} into the proteins of mammary gland and casein. This might mean either (i) that phosphorylation of casein and mammary gland protein is taking place at the amino acid stage or (ii) the decreased Pi^{32} incorporation is due to depletion of ATP. Schull(22) observed that ethionine administration decreased glycogen phosphorylase activity of liver which was attributed to the decreased ATP content. Such a reduction was reversed by the administration of ATP or adenine. In the present experiments, this possibility has been ruled out since adenine administration could not reverse the ethionine effect on the incorporation of Pi^{32} into casein or the proteins of mammary gland. Another possibility is that phosphorylation at the protein level is mediated by some hormone which might be influenced by ethionine.

However, the existence of hormonal influence can be clarified to certain extent by using tissue slices for studying the incorporation of amino acids and Pi^{32} . In these experiments the incorporation of Pi^{32} was inhibited to about only 20 to 30% as against 80-90% of inhibition of amino acid incorporation. Ethionine was much less efficient in inhibiting the protein synthesis but here again the extent of inhibition of Pi^{32} is only 1/3 of the extent of inhibition of amino acid incorporation. This could be interpreted to mean that ethionine inhibition of phosphorylation in vivo is not subjected to hormonal influences.

When this investigation was in progress, Turkington and Topper(21) using mouse mammary explants

compounds which may be present as contaminants such as the nucleotides and phosphoserine had entirely different R_f values as represented in the Table 7.

Table 7. R_f VALUES OF THE VARIOUS MONONUCLEOTIDES, SERINE, SERINOL, PHOSPHOSERINE AND PHOSPHOSERINOL.

	R_f value
Serine	0.32
Serinol	0.87
Phosphoserine	0.045
Phosphoserinol	0.41
AMP	0.08 to 0.11
GMP	
CMP	
UMP	

Discussion

The question as to whether phosphoserine in phosphoprotein arises from a step prior to peptide bond formation or after the peptide or complete protein is synthesised is still not clearly understood. Although overwhelming evidences are available for the phosphorylation at peptide or protein level they do not rule out the possibility of phosphorylation at other sites.

Considerable work has been carried out on the enzyme phosphoprotein kinase which phosphorylates casein. The enzyme has been identified in many different tissues by different groups of workers (5,9,10,19). The properties of the protein kinases can be summarized as follows: (i) casein and phosphovitin are the main substrates for this enzyme; (ii) partially dephosphorylated proteins are better acceptors; (iii) serine as free amino acid is not phosphorylated and (iv) small peptide also can be phosphorylated.

However, completely dephosphorylated casein cannot be phosphorylated by this enzyme. So far non-phosphorylated precursors either in milk or in mammary gland have not been isolated. Therefore, phosphorylation of casein to a certain extent must take place by other mechanisms before it can be phosphorylated by protein kinase. According to the current concepts of

Preiss et al(17), and goda et al. However, no positive results were obtained. This may not necessarily prove the absence of phosphoseryl-S-RNA, since the methods employed were not highly satisfactory.

The R_f value of phosphoseryl adenosine is assumed to be between phosphoserine and adenosine. However, it is possible that phosphoseryl adenosine may not move very far from the origin. Owing to the lack of an authentic sample of phosphoseryl adenosine, it was not possible to determine the correct R_f value.

In conclusion the results presented in this communication indicate that phosphorylation of proteins in mammary gland is different from the phosphorylation of protein in other tissues such as liver, kidney and blood.

While the phosphorylation of liver proteins takes place after the complete protein is formed, the phosphorylation of protein from mammary gland takes place before the formation of complete protein. Attempts to locate the phosphorylation at earlier steps indicated the possible presence of phosphoseryl SRNA in rat mammary gland.

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ABSTRACT

Evidence has been presented to show that the phosphorylation of protein of milk to form casein can take place not only by phosphorylation by protein kinase but by other mechanisms as well. No pool of phosphoserine has been detected in the lactating mammary gland under conditions wherein the biosynthesis of protein is inhibited. The data obtained indicate the possible presence of phosphoseryl SRNA in the rat mammary gland. By use of inhibitors of protein synthesis such as ethionine, puromycin and cycloheximide, it has been shown that while the phosphorylation of liver protein occurs after the complete protein is formed, the phosphorylation of protein from mammary gland takes place before the formation of the complete protein.

showed that phosphorylation is relatively insensitive to the presence of puromycin during the first two hours of incubation and as the incubation time increased, the rate of incorporation of Pi^{32} would be equal to that of C^{14} -amino acid incorporation in presence of puromycin. In our experiments not only puromycin but also, ethionine and cycloheximide partially inhibited the phosphorylation of mammary gland protein. The higher inhibition of phosphorylation in whole animals could be due to the reason that phosphorylation is subjected to the effect of ethionine for 5 hours where as in tissue slice experiments it is only for $\frac{1}{2}$ hr. and 2 hrs. similar to the experiments of Turkington and Topper (21).

The results compiled above from this laboratory as well as that of others indicate that phosphorylation at the peptide level before the complete protein is formed is probably by the enzyme protein kinase. However these results do not rule out the possibility of phosphorylation at earlier sites. Therefore, a detailed study was undertaken to evaluate the first three possible sites for the phosphorylation.

The phosphorylation of serine has not been shown so far. But phosphoserine can be formed from phosphoenol pyruvic acid. If phosphoserine is incorporated into protein, there must be a pool of phosphoserine in lactating mammary gland. Under conditions of inhibition of protein synthesis, phosphoserine must accumulate like other amino acids. However, phosphoserine could not be identified in normal lactating mammary gland or when ethionine was injected. Second possibility is that phosphoserine formed is rapidly incorporated into S-RNA. It is known that protein synthesis is very rapid in lactating mammary glands. Therefore, incorporation of chemically prepared phosphate- P^{32} into S-RNA was followed using the amino acid activating system of rat mammary gland and the incorporation of phosphoserine into S-RNA fraction was observed.

The next obvious step would be to look for phosphoserine in the S-RNA fraction. Phosphoserine was identified in S-RNA fraction. However, to prove unequivocally the presence of phosphoserine in the -CCA terminal of S-RNA should be shown, because a number of studies have shown that amino acids can attach on S-RNA at non-specific sites(16). Therefore, experiments were conducted following the method of

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SUMMARY

Evidence for two types of phosphorylation of proteins is presented. Ethionine inhibited the in vivo incorporation of both amino acids and P_i^{32} into casein to the same extent, where as it inhibited the amino acid incorporation and not of P_i^{32} into proteins of liver and kidney.

In vitro addition of puromycin and cycloheximide to liver or lactating mammary gland slices inhibited the C^{14} -valine incorporation into proteins to an extent of 90% and 80% respectively. Using mammary gland slices, in vitro addition of these two antibiotics inhibited the incorporation of P_i^{32} to a less but significant extent; whereas they did not inhibit the incorporation of P_i^{32} into liver proteins, when added to liver slices.

Although in vitro addition of ethionine to liver slices inhibited C^{14} -valine incorporation into proteins to an extent of 69%, its addition to mammary gland slices, inhibited the C^{14} -valine incorporation only to about 29%.

The presence of phosphoseryl-sRNA is indicated though not conclusively, in lactating mammary gland.

From these results it is suggested that there are two types of phosphorylation of proteins, one occurring in liver and kidney which is not sensitive to the inhibitors of protein synthesis and is present in lactating mammary gland.

SUMMARY

Attempts have been made to fractionate cow and buffalo casein and the purification of casein has been followed using acrylamide gel electrophoresis. The preparation of K-casein from buffalo milk has also been made.

Comparative studies have been carried out on the physico-chemical properties of casein from cow and buffalo milk. The data presented show that buffalo casein has all the four major components, namely alpha-, beta-, gamma- and K-casein corresponding to the respective components in cow casein. The electrophoretic mobilities of alpha-, beta- and gamma-casein are almost same as that of cow casein in Agar gel and acrylamide gel electrophoresis. The mobility of K-casein in acrylamide gel electrophoresis after reduction by mercaptoethanol is same as that of cow K-casein. Other similarities were found in amino acid composition. The main difference appears to be in the case of sulphur containing amino acid namely cystine. The difference in cystine content found in whole casein appears to be mainly due to difference in the cystine content of K-casein. The higher cystine content of buffalo K-casein may be one of the reasons for the slow release of sialic acid from buffalo casein compared to cow casein by the action of rennet.

The considerable variation in casein components found in different breeds of cow are not present in buffalo casein. There was no difference in the pattern of casein components from local buffaloes and Murrah buffaloes.

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