STUDIES ON THE REGULATION OF ENZYMES RELATED TO FATTY ACID SYNTHESIS IN GOAT MAMMARY GLAND USING EXPLANT CULTURE SYSTEM

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September 27, 1985.

I certify that the work reported in this thesis entitled "STUDIES ON REGULATION OF ENZYMES RELATED TO FATTY ACID SYNTHESIS IN GOAT MAMMARY GLAND USING EXPLANT CULTURE SYSTEM" was carried out by Mr. Hitesh Kumar Jindal under my guidance for the requirement of Degree of Doctor of Philosophy in ANIMAL BIOCHEMISTRY in the Faculty of Dairying, Animal Husbandry and Agriculture of Kurukshetra University, Kurukshetra.

(R.S. PANDEY)

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(HITESH K. JINDAL)

DEDICATED

To My

GRANDFATHER

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CHAPTER - 1.

I N T R O D U C T I O N

INTRODUCTION

The mammary gland is a unique specialization of mammalian class of animals. It is distinct from other tissues as it undergoes changes of most striking kind, both structurally and functionally during different physiological stages. The mammary gland development results in the production of lactating tissues that have the capacity for enormous rates of milk secretion. The high biosynthetic capacity is a consequence of the cyto differentiation and proliferation of the mammary lobulo-alveolar cells. It has long been established that cell differentiation is hormonally controlled and the differentiated stage of a given cell type can be hormonally maintained (Rutter et al., 1973; Kuhn, 1977; Tucker, 1979; Shiu and Friesen, 1980; Akers et al., 1981). The multiple regulatory processes which permit the mammary cells to develop and function in response to pregnancy occuring at discrete periods in the life of the animal, are stimulated by a large number of systematic hormonal signals. Although the complexity of hormonal signals has been defined by studies in vivo (Folley, 1956; Lyons, 1958), the analysis of processes requires a more defined system such as tissue culture or organ culture.

Explant culture of mammary gland from various stages of pregnancy and lactation has been used to study several aspects of mammary development and differentiation. Tissue culture techniques particularly with the mammary explants,

have been used extensively in order to understand the role of individual hormones in initiation of lactogenesis (Topper and Oka, 1974). The effect of hormones (insulin, prolactin and cortisol) on the synthesis of milk proteins and lactose has been studied in considerable details using explants of mammary gland (Forsyth, 1971; Topper and Oka, 1974; Houdebine and Gaye, 1975). It has been demonstrated by Hallowes et al., (1973) that insulin, prolactin and cortisol are necessary for maximal fatty acid synthesis in mammary explants from mid-pregnant rats. It has also been observed that the explants of mammary gland from mid-pregnant rabbit respond to hormones (insulin, prolactin and cortisol) with an increase in the activity of acetyl-CoA carboxylase (Manning et al., 1976a), fatty acid synthetase (Speake et al., 1975, 1976a,b; Lynch and Dils, 1976; Forsyth, 1971), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Leader and Barry, 1969; Oka and Perry, 1974; Betts and Mayer, 1977) and medium-chain fatty acid synthesis in mouse mammary explants(Borst, 1980). But tissues from just before or just after parturition show a much poorer stimulation of lipogenesis. Cameron et al., (1983) reported that lipid biosynthesis in cultured mammary tissue from mice during mid-pregnancy was maximally stimulated by the combined action of insulin, prolactin and cortisol. However, fewer information is available on the effect of these hormones on the enzymes related to fatty acid synthesis and reducing equivalents generating enzymes in explants from ruminant or goat mammary gland.

The acetyl-CoA carboxylase which is the first enzyme related to fatty acid synthesis in lipogenic pathway, has long been recognised as a potentially rate limiting enzyme of the pathway leading to fatty acid synthesis. It has been purified from rat mammary gland (Miller and Levy, 1975; Ahmad et al., 1978; Ahmad and Ahmad, 1981; Ahmad et al., 1982), rabbit mammary gland (Manning et al, 1976b) and bovine adipose tissue (Moss et al., 1972). Fatty acid synthetase has also been purified to homogeneity from rat mammary gland (Smith and Abraham, 1970, 1971a,b), rabbit mammary gland (Carey and Dils, 1970 and Strong and Dils, 1972) and bovine mammary gland (Knudsen, 1972; Kinsella et al., 1975). The anti bodies have been raised against these enzymes purified from different sources by different workers like from rabbit mammary gland (Ahmad et al., 1978), rat mammary gland (Smith, 1973) and rat liver (Volpe et al., 1973). The antibodies so produced have been used to estimate the amount of these enzymes in mammary explants from mid-pregnant rabbits. The reports have conclusively shown that the changes in the activities of these enzymes in the presence of hormones are due to the changes in the enzyme amounts. The maximum increases in the amounts of the enzymes were only brought about by the culture of explants in the presence of hormones (insulin, prolactin and cortisol). The increase in the activities of fatty acid synthetase (Speake et al., 1975; 1976a,b), acetyl-CoA carboxylase (Mayer, 1978) and 6-phosphogluconate

dehydrogenase (Betts and Mayer, 1977) were found to be due to the increase in the amounts of these enzymes.

A scanty information is available on the purification of the above enzymes and their quantitative estimation employing immunological technique to elucidate the action of hormones on the activities as well as on the amounts of the lipogenic enzymes associated with fatty acid synthesis and reducing equivalents (NADPH) generating enzymes in the explants from mid-pregnant goat mammary gland.

In view of the above consderations, the present project has been designed to

(1) Study the hormonal (insulin, prolactin and cortisol) regulation of the enzymes related to fatty acid synthesis viz. acetyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthetase, medium-chain acyl thioesterase, long-chain acyl thioesterase and reducing equivalents generating enzymes viz. glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase and NADP-isocitrate dehydrogenase in mid-pregnant goat mammary gland using explant culture system.

(2) Purify some of the lipogenic enzymes to homogeneity for the preparation of monospecific antisera and to estimate immunologically the amounts of these enzymes, in explants from mid-pregnant goat mammary gland under the influence of insulin, prolactin and cortisol.

CHAPTER – 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The mammary gland is a unique tissue in the body which undergoes tremendous metabolic adaptations during prepartum and lactation. The control of mammogenesis, lactogenesis and lactation are sequential events in the differentiation process, whereby mammary cells are converted from a nonsecretory to a secretory stage. It undergoes differentiation to prepare itself for the task of copious secretion of milk with unique components like casein, lactose and milk fat, at about the time of parturition. With the approach of onset of lactation, extensive changes in enzyme activities occur in mammary tissue for synthesis of milk components, which were either lower or absent during virgin or pregnant state. Hormones are primary physiological factors, which act and interact in a complex fashion to regulate mammary secretory cell development, initiate secretory process and maintain lactation. Thus lactogenesis is a cascade of events exhibited by appearance or increase in enzyme activities specific for synthesis and secretion of milk components like lactose, casein or triglycerides, with marked increase in the mammary nucleic acids (RNA). Increased secretion of prolactin, glucorticoids, estrogens and placental lactogens are also observed during this process and are related to these events.

Baldwin and Yang (1974) have presented a model in which enzymes are placed into three groups namely, 1. Constitutive enzymes whose activities are not hormone dependent, 2. Enzyme whose synthesis is in part constitutive and in part hormone dependent, 3. Enzymes whose synthesis is almost entirely subjected to specific hormonal regulation. The synthesis of the enzymes involved in milk fat biosynthesis is considered to be under the partial or complete control of hormones (Baldwin and Yang, 1974).

2.1 Lipid biosynthesis in mammary gland:

The mammary gland is one of the most active tissues in the body with regard to lipid synthesis. Apart from the high rate of synthesis, it is unique in the synthetic process viz. production of short and medium-chain fatty acids. The fatty acids comprising milk triglycerides arise from two sources, circulating lipids in the blood and de novo synthesis within mammary cell. In goat and cow the fatty acids of milk fat with carbon chain from C_4 - C_{14} as well as part of C_{16} arise from de novo synthesis (Popjak et al., 1951a,b; Palmquist et al., 1969), while rabbit mammary tissue produces large amounts of C_8 and C_{10} fatty acids (Carey and Dils, 1972). Bressler and Wakil (1961) and Wakil (1962) showed that the principal mechanism of fatty acid synthesis in many animal tissues involved two basic reactions. The first reaction concerns carboxylation of acetyl-CoA to malonyl-CoA, which is catalyzed by acetyl-CoA carboxylase enzyme. The second reaction is catalyzed

by a group of enzymes, collectively called as fatty acid synthetase, for synthesis of long-chain fatty acids by repetitive addition of two carbon units in the form of malonyl-CoA. Ganguly (1960) established that these two reactions were mainly responsible for the de novo synthesis of fatty acids in the cytosolic fraction of bovine mammary gland. The literature on lipid biosynthesis in mammary gland has been divided into the following topics:

(a) Source of carbon for fatty acid synthesis,

(b) Source of reducing equivalents (NADPH) required for fatty acid synthesis,

(c) de novo fatty acid synthesis,

(d) Hormonal regulation of overall lipogenesis.

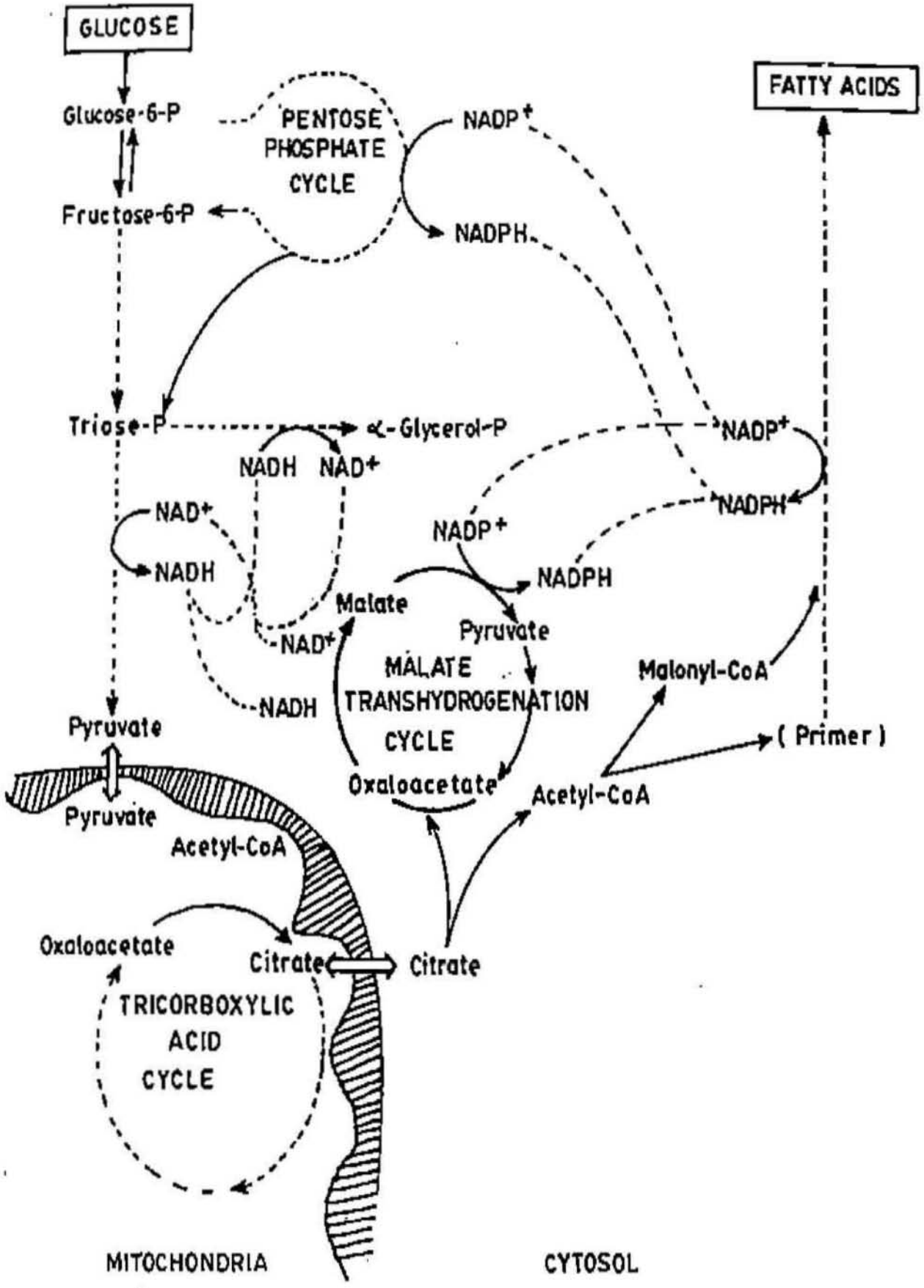
2.1.1 Source of carbon for fatty acid synthesis:

Fatty acid synthesis involves a source of substrates and associated enzymes for their conversion to acetyl-CoA and malonyl-CoA followed by orderly addition of this compound to a primer (acetyl-CoA and/or propionyl-CoA) until the newly synthesized fatty acid is released from the fatty acid synthetase complex.

Cytosol is the site of de novo synthesis of fatty acids both in non-ruminants (Bartley et al., 1965; Smith and Dils, 1966; Bauman and Devis, 1974) and in ruminants (Dekay et al., 1976). In vivo and in vitro studies demonstrate that non-ruminants utilize glucose as a carbon source for fatty acid synthesis. Glucose is converted to pyruvate which enters the mitochondria where it undergoes oxidative decarboxylation to acetyl-CoA. Since acetyl-CoA

Plate 1. Pathways of fatty acid synthesis in non-ruminant mammary tissue.

(Source: Bauman, D.E. and Davis, C.L. (1974) In "Lactation: A comprehensive Treatise", Vol.II (eds. B.L.Larson and V.R.Smith) p 36, Academic Press, New York.)

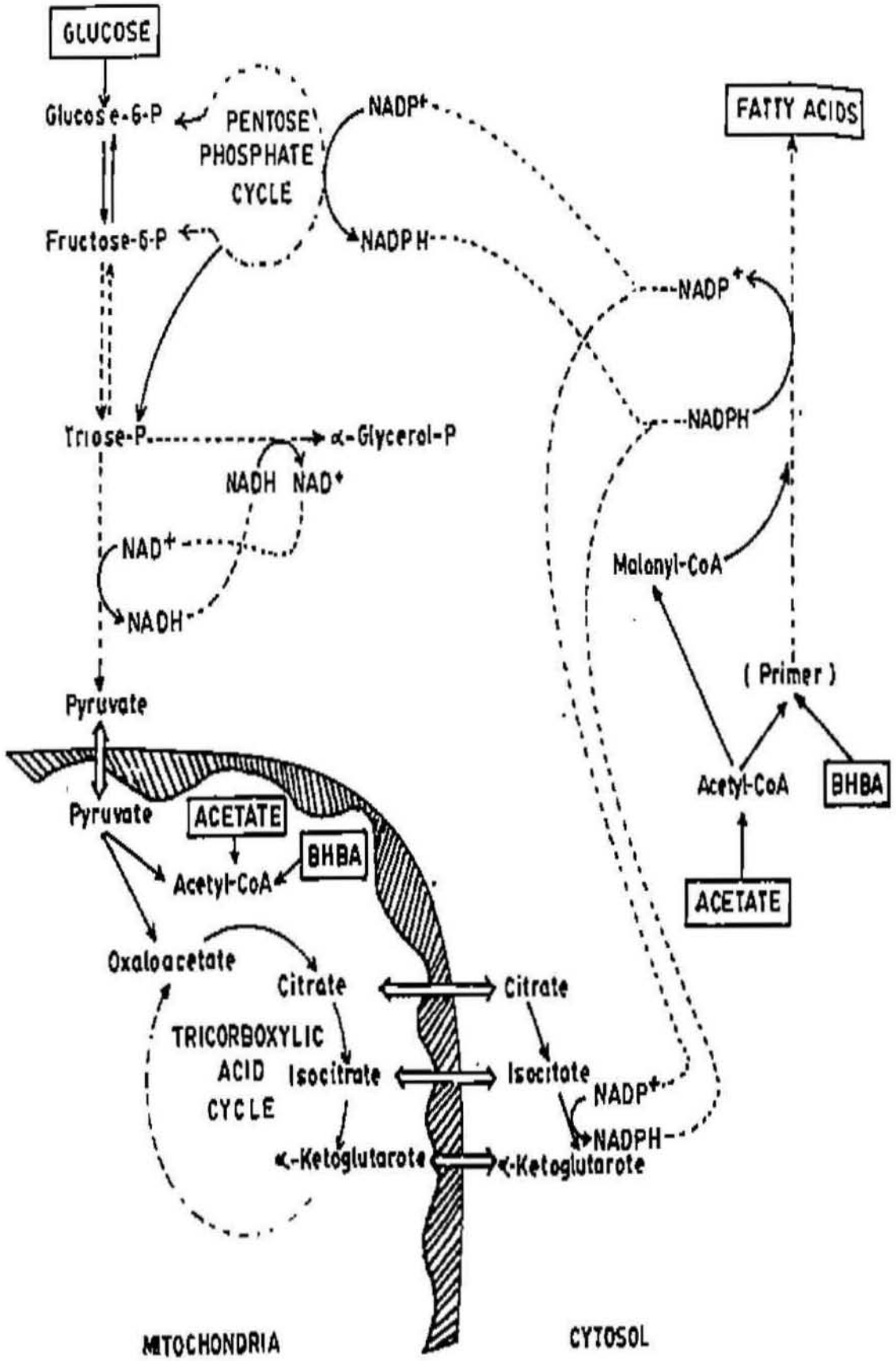

GLUCOSE
Glucose-6-P
Fructose-6-P
PENTOSE PHOSPHATE CYCLE
NADP+
NADPH
FATTY ACIDS
Triose-P
α-Glycerol-P
NADH
NAD+
NADP+
NADPH
NAD+
NADH
NADP+
NADPH
Malate
Pyruvate
NAD+
NADH
MALATE TRANSHYDROGENATION CYCLE
Malonyl-CoA
(Primer)
Pyruvate
Pyruvate
Oxaloacetate
Acetyl-CoA
Acetyl-CoA
Oxaloacetate
Citrate
Citrate
TRICORBOXYLIC ACID CYCLE
MITOCHONDRIA
CYTOSOL

can not diffuse into the cytosol where fatty acid synthesis occurs, it circumvents the mitochondrial barrier by condensing with oxaloacetate to form citrate (Lowenstein, 1968). Citrate passes into the cytosol and is cleaved by the enzyme ATP-citrate lyase to form extra mitochondrial oxaloacetate and acetyl-CoA (Spencer and Lowenstein, 1966; Bhaduri and Srere, 1963). Acetyl-CoA is then used for fatty acid synthesis and oxaloacetate thus released is used to regenerate pyruvate through coupled reactions involving the two malate dehydrogenases, NAD-malate dehydrogenase and NADP-malate dehydrogenase (Plate 1). The activity of ATP-citrate lyase in non-ruminant mammary tissue increases dramatically at the onset of lactation and decreases with weaning (Howenitz and Levy, 1965; Baldwin and Milligan, 1966; Spencer and Lowenstein, 1966; Jones, 1967; Kuhn and Lowenstein, 1967; Watson and Lowenstein, 1970).

On the other hand ruminant mammary tissue utilizes acetate, propionate and β-hydroxybutyrate (rumen microbial fermentation products) for de novo synthesis of fatty acids (Folley and French, 1950; Balmain et al., 1954; Hardwick et al., 1963; Bauman et al., 1973; Parker and Smith, 1974). The key to this species difference came from studies with perfused goat mammary gland (Hardwick et al., 1963; Hardwick, 1966) in which it was shown that although glucose was not incorporated into fatty acids, it was utilized in the synthesis of milk citrate and casein glutamate (Plate2). These results led Hardwick (1966) to propose that in ruminant mammary tissue the mitochondrial and cytosolic

Plate 2. Pathways of fatty acid synthesis in ruminant mammary tissue

(Source: Bauman D.E. and Davis, C.L. (1974), In "Lactation: A Comprehensive Treatise", Vol.II (eds. B.L. Larson and V.R. Smith) P.38. Academic Press, New York.


GLUCOSE
Glucose-6-P
PENTOSE PHOSPHATE CYCLE
NADP+
NADPH
Fructose-6-P
FATTY ACIDS
NADP+
NADPH
Triose-P
α-Glycerol-P
NADH NAD+
NAD+
NADH
Malonyl-CoA
(Primer)
Pyruvate
Pyruvate
ACETATE
BHBA
Acetyl-CoA
Acetyl-CoA
BHBA
ACETATE
Oxaloacetate
Citrate
Citrate
TRICORBOXYLIC ACID CYCLE
Isocitrate
Isocitrate
NADP+
NADPH
α-Ketoglutarate
α-Ketoglutarate
MITOCHONDRIA
CYTOSOL

pools of acetyl-CoA were not in equilibrium. Both glucose and acetate contributed to the intramitochondrial acetyl-CoA pool, but only acetate contributed to the extra mitochondrial acetyl-CoA pool where fatty acid synthesis occurred (Plate 2)

A comparison of the activities of enzymes involved in citrate cleavage pathway (i.e. generation of extra mitochondrial acetyl-CoA) reveals marked species differences between ruminants and non-ruminants. Substantial activity of ATP-citrate lyase has been found in the tissues of all non-ruminant species investigated. However, the activities of this enzyme as well as of NADP-malate dehydrogenase are extremely low in cow (Bauman et al., 1970). Sheep (Bauman et al., 1973) and goat (Hardwick, 1966; Reddy and Ray, 1982). Therefore, the inability of the ruminant mammary gland to utilize glucose carbon for fatty acid synthesis is due to the absence of cytosolic ATP-citrate lyase which is essential for translocation of mitochondrial acetyl-CoA (derived from glucose) to cytosol for fatty acid synthesis (Plate 2).

It is now established from the in vivo experiments that acetate and β-hydroxybutyrate originating from microbial fermentation in the rumen are the predominant carbon precursors for fatty acid synthesis in ruminant mammary gland (Popjak et al., 1951a,b; Palmquist et al. 1969). It has been shown that these two metabolites contribute almost equally to the initial four carbons of the fatty acids synthesized by goat and cow mammary tissue. However, acetate is the main supplier of the remaining

carbon in the fatty acid chains. Part of β-hydroxy butyrate has been shown to enter chain elongation process in the ruminant mammary tissue as an intact carbon unit (Kumar et al., 1965; Luick and Kameoka, 1966; Bines and Brown, 1968; Smith and McCarthy (1969). Other studies have indicated that ruminant mammary tissue initially cleaves a significant portion of β- hydroxybutyrate to C_2 units (acetate) prior to its utilization for fatty acid synthesis (Kumar et al., 1959; Lauryssens et al., 1960; Linzell et al., 1967). The utilization of β-hydroxybutyrate for fatty acid synthesis, other than the initial primer C_4 unit requires its conversion to acetyl-CoA.

The lack of utilization of glucose carbon for fatty acid synthesis in ruminants appears to be linked with evolutionary adaptations which have resulted in a unique process of digestion of feed stuffs rich in cellulose common to ruminants. Ingested carbohydrates are fermented extensive in the rumen to acetate, proprionate and butyrate, resulting in little absorption of glucose from the gut. Thus, the glucose which is needed for specific functions by the mammary gland (milk lactose, NADPH generation, glyceride glycerol formation etc.) must be supplied almost entirely from gluconeogenesis by the liver and kidney. This places glucose at a premium in the tissues of these animals. As a consequence, acetate and β-hydroxybutyrate, because of their availability, become the predominant carbon sources for fatty acid synthesis in the mammary gland as well as other lipogenic tissues of ruminants (Hanson and Ballard,

1967, 1968; Ingle et al., 1972).

2.1.2 Source of reducing equivalents (NADPH) for fatty acid synthesis:

Apart from the source of carbon atoms, the other factor needed for active fatty acid synthesis is a readily available supply of reducing equivalents in the form of NADPH. The NADPH is generated mainly by the first two steps of pentose phosphate pathway. The other enzymes involved in the contribution of reducing equivalents are cytosolic NADP-malate dehydrogenase and NADP-isocitrate dehydrogenase (Bauman et al., 1970). All these enzymes are generally present in the cytosol of synthesizing mammary cell.

There are some differences in the source of reducing equivalents (NADPH) for fatty acid biosynthesis in ruminants and non-ruminants as might be expected when different carbon sources are utilized for oxidation to provide NADPH. Two essentially similar schemes have been identified for generation of NADPH in the ruminants (Plate 2) and non-ruminants (Plate 1) (Bauman et al., 1970; Gumaa et al., 1973; Bauman and Davis, 1974).

In non-ruminants, the pentose phosphate pathway and transhydrogenation cycle are seen as potential producers of NADPH (Bauman and Davis, 1974). It is postulated that in lactating rat mammary tissue the pentose phosphate pathway is the predominant system. The malate transhydrogenase system plays a lesser role in mammary tissue than

in adipose tissue (Plate 1) and cytosolic isocitrate dehydrogenase is much less important than in the case of ruminants (Gumaa et al., 1973).

In ruminants, the cytosolic NADP-isocitrate dehydrogenase is allocated a key role in NADPH biosynthesis (Bauman et al., 1970, 1973; Gumaa et al; Reddy and Ray, 1982) although pentose phosphate pathway also plays an important role here (Plate 2). The quantitative significance of pentose phosphate pathway will depend upon glucose availability in ruminant mammary gland. Gumaa et al., (1973) have pointed out that in a tissue such as ruminant mammary gland, which is adapted to conserve glucose, pentose phosphate pathway is the optimal system in relation to lipogenesis.

With the onset of lactation pronounced increases in the activities of enzymes in particle free supernatant fractions of mammary cells have been noticed. Activities of acetyl-CoA carboxylase, citrate cleavage enzymes, enzymes of HMP shunt were found to increase several folds in rat, mice and rabbit mammary tissue as compared to thp pregnant levels (Howanitz and Levy, 1965; Baldwin and Milligan, 1966; Bartly, et al., 1966). The peak enzyme activities were observed at mid-lactation (Emery and Baldwin, 1967; Gul and Dils, 1969). The peak enzyme activities were also reported to remain elevated during late lactation, though a drop in milk yield was noticed (Hartmann and Jones, 1970). A biphasic adaptation of mammary tissue for fatty acid synthesis was observed by

Mellenberger and Bauman (1974) in rabbit mammary tissue as evidenced by increased dehydrogenase activities during mid-pregnancy and at post partum. A temporal relation between fatty acid synthetic ability and acetyl-CoA carboxylase activities was also observed by the same investigators. Weaning results in precipitous fall in the activities of the acetyl-CoA carboxylase but much slower decline was observed for dehydrogenase activity.

In ruminant mammary gland 18 to 44-fold increase in the activities of dehydrogenase enzymes between 3 to 14 week prepartum to 2 week postpartum were noticed (Shirley et al., 1973). A similar trend but of lesser magnitude was observed by Mellenberger et al., (1973) in bovine mammary tissue between 30 and 7 day prepartum and 7 and 40 day postpartum studies. However, Currie (1972) reported erratic but statistically significant changes in the enzymatic activity in the bovine mammary samples obtained by biopsy throughout lactation. Increased levels of dehydrogenases, especially isocitrate dehydrogenase were reported in lactating bovine mammary tissue as compared to non-lactating mammary tissue or mammary tissue obtained from virgin animals (Weldschmidt and Rilling, 1973). The activities of glucose-6-phosphate dehydrogenase and lipoprotein lipase were also found to vary in parallel with milk fat secretion during early lactation in goats (Chilliard et al., 1978).

2.1.3 de novo fatty acid synthesis

Notwithstanding species variations, a major quantity of fatty acids is synthesized within mammary cell. Cytosol is the site of de novo synthesis of fatty acids both in nonruminants (Dils, 1977) and in ruminants (Dekay et al., 1976). In animals, the seven enzymes in the synthesis of fatty acids are held together tightly, in a multienzyme complex known as fatty acid synthetase (FAS) which behave as a single unit (Bloch and Vance, 1977). This enzyme complex is highly active in the lactating mammary gland, where it is involved in the production of substantial amount of milk fat (Chatterjee et al., 1979). Acetyl-CoA carboxylase catalyses the first committed reaction - the carboxylation of acetyl-CoA to malonyl-CoA in de novo fatty acid synthesis and is considered a likely site for regulation of lipogenesis in animal tissues (Lane et al., 1974). After formation of malonyl-CoA, its condensation with acetyl-CoA and further reduction steps are catalysed by fatty acid synthetase. The mechanism of synthesis of fatty acid in mammary tissue is similar to that of other tissues, except that the product formed are short and medium-chain length acids which are characteristics of milk fat.

The importance of these enzymes in mammary lipid metabolism can be seen by the characteristic changes in their activities during later stages of pregnancy, lactogenesis and involution of the gland. Hartmann and Jones (1970) and Short et al. (1977) noted 2 to 10 fold

increase in the activities of acetyl-CoA carboxylase and acetyl-CoA synthetase during early lactation in rabbit mammary tissue as compared to the activities observed in mammary tissue obtained from non-pregnant and pregnant rabbits. Mellenberger and Bauman (1974) also observed a dramatic rise in acetyl-CoA carboxylase activity at onset of lactation. Similar observations in the rats (Howanitz and Levy, 1965; Mackall, 1976; Mackall and Lane, 1977) indicated that acetyl-CoA activity which remained low throughout gestation, increased to values 30 to 40 times at parturition and during early lactation. In mouse mammary tissue, in contrast to the rat, the lipogenic activity could be detected in late gestation which reached maximum level at mid-lactation. Changes in the lipogenic activity in ruminant mammary tissue has been investigated by Kinsella and Heald (1972); Mellenberger et al. (1972, 1973) who obtained mammary tissue during prepartum and postpartum. It has been observed that 5 to 25-fold increase in the activities of acetyl-CoA carboxylase and fatty acid synthetase were noticed with initiation of lactation. Chilliard et al. (1978) observed the changes in the activities of acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, malic enzyme and lipoprotein lipase which varied parallel with milk fat secretion after parturition in goats and the proportion and chain length of fatty acids formed in goat mammary tissue homogenate, were similar to that found in goat milk (Grunnet and Knudsen, 1979a).

Normally the fatty acid synthetase produces the long-chain fatty acids as end products. However, short and medium-chain fatty acids occur in milk fat. The fatty acids are cleaved from their thioester linkages by thioesterases that are a composite part of the fatty acid synthetase complex. The mammary gland specific thioesterase appears to be responsible for the ability of the tissues to synthesise medium and short-chain fatty acids characteristic of milk fat (Knudsen, 1976; Libertini et al., 1978; Smith and Ryan, 1979; Smith, 1980; Smith and Stern, 1981; Abdinejad et al., 1981). A chain length modifying protein has been prepared from rabbit mammary gland, which may loosely but specifically interact with fatty acid synthetase (Carey, 1977). An acyl-thioester hydrolase was isolated from cytosol of the lactating rabbit mammary gland. This enzyme terminates fatty acid synthesis at medium-chain ($C_{8:0}$ - $C_{12:0}$) fatty acids when incubated with fatty acid synthetase and rate limiting concentration of malonyl-CoA esters of chain length ($C_{10:0}$ - $C_{16:0}$) when these were used as model substrates (Knudsen et al., 1976). The appearance of these acylthioesterases becomes quite profound just prior to parturition (Chivers et al., 1976; Knudsen and Dils, 1975; Knudsen et al., 1975). The presence of short and medium-chain acyl-thioesterases has been demonstrated in rabbit and rat mammary gland. Its presence in the goat mammary tissue is, however, not well established (Grunnet and Knudsen, 1979 a,b) but the presence of long-chain acyl-thioesterase has been observed

(Smith et al., 1976; Agradi et al., 1976).

2.1.4 Hormonal control of over all lipogenesis:

The studies during pregnancy and lactation show that the mammary secretory activity increases but the most rapid shift occurs from non-lactating to lactational stage around the time of parturition. The gradual increase in the total secretory activity is probably related to the total amount of secretory tissue present as well as the activity of these cells to secrete milk fat (Jeffer, 1935). Normally, it is only during pregnancy that the previously determined mammary epithelial cells begin to realize their differentiative potential. It is seen that the progression of changes, both structural and functional which the epithelial cell experiences in this stage of ontogeny is a response to several hormones (Hershako et al., 1971; Rutter et al., 1973). It is ubiquitous that the initiation and maintenance of lactation are dependent upon hormonal requirements vary among species, but in general minimal needs include prolactin and adrenal glucocorticoids and relative absence of progesterone. Hormone actions at the cellular levels can be divided into those that are rapidly mediated (acute responses) and those that are slowly mediated (chronic responses) according to Mayer (1978). Several acute hormone responses, such as the regulation of glycogen metabolism or lipolysis have been delineated in detail. These responses to hormones are very rapid and are associated with changes in cellular concentration of cyclic-AMP (Hers, 1976). The chronic hormonal

regulation of cell metabolism occurs over periods of hours to weeks, and usually involves selective changes in gene expression resulting in the accumulation of enzymes or proteins necessary for some differentiated function (Chan and O'Malley, 1976). Hormones are required in mammary gland *in vivo* in regulating the distinct parts of lipogenesis (a) The rate of fatty acid biosynthesis. (b) The chain length composition of fatty acids produced.

Detailed measurements of the changes in lipogenic flux in rabbit mammary gland development have been made by Strong and Dils (1972). The results show a biphasic increase in the lipogenic rate with an initial lipogenic stimulus in mid-pregnancy (16-18 days) and a second stimulus occurs just before parturition. It has been suggested that in the rabbit the depressed plasma progesterone on 21 day of pregnancy combined with a rise in concentration of free glucocorticoids, may coincide with the first lipogenic stimulus in rabbit mammary gland. From studies with explants of mammary gland from pseudopregnant rabbit, it is apparent that prolactin and insulin are also required for the first lipogenic stimulus in mammary gland (Denamur, 1971).

Hallowes *et al.*, (1973) have demonstrated that insulin, prolactin and corticosterone are necessary for maximal fatty acid synthesis in mammary explants from mid-pregnant rats. It has further been observed that the explants of mammary gland from mid-pregnant rabbit respond

to hormones (insulin, prolactin and cortisol) with an increase in the activity of acetyl-CoA carboxylase (Manning et al., 1976a) and an increase in the amount of fatty acid synthetase (Speake et al., 1975, 1976a,b; Lynch and Dils, 1976; Forsyth, 1971), 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase (Leader and Berry, 1969; Rivera and Cummins, 1971; Green et al., 1971; Oka and Perry, 1974; Betts and Mayer, 1977), acetyl-CoA synthetase activity (Merineze and Cook, 1971) and medium chain fatty acid synthesis in mouse mammary gland explants (Borst, 1980). But tissue from just before or just after partirition show a much poorer stimulation of lipogenesis or none at all. In explants from lactating tissue, there are decreases in rate of fatty acid biosynthesis, the percentage of medium-chain fatty acids synthesized compared to that in the earlier stages of development. However, the decrease observed in the absence of hormones is significantly greater than in the presence of hormones.

Response of the mammary gland to hormones progressively decreases during pregnancy and in lactation. However, the interpretation of these responses is complex, since it undoubtedly results from several factors including the accumulation of the milk within the alveolar lumina in explants, which simply prevents further response to hormones in the tissue by hydrodynamic feed back similar to that which is believed to occur during mammary involution in vivo (Jones, 1967).

2.1.4.1 Effect of insulin:

It has been revealed through histological studies that insulin is required for good survival of postnatal mammary tissue in vitro in many species (Trowell, 1959; Elias and Rivera 1959; Walters and Mclean, 1968). It is also essential for the maintenance and survival of the nonruminant secretory cells and the prolonged insufficiency of insulin results in the significant loss of mammary secretory cells. Changes in enzyme activities invclved in lipogenesis and ultimately decreased synthesis of fatty acids and glyceride glycerol from glucose and acetate (Baldwin and Louis, 1975). By utilizing tissue slices and explants from mammary tissues of mice (Jones and Forsyth, 1969; Leader and Barry, 1969; Rivera and Cummins, 1971; Wang et al., 1971, 1972 a,b; Denamur, 1971; Cameron, 1974; Oka and Perry, 1974), rats (Hallowes et al., 1973) and rabbits (Heitzman, 1968; Speake et al., 1975, 1976 a,b; Manning et al., 1976a; Betts and Mayer, 1977) the role of insulin has been established for the optimal rate of milk fat synthesis and its requirements for glucose oxidation via pentose phosphate pathway. Only limited data, however, is available on possible insulin action, in the regulation of ruminant mammary lipid metabolism.

Injection of insulin in the lactating cows (Baldwin et al., 1972) brings about a significant increase in the glucose oxidation and its conversion to glyceride glycerol. The mammary tissue slices from buffaloes (Bhatia et al., 1979) and goat (Skarda et al., 1977, 1978)

incorporated increased levels of C^{14}-acetate into total lipids in the presence of insulin and other hormones. However, no effect of insulin could be observed in cows in other experiment (Bauman et al., 1973).

Geelen et al., (1978) have concluded that the part of the action of this hormone is located at a point beyond the formation of pyruvate. Three lines of evidences support this conclusion. First of all, the insulin stimulates the incorporation of C^{14}-acetate into fatty acids. Secondly, the activity of acetyl-CoA carboxylase, the rate limiting enzyme of fatty acid biosynthesis is stimulated by the addition of insulin (Lee et al., 1973). Thirdly, insulin rapidly elevates the level of cellular content of malonyl-CoA, the product of acetyl-CoA carboxylation (Geelen et al., 1978). The observations substantiate the rate of acetyl-CoA carboxylase as a target in the short term control of fatty acid synthesis by insulin. Denton (1974) reported two-fold increase in the initial activity of acetyl-CoA carboxylase when the explants were treated with insulin. However, the insulin appeared to have no effect on the total activity of the enzyme. Volpe and Vagelos (1974) also reported the fatty acid synthetase activity in relation to administration of insulin and glucose or fructose feeding. The results indicated that the insulin wes not necessary for the regulation of fatty acid synthetase activity in liver but it may be necessary in the adipose tissue. Acetyl-CoA carboxylase activation occurs with insulin when inhibitors of protein synthesis are employed in the medium (Lee et al.,

1973). The rate of fatty acid synthesis in culture or suspension has been shown to be stimulated with added insulin and defused with glucagon (Geelen et al., 1978). The decreased level of insulin decreased the fatty acid synthesis in liver (Robinson et al., 1978). Insulin in combination with triiodothyronine, however, increased the activity of acetyl-CoA carboxylase activity and fatty acid synthetase activity in rats (Sophia et al., 1972).

In isolated rat mammary secretory cells insulin stimulates fatty acid synthesis from pyruvate three times and decreased lactate conversion to fatty acids 20 to 30% and stimulated glucose conversion to fatty acids 1.2 to 1.5 times. Incubation of glucose and pyruvate together depressed fatty acid synthesis from glucose. Insulin acts at important site other than or in addition to glucose transport in regulating mammary secretory cell metabolism and particularly fatty acid synthesis. Its action in fatty acid synthesis is dependent on the culture redox state. Insulin increases fatty acid synthesis in cells with a low redox state (Yang and Baldwin, 1976).

Green et al.,(1971) reported that glucose 1 mg/ml (ml of media) and insulin 5 ug/ml (of media) are needed for maximum increase in the activity of the enzyme glucose 6-phosphate and 6-phosphogluconate dehydrogenases. The increase in the enzyme activity results from a stimulated uptake of glucose by the mammary tissue, and that they are caused by a metabolic product of glucose stimulating the formation of mRNA for the two enzymes.

The central anabolic role of the hormones in the regulation of intermediary metabolism makes it difficult to distinguish between these actions of the hormones directed at the mammary gland and those affected or mediated via its influence on overall metabolism of lactating animals. It was shown that insulin could stimulate fatty acid synthesis in mammary explants from mid-pregnant mice (Moretti and Abraham, 1966) but the highest rate of fatty acid synthesis was observed when insulin was initially present in the medium rather than when it was added after a period of 24 hours (Wang et al., 1972a). Although insulin stimulates fatty acid synthesis and improves the viability of the tissue, it did not affect the pattern of fatty acid synthesized (Dils et al., 1972, 1974 and Borst, 1980). Nepokroeff et al. (1974) have indicated an increase in the fatty acid synthetic activity in the rat liver.

2.1.4.2 Effect of corticosteroids:

Glucocorticoid is one of the so called lactogenic hormones, both in vivo and in vitro in many species. Its role in the formation of rough endoplasmic reticulum, casein mRNA and casein have been discussed. It has been shown that glucocorticoids are required for maximal ductal growth (Topper and Freeman, 1980). The mammary tissue of dairy cows contain specific sites for glucocorticoids (Gorewit and Tucker, 1976 a,b; Tucker et al. 1971; Denamur, 1971; Collier et al., 1977) and these binding sites are more in number in lactating than in

non lactating mammary tissue. A number of studies indicate that the glucocorticoids are not acute or short term effectors of mammary metabolism but do act over a long term such that glucocorticoids insufficiency results in depressed lactational performance, changes in pattern of mammary nucleic acid synthesis and rates of synthesis of several key mammary biosynthetic enzymes. The effect of adrenalectomy, hypophysectomy and glucocorticoid replacement therapy upon lactating rat mammary enzyme levels have been investigated (Willmer, 1960; Korsrud and Baldwin, 1969, 1972 a,b,c; Yang and Baldwin, 1976; Plucinski and Baldwin, 1976). The results suggest that glucocorticoids are intimately involved in the regulation of mammary enzyme levels, that the effects are specific: that the enzymes affected are primarily involved in milk synthesis and that the extent of dependence upon glucocorticoids for the development or maintenance of normal enzyme activities varies such that the apparent enzyme inductions by glucocorticoids range from two to ten-fold (for malic enzyme and fatty acid synthetase, respectively). Although it was established long ago that glucocorticoids are essential for maximal lactational performance in ruminants (Baldwin and Louis, 1975), only limited data are available regarding possible glucocorticoid action upon mammary function. Ely and Baldwin (1976) investigated the effects of adrenalectomy and glucocorticoids replacement therapy upon enzyme activities in mammary tissue of lactating sheep. In contrast to rat, it has been

found that glucocorticoid insufficiency resulting from adrenalectomy did not result in decreased enzyme activities suggesting that the ruminant mammary function is less dependent upon glucocorticoids than in the rat.

Increased concentrations of glucocorticoids in humans and animals are associated with enhanced hepatic lipogenesis and increased hepatic and plasma lipids (Steinberg et al., 1952; Stern et al., 1973; Casaretto et al., 1974; Reaven et al., 1974; Diamant and Shafrir, 1975; Bagdade et al., 1976a,b; Krik et al., 1976). In addition, physiological concentrations of glucocorticoids in conjunction with insulin are required for the enhanced lipogenesis associated with refeeding after starvation (Berdanier and Shubeck, 1979; Bouillon and Berdanier, 1980). The relative role of these two hormones in this hyperlipogenic response are known. It has been suggested that the increased insulin concentrations associated with glucocorticoids excess, or a synergism between glucocorticoids and insulin, may mediate the enhanced hepatic lipogenesis (Krik et al., 1976; Bardanier and Shubeck, 1979).

Wang et al. (1972 a,b) reported that various hormones do not have any effect on lipogenesis singly. Cortisol singly had no effect but in combination with other hormones, it stimulated linogenesis. Korsrud and Baldwin (1969) also reported that administration of cortisol and prolactin were required to increase the normal levels of the activities of glucose-6-phosphate

dehydrogenase and 6-phosphogluconate dehydrogenase. Forsyth et al., (1972) reported that corticosterone antagonised the effect of prolactin in stimulating the synthesis of medium chain fatty acid.

2.1.4.3 Effect of prolactin:

Prolactin is necessary for the initiation and differentiation in early pregnancy and it also plays a dominant role in the epithelial growth after parturition (Topper and Freeman, 1980).

Topper and Oka (1974) concluded that prolactin is the hormone required for the sensitization of the epithelial cells upto mitogenic effect of insulin and other serum factors that may be mitogenic. Prolactin is required for the production of biosynthetic products of the mammary gland (casein and lactalbumin).

Cameron et al., (1983) reported that lipid biosynthesis in cultured mammary tissue from mice during mid-pregnancy was maximally stimulated by the combined action of prolactin with insulin and corticoids. The action of prolactin was specific for the formation of triglycerides but not other lipid classes. Prolactin increased fatty acid synthesis in pregnant mouse mammary gland explants (Mayne and Berry, 1970) and these fatty acids were found to be similar to those produced by lactating gland of mouse (Wang et al., 1972a).

Forsyth et al., (1972) showed that culture of mammary explants from mid-pregnant rabbits with prolactin

gives a rapid increase in the preparation of medium-chain fatty acids synthesized, but lipogenic rates are relatively low. The explanation for this could be that prolactin alone stimulates accumulation of the enzymes involved in producing medium-chain fatty acids but makes much less accumulation of other lipogenic enzyme, fatty acid synthetase when the mammary explants from mid-pregnant rabbits are cultured with prolactin alone (Speake et al., 1976b). However, culture of explants with insulin, prolactin and cortisol results in the maximum increase in the activity and rate of synthesis of fatty acid synthetase. The data put forward by Forsyth et al., (1972) support the concept that prolactin alone or insulin and prolactin together cause incomplete differentiation of mammary explants as measured by low fatty acid synthetase activity, rate of enzyme synthesis, or lipogenic rate. However, maximal amounts of medium-chain fatty acids are produced by these hormones. Much greater cyto-differentiation occurs in the tissues in response to insulin, prolactin and cortisol as measured by high fatty acid synthetase activity and rate of enzyme synthesis, or lipogenic rate (Forsyth et al., 1972) but the increased cytodifferentiation is accompanied by decreased rates of medium-chain fatty acid synthesis. The detailed mechanism is presently unknown but is probably mediated at the transcriptional or translational levels.

The mechanism by which prolactin exerts its action is obscure. It first interacts with the prolactin

receptors on the plasma membrane. The number of these receptors is under positive control of prolactin itself (Bohnet et al., 1978; Sheth et al., 1978).

2.1.4.4 Combined effect of insulin, prolactin and corticosteroids:

Mammary tissue undergoes a structural and biochemical differentiation during organ culture in the presence of the hormone combination of insulin, prolactin and cortisol.

Insulin could stimulate fatty acid synthesis in mammary explants from mid-pregnant mice (Moretti and Abraham, 1966) and the rate of fatty acid synthesis could be better maintained over 2 days by culture with insulin, corticosterone and prolactin. The appearance of lipid droplets in mammary explants treated with this hormone combination can be observed histologically in a number of species including the rabbit (Bronewell, 1965; Mills and Topper, 1970). The experiments of strong et al. (1972) showed that if explants from pseudo-pregnant rabbits were cultured with insulin, corticosterone and prolactin for 6-7 days, they could synthesize triglycerides enriched in the medium-chain fatty acids characteristics of rabbit milk. Freshly excised explants from pseudo-pregnant rabbits synthesize triglycerides and phospholipids containing long-chain fatty acids. The maximum rate of fatty acid synthesis observed with such explants after culture with insulin, corticosterone and prolactin was similar to that seen with freshly excised explants from lactating rabbit mammary gland

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by insulin and prolactin associated with or not with cortisol. Casein synthesis was stimulated only when prolactin was present in the culture medium. Prolactin alone was able to significantly support the induction of casein synthesis. The results indicate that insulin, prolactin and cortisol are involved in maintenance of goat mammary tissue in culture but prolactin essentially stimulates milk synthesis (Skarda et al., 1982a). The magnitude of the effect of prolactin on lipid synthesis was optimal when cortisol concentration between 0.1 to 0.5 µg/ml were employed. These concentrations of cortisol were reported by Ono and Oka (1980) to optimise prolactin stimulation of casein synthesis. The observations suggest the possibility that the mechanism by which prolactin stimulates lipids and protein biosynthesis in mammary gland may have some aspects in common (Cameron et al., 1983).

Green et al. (1971) studied the hormonal regulation of glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in mammary explants from mice. Insulin, glucose, amino acids and inorganic salts were the minimal requirements needed tc increase the enzyme activities in explants from lactating mice. Rivera and Cummins (1971) also showed that sustained maximal increase in the enzyme activities of glucose-6-phosphate dehydrogenase and 6-phosnho gluconate dehydrogenase were obtained when mammary explants from mid-pregnant mice were cultured with insulin, corticosterone and prolactin. Hormones were required in the medium for several hours in order to produce the maximal increase in

enzyme activities which were measured after 48 hours in culture. Oka and Perry (1974) have shown that insulin, prolactin and cortisol were required for a maximal increase in the activities of glucose-6-phosphate dehydrogenase in mammary explants from mid-pregnant mice. Cortisol enhanced the effect of insulin in causing an increase in the enzyme activity.

The mechanism of the change in enzyme activities has to be identified i.e. whether the increase in the enzyme activities is due to activation of preexisting enzyme or to an increase in the concentration (amount) of the enzymes in the tissue (Mayer, 1978). To measure the changes in the amount of 6-phosphogluconate dehydrogenase into the mammary explants from mid-pregnant rabbits, 6-phosphogluconate dehydrogenase has been purified from Rabbit mammary gland (Betts and Mayer, 1975) and a monospecific antisera has been produced. The antisera so produced has been used to estimate the amount of 6-phospho-gluconate dehydrogenase in mammary explants from mid-pregnant rabbits. The results conclusively have shown that changes in the activity of the enzyme in the presence or absence of hormones are due to changes in enzyme concentration (Betts and Mayer, 1977). The increases in the concentration of the enzyme were only brought about by culture of explants in the presence of hormones (insulin, prolactin and cortisol).

Similarly, the increases in the activities of fatty acid synthetase (Speake et al., 1975, 1976 b) and

acetyl-CoA carboxylase (Mayer, 1978) are found due to the increases in the amounts of the acetyl-CoA carboxylase (Mayer, 1978) and fatty acid synthetase (Speake et al., 1975, 1976b). Approximately six folds increase in the apparent rate of synthesis of fatty acid synthetase was observed when the explants of mammary gland from mid-pregnant rabbits were cultured with insulin, prolactin and cortisol.

2.1.4.5 Effect of insulin, prolactin, cortisol and glucose:

The role of glucose in triggering the increase in the amount of 6-phosphogluconate dehydrogenase in explants of rebbit mammary gland has been defined (Betts and Mayer, 1977). In the absence of glucose, no increase in the amount of enzyme occurred in the presence or in the absence of hormones. When explants were cultured in the presence of glucose. increases in the amount of enzyme occurred with or without hormones (insulin, prolactin and cortisol). Transfer of explants to glucose free medium resulted in a decrease in the amount of enzyme, both in the presence and absence of hormones. When explants were cultured in the absence of glucose, little increase in the amount of enzyme occurred in the presence or in the absence of hormones but if the explants were transferred to a glucose-containing medium an increase occurred in the amount of the enzyme in the presence, but not in the absence of hormones. The regulation of 6-phosphogluconate dehydrogenase in mammary explants is evidently complex. However, the results do show the obligatoryrequirement for glucose to produce an increase in enzyme amount and the potentiating effect of

insulin, prolactin and cortisol on glucose triggered increase in the enzyme amount. Glucose used in media could be replaced by mannose or fructose (Green et al., 1971). With higher glucose concentrations in the culture media, insulin did not potentiate the glucose-mediated increase in enzyme activity. Glucose was necessary initially in order to trigger the increase in enzyme activity, which occurred after 12 hours in culture (Leader and Barry, 1969). It has been suggested by Leader and Barry (1969) that increase in the activities of glucose-6-phosphate dehydrogenase result from an increased uptake of glucose by the mammary tissue and that the increases in activity are caused by metabolic product of glucose that stimulates the formation of mRNA during a few hour period after glucose is added to the culture medium. Leader and Barry (1969) have also shown that the increases in the enzyme activity both in culture and in the living animal at parturition are induced by an influx of glucose that is restrained during pregnancy by the growth hormone like action of placental lactogen. Green et al. (1971) have shown that when glucose was not added until 4 hours during explant culture of mid pregnant rabbit mammary gland, actinomycin D added at this time did prevent the rise in enzyme activity suggesting that synthesis of essential RNA only occurs on the addition of glucose.

Bolton (1971) has reported that when explants from pseudo-pregnant rabbit mammary gland were cultured for 8 days with insulin, prolactin and corticosterone, the

incorporation of 1-C^{14} glucose and 6-C^{14} glucose with total lipids increased seven-fold over control culture without prolactin. Insulin stimulated fatty acid synthesis from C^{14}-glucose (Moretti and Abraham, 1966) but that the further addition of corticosterone and prolactin in culture medium did not affect glucose incorporation into fatty acids (Mayne and Barry, 1970).

In <u>in vitro</u> experiments, insulin has been shown to increase membrane transport of glucose in the adipose tissue. Simultaneously conversion of glucose to glyceride glycerol and fatty acid with accompanying formation of CO_2 through HMP shunt pathway and oxidation of glucose by TCA cycle are also enhanced. Increased rates of glucose oxidation through HMP shunt pathway have been attributed to the generation of reduced NADP, which is required for the effective fatty acid biosynthesis (Mclean, 1958). Insulin increases the concentration of glycerol phosphate and may suppress lipolysis. It has been suggested that consequent to triglyceride formation, the tissue concentration of fatty acyl-CoA is lowered and thereby acetyl-CoA carboxylase is activated. In severe glucose deficiency in rats, fatty acid biosynthesis is markedly reduced and normal rates are not restored even with insulin <u>in vitro</u>.

2.2 Distribution, purification and some properties of lipogenic enzymes:

2.2.1 Acetyl-CoA carboxylase:

Acetyl-CoA carboxylase has long been recognized as a potentially rate-limiting enzyme in the process of fatty acid synthesis because of its low activity in various tissues as compared with other enzymes involved in lipogenesis (Korchak and Masora, 1962; Howantiz and Levy, 1965). The acetyl-CoA carboxylase in extracts of pigeon liver (Margolis and Baum, 1966) and rabbit mammary gland (Smith et al., 1966) was found distributed between the microsomal and supernatant fractions. Margolis and Baum (1966) and Easter and Dills (1968) have concluded from their experiments that all the acetyl-CoA carboxylase in pigeon liver and in rabbit mammary gland, respectively, is membrane bound in vivo.

Acetyl-CoA carboxylase has been isolated in homogenous form (Gregolin et al., 1966; 1968; Numa et al., 1966; Majerus and Kilburn, 1969) and crystalized (Goto et al., 1967) from chicken liver. This enzyme has also been purified from rat liver (Majerus et al., 1968; Nakanishi and Numa, 1970; Inoue and Lowenstein, 1972), lactating rat mammary gland (Miller and Levy, 1975; Ahmad et al., 1978; Ahmad and Ahmad, 1981; Ahmad et al., 1982), rat adipose tissue (Vagelos et al., 1963), rabbit mammary gland (Manning et al., 1976 b), bovine adipose tissue (Moss et al., 1972), pigeon liver (Waite and Wakil, 1962), E. coli (Alberts and Vagelos, 1968) and

wheat germ (Hatch and Stumpf, 1961).

It has been established that the carboxylase from animal tissues, either in homogenous form or in cell free extracts requires the presence of tricarboxylic acid activator e.g. citrate or isocitrate (Martin and Vagelos, 1962; Kleinschmidt et al., 1969; Lane et al., 1974 and Volpe and Vagelos, 1976), without activator, homogenous liver enzyme exists as a protomer (Moss and Lane, 1972; Guchhait et al., 1974). The addition of citrate leads to the formation of catalytically active form of enzyme (Lane et al., 1975). Activation of the enzyme by Mg^{++} (Greenspan and Lowenstein, 1968) and α-Glycerol 3-phosphate (Rasmussen and Klein, 1967) has also been reported. Phosphorylation of the enzyme inactivates whereas dephosphorylation activates the inactive acetyl-CoA carboxylase (Lee and Kim, 1977; Brownsey et al., 1977,1981; Hardie and Guy, 1980; Hardie and Cohen, 1978a,b, 1979; Lent et al., 1978). The activity of acetyl-CoA carboxylase increases in parallel with the increased lipogenic activity of the gland during the transition from early to peak lactation (Mackall and Lane, 1977; Martyn and Hansen,1981).

2.2.2 Fatty acid synthetase:

This enzyme is found in nature in two different organisational forms. In plants and primitive bacteria, the seven enzymes of fatty acid synthetase complex exist as discrete monofunctional proteins which can be isolated individually. However, in the more advanced microorganisms, yeasts, mold and animals, the component enzymes are

integrated into a multienzyme complex, which has been purified as a single protein (Bloch and Vance, 1977). Fatty acid synthetase has been purified to homogeneity from rat mammary gland (Smith and Abraham, 1970; 1971a,b), rat liver (Burton et al., 1968; Nepakroeff et al., 1975), rabbit mammary gland (Carey and Dils, 1970; Strong and Dils, 1972), bovine mammary gland (Knudsen, 1972; Kinsella et al., 1975), pigeon liver (Hsu et al., 1965) and human liver (Roncari, 1974a). The fatty acid synthetase from animal sources is obtained as a protein of approximately 500,000 molecular weight (Kumar et al., 1972; Yun and Hsu, 1972). Dissociation of this synthetase into 200,000 - 250,000 molecular weight subunits has been demonstrated with both low ionic strength buffer (Kumar et al., 1972; Yun and Hsu, 1972) and prolonged storage at 0 - 4°C (Muesing et al., 1975). Jacob et al. (1968) studied the covalent binding sites of the acetate and malonate to the soluble pigeon liver fatty acid synthetase. Reports of Yu and Burton (1975) confirmed the requirement of 4-phosphopantetheine group for fatty acid synthetase activity. It possesses the properties similar to those of acyl carrier protein (ACP) isolated from E. coli (Qureshi et al., 1974), yeast (Willecke et al., 1969) and dog liver (Roncari, 1974b).

The multienzyme complex from different species showed remarkable physical and chemical similarities. The molecular weight of the native enzyme has been found to be 500,000. Fatty acid synthetase from rabbit mammary

gland has been found to have pH optimum of 6.5 - 6.8 (Hansen et al., 1970; Carey et al., 1972). The major product formed is palmitic acid. The enzyme has been shown to be cold labile. Upon ageing in the cold, the native enzyme dissociates into two identical half molecular weight subunits which are inactive (Smith and Abraham, 1971b).

2.3 Immunological properties of lipogenic enzymes

Antibodies have been developed against some of the lipogenic enzymes like acetyl-CoA carboxylase (Mackall and Lane, 1977; Ahmad et al., 1978, 1981), fatty acid synthetase (Smith, 1973; Volpe et al., 1973; Buckner and Kolattukudy, 1976) and 6-phosphogluconate dehydrogenase (Betts and Mayer, 1975). Fisher and Goodridge (1978), Smith (1973), Volpe et al. (1973) and Gharbhi-Chihi et al. (1983) have reported the formation of a single precipitin line on immunodiffusion of the purified fatty acid synthetase from different sources with the corresponding antibodies developed in rabbits. Ahmad et al. (1978) and Betts and Mayer (1975) have also reported the formation of a single precipitin line on immunodiffusion of the purified acetyl-CoA carboxylase and 6-phosphogluconate dehydrogenase, respectively with the antibodies developed against these enzymes.

CHAPTER - 3

MATERIALS AND METHODS

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3.1 Chemicals:

Acetyl-CoA, malonyl-CoA, dodecanoyl-CoA, palmitoyl-CoA, coenzyme A, glucose-6-phosphate, 6-phosphogluconate, isocitrate, nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), adenosine triphosphate (ATP), dithiothreitol (DIT), dithioerythritol (DTE), 5'-5'-dithiobis (2-nitrobenzoic acid) (DTNB), glutathione reduced, bovine serum albumin, (Tris hydroxymethyl amino methane), HEPES buffer(N-2 hydroxyethyl piperazine N-2 ethane sulfonic acid), 2,5-diphenyloxazone (PPO), Phenyloxazolyl phenyl oxazolylphenyl (POPOP), acrylamide, NN'-methylene bisacrylamide, N, N, N', N'-tetramethyl ethylene diamine, Coomassie brilliant blue, imidazole, DEAE-Cellulose, Sephadex G-200, Sepharose-2B, calcium phosphate gel, insulin (porcine pancrease) prolactin (sheep pituitary gland) and cortisol were purchased from Sigma Chemical Company, MO, U.S.A.

C^{14}-sodium bicarbonate and C^{14}-sodium acetate were obtained from Bhabha Atomic Research Centre, Trombay, Bombay. Medium 199 was purchased from Hindustan Dehydrated Media, Bombay. Streptopenicillin (2×10^6 units/5 ml) was obtained from M/s Alembic Chemical Works Co. Ltd. Baroda. Silicone oil was obtained from M/s S.D. Fine Chemicals Pvt.

Ltd. Boiser, Lens papers (Microklin Brand) were obtained from M/s B.K. Dutta & Co. Calcutta. Nutrient broth medium was purchased from OXOID Ltd., Basingstoke, Hants, England. Agarose was obtained from Loba Chemie Indo-Austranal Co. Bombay. All other chemicals were either from BDH or Polypharm and were of analytical grade.

3.2 Experimental animals:

Mixed breed of goats of approximately same age which were in first or second lactation, were selected from the herd maintained at this Institute. The milk yield of these animals was about 1 Kg per day. These animals were kept under identical conditions of feeding and management throughout the experimental period.

3.3 Collection of mammary tissue samples:

Mammary tissue samples were obtained from mid-pregnant goats by surgical biopsy technique. The goats were given 2 ml (50 mg) of largactil (M & B) as tranquillizer. The milk was completely removed from the udder before operation. The area of the udder to be operated, was cleanly shaved and applied with an antiseptic lotion. Local anaesthetic, procaine hydrochloride (3-4 ml) was injected subcutaneously. An incision of about 1-1$\frac{1}{2}$" was made on the skin and the underlying connective tissue was exposed. The mammary gland (about 2 gm) was taken out and immediately kept in ice-cold pre-sterilized normal saline (.9% NaCl w/v). The whole process was performed under well maintained aseptic conditions. The haemorrhage was controlled by plugging the cotton and by applying

adrenaline drops. The incision was closed by suturing the different layers and was treated as open wound.

3.4 Processing of the tissue:

The adipose and connective tissues were removed and the glandular tissue washed repeatedly with normal saline. The tissue was cut into small pieces (approximately 1 mg each) and were dipped in small quantity of media for culturing purpose.

3.5 Method of explant culture:

The explant culture of the goat mammary tissue was carried out essentially according to the method of Tooper and Oka (1974). The procedure involved the following steps.

3.5.1 Preparation of culture media:

The culture media contained 1.1 gm chemically defined synthetic medium, Medium - 199 supplemented with 0.4766 gm of HEPES buffer; 0.0175 gm of sodium bicarbonate; 10 mg pot. sorbate and 1.1 ml of sodium hydroxide (1 M) dissolved in a final volume of 100 ml with distilled water. The pH of the content was adjusted to 7.2. The media was sterilized and 1000 units of streptopenicillin were added per 10 ml of media, before use for culture.

3.5.2 Preparation of siliconized paper:

Siliconized lens papers were used as a support for the small tissue explants to float on the media during the culture. Prior to siliconization, the lens papers were suspended in ethyl ether for 30 minutes and then ether was

removed by aspiration. This was done three times. The same procedure was then performed with 95% ethyl alcohol. Finally the papers were washed four times for 15 minutes each with glass distilled water and dried at 37°C. Siliconization was then accomplished by submerging the dried paper in silicon oil: hexane (1: 1000 v/v) for 10 minutes at room temperature. After removal of silicone solution by aspiration, excess silicone was eliminated by treatment with hexane as described above. Again the papers were dried at 37°C and placed in covered petri dishes. Before use, the papers were heated for 1 hr at 150°C in dry oven. This final heating served two purposes. First the lens papers were sterilized and secondly the impregnated silicone was modified so that it permitted the paper to float.

3.5.3 Culture of explants:

Small pieces of the explants (about 25 explants approximately 1 mg each) were transferred on a siliconized paper which was floating on the medium in the culture dish (80 mm dia). Each culture dish contained 10 ml of constituted medium 199, glucose and specific hormones at various levels wherever necessary. Four different sets, consisting of 6 to 7 culture dishes each were made. One set, in which hormones or glucose were not added to the culture medium, functioned as control. Rest three sets were experimental (combination of hormones, glucose and combination of hormones in addition with glucose treated respectively). The whole process was carried out under aseptic conditions in BBL Biological Cabinet (BBL Micro-

biology System, Division, Becton Dickinson and Co., USA) equipped with U.V. lamp and laminar flow of sterilized air.

3.5.4 Maintenance of culture:

Incubation was carried out in an sterilized incubator (previously swabbed with alcohol) normally for 24 hrs, unless stated otherwise, at 37°C in the presence of a constant flow of O_2 + CO_2 (95:5). All the above operations were carried out under sterile conditions. After culturing the lens papers were removed from the culture dish and gently blotted to remove adhering medium. The explants were then carefully collected with a fine forcep and processed for enzyme assays.

3.6 Monitoring the bacterial contaminsation of the culture media:

Before culturing and after harvesting, the culture media were tested routinely for the bacterial and fungal contamination. Sterilized nutrient broth (The OXOID Manual, 1982), which contained all the nutrients required for the growth of bacteria and fungi, was used for testing the contamination in the culture media. To about 10 ml of pre-sterilized nutrient broth, 0.5 ml of culture medium was added with a presterilized pipette. The tube was then vortexed and incubated at 37°C. In the event of any growth in the medium upto 48 hrs of incubetion, the experiment was discarded.

3.7 Preparation of cytosol:

The tissue samples, collected from different sets

of petri dishes, were washed many times with the ice-cold 0.01 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 1 mM DTE and 1 mM EDTA till the colour of media was disappeared. The tissue was homogenized in four volumes of the ice-cold buffer in a Duall (R) - 21 glass homogenizer (Kontes Glass Co. U.S.A.). The homogenate was centrifuged at 5000 x g for 10 minutes in refrigerated centrifuge (Janetzki K-24) at 4°C to remove nuclei and unbroken cells. The supernatant was taken after discarding the floating fat layer and filtered through cheese cloth. It was again centrifuged at 105000 x g for 60 minutes in Beckman L-Ultracentrifuge at 4°C. The pellet and the floating fat layer were discarded. The supernatant was filtered through four layers of cheese cloth if necessary. The particle free supernatant (cytosol) was used for determination of the activities of the various enzymes.

3.8 Protein estimation:

The total protein in the cytosol was estimated according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

3.9 Enzyme assays:

The enzymes assayed were: Acetyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthetase, medium-chain and long-chain acyl thioesterases, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-isocitrate dehydrogenase. All the enzymes were assayed at 37°C under optimum conditions where the activity was linearly related to protein (enzyme)

concentration and time of incubation. The enzyme assays were done in duplicate. The dehydrogenases were measured spectrophotometrically at 340 nm and the increase in absorbance was measured. The extinction coefficient (f) of NADPH 6.22 x 10^6 cm^2/mole was taken for the calculation of the results.

3.9.1 Acetyl-CoA synthetase:

This enzyme was assayed as described by Paul (1962) with some modification (Roughan and Slack, 1977). The enzyme was assayed by the formation of radioactive acetyl-CoA from radioactive acetate. The assay tubes consisted of Tris-HCl buffer (pH 7.4), 100 mM; $MgCl_2$, 5 mM; KF, 50 mM; glutathione (reduced), 10 mM; ATP, 10 mM; CoA, 0.5 mM; 2-C^{14}-sodium acetate (2 μCi/μ mole), 10 mM and the enzyme in variable amounts in a final volume of 0.2 ml. The tubes were incubated at 37°C for 5 min. Aliquots of 100 ul were spotted on the Whatman No.3 filter paper discs. The discs were dried well and were immersed in 5 ml of 0.25% (w/v) trichloroacetic acid in ethanol:ether mixture (1:4) for 5 min. The solvent was decanted. Washing was repeated three times. The discs were finally washed in 5 ml of ether. The discs were air dried and transferred to scintillation vials containing 10 ml of Bray's solution (Bray, 1960) (POPOP, 100 mg; PPO, 2.0 g; ethylene glycol, 10.0 ml; methanol, 50.0 ml; naphthalene, 30 gm and dioxane, 440 ml). The residual radioactivity was measured in Scintillation Counter. An enzyme blank was prepared simultaneously.

3.9.2 Acetyl-CoA carboxylase:

Acetyl CoA carboxylase was assayed using C^{14}-bicarbonate fixation method as described by Markall and Lane (1977) with some modifications. The reaction mixture (0.4 ml) consisted of imidazole-HCl buffer, pH 7.5, 100 mM; glutathione, 3 mM; potassium citrate, 20 mM; magnesium chloride, 20 mM; BSA, 0.6 mg; ATP, 3 mM; acetyl-CoA, 1 mM; C^{14}-sodium bicarbonate (0.5 μCi/μ mole), 20 mM and enzyme protein (0-250 ug). Initially the enzyme was preincubated at 37°C for 30 minutes with all the components except ATP, acetyl-CoA and C^{14}-bicarbonate. After preincubation of the enzyme, the reaction was started by the addition of rest of the components. The complete reaction mixture was incubated at 37°C for 5 min and the reaction was terminated by adding 0.05 ml of 4N perchloric acid ($HClO_4$). The tubes were placed in an ice bath and excess ClO_4^- was precipitated by adding 0.1 ml of 2 M KCl. The precipitated protein and $KClO_4$ were allowed to settle down (or centrifuged for 10 minutes at 2000 g) and 0.1 ml sample of the clean supernatant was spotted on Whatman No.1 filter paper disc of 2.2 cm diameter. The discs were completely dried with a stream of warm air which removes any unreacted $H^{14}CO_3$ as $^{14}CO_2$. The discs were placed into scintillation vials and Scintillation fluid was added. The samples were then counted in a Scintillation Spectrometer.

3.9.3 Fatty acid synthetase:

The malonyl-CoA dependent oxidation of NADPH was measured spectrophotometrically according to the method of Dils and Carey (1975). The assay mixture, in a final volume of 1.5 ml, consisted of potassium phosphate buffer (pH 6.8), 200 mM; DTE, 1 mM; EDTA, 1 mM; NADPH, 0.24 mM; acetyl-CoA, 30 μM; malonyl-CoA, 50 μM and enough enzyme protein to bring about a change of 0.05 to 0.15 O.D. per minute. The enzyme was preincubated with buffer, DTE and EDTA for 40 min at 37°C after which the other components excluding malonyl-CoA were added. The contents were mixed and transferred to the cuvette where the reaction was initiated by the addition of malonyl-CoA. The decrease in absorbance at 340 nm was measured. The extinction coefficient of NADPH, 6.22×10^6 Cm^2 per mole was taken for calculation of results. A unit of enzyme activity was defined as nmoles of NADPH oxidised $minute.^{-1}$

3.9.4 Thioesterase I and II or (long-chain and medium-chain-acyl thioesterases):

Both thioesterase I and II were measured by the spectrophotometric method of Knudsen et al. (1976). Thioesterase I was measured by the release of thio group from palmitoyl-CoA, whereas in the case of thioesterase II the release of thiol group from dodecanoyl-CoA was measured. Spectrophotometric measurement of the complex formed by the reaction of DTNB (5'5'-dithiobis (2-nitro-benzoic acid) with the released thiol groups was carried out. The assay mixture (1.5 ml) contained 0.4 M Tris HCl

buffer (pH 7.4), EDTA, 1 mM; DTNB, 0.2 mM (in 10 mM phosphate buffer, pH 7.4) and enzyme protein. The reaction was started by adding 40 µM acyl-CoA and follwed in a Spectrophotometer at 413 nm. The amount of thiol released was calculated from molar extinction coefficient $\underline{E}$ of the complex, 1.36×10^4 litre $mole^{-1}$ cm^{-1} (Means and Feeney, 1971). A unit of enzyme activity is described as the liberation of 1 n mole of CoA $minute^{-1}$.

3.9.5 NADP-isocitrate dehydrogenase: This enzyme was assayed according to the method of Cleland et al. (1969). The assay mixture in a final volume of 1.5 ml had the following components: Tris buffer (with EDTA 1 mM and DTE 0.3 mM, pH 7.4), 33 mM; $MnSO_4$, 1.33 mM; NADP, 0.1 mM; isocitrate, 1.33 mM and enzyme preparation. The reaction was initiated by the addition of the substrate.

3.9.6 6-Phosphogluconate dehydrogenase:

The enzyme was assayed as described by Bauman et al. (1970). The assay mixture in the final volume of 1.5 ml consisted of Tris buffer (pH 7.4) 67 mM, $Mg\ Cl_2$, 10 mM; NADP, 0.1 mM; 6-phosphogluconate, 1.4 mM and the appropriate amount of the enzyme preparation. The reaction was allowed to proceed with all the components except substrate until the reaction ceased at which time the substrate was added. The increase in absorbance at 340 nm was noted for atleast 3 minutes.

3.9.7 Glucose-6-phosphate dehydrogenase:

This enzyme was assayed by the double method of Bauman et al., (1970). In one cuvette all the components of 6-phosphogluconate dehydrogenase assay mixture were taken and in the second cuvette, in addition to the above assay mixture, glucose-6-phosphate (1.4 mM) was also added in the final volume of 1.5 ml. The rate of NADPH formation in cuvette-1 provides an estimate of 6-phospho gluconate dehydrogenase activity, whereas in cuvette-2 the rate included both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities. The difference in the rate of reaction in cuvette-2 to cuvette-1 gives the activity of glucose-6-phosphate dehydrogenase.

3.10 Purification of fatty acid synthetase:

Fatty acid synthetase was purified according to the method of Dils and Carry (1975), following the steps mentioned as below:

3.10.1 Preparation of particle free supernatant solution

All operations were carried out at 0 - 4°C. Mammary tissue (about 500 g wet weight) was obtained from lactating goats from the local slaughter house. The mammary tissue was cut into strips about 0.5 mm wide and thoroughly washed with 0.15 M KCl to make it milk free, followed by fine mincing with scissors. The tissue was homogenized with 3-6 volume of Tris-Hcl buffer 0.01 M containing 0.25 M sucrose, 1 mM DTT and 1 mM EDTA (pH 6.8) in a Potter Elvehjam homogenizer. The homogenate was filtered through a single

layer of cheese cloth and subjected to low speed centrifugation (5000 x g) to remove cell debris and other particles. The floating fat layer was carefully removed. The supernatant was recentrifuged at 35,000 rpm (105,000 x g) for 60 minutes. The floating fat layer was carefully removed. The particles free supernatant contained the fatty acid synthetase activity.

3.10.2 First ammonium sulfate fractionation:

The supernatant solution was thawed slowly at 4°C. Solid ammonium sulfate was added at the rate of about 1 gm/min/100 ml to bring the saturation to 25%. The sediment was removed by centrifugation at 15,000 x g for 15 min. More ammonium sulphate was added in a similar manner to the supernatant to achieve a 40% saturation. The protein precipitated between 25 - 40% saturation was collected and was dissolved in minimum amount of phosphate buffer (0.1 M, pH 7.0) containing 1 mM EDTA and 1 mM DTE. This was dialysed against the same buffer for 10 hrs.

3.10.3 Calcium phosphate gel treatment:

Maximum purification is generally achieved with 0.4 to 0.5 mg of gel/milligram of the protein depending upon the batch of the gel. The protein solution was gently stirred and mixed with calcium phosphate gel for 2 minutes. The gel was removed by centrifugation at 1500 x g for 15 minutes. The gel was washed twice with 50 mM potassium phosphate buffer (pH 7.0) and the three supernatants were combined.

3.10.4 DEAE-Cellulose chromatography:

DEAE-Cellulose was washed successively with 0.5N NaOH, water, 0.5 N H_3PO_4, water and 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM DTE. The slurry was poured into glass column (2.2 x 25 cm) to a bed height of about 23 cm. The supernatant from the step 3.10.3 was applied to the column. Normally not more than 350 mg of protein was chromatographed on the column. The enzyme was eluted with a linear gradient of 50 - 250 mM of potassium phosphate buffer (500 ml) at a flow rate of 1.5 to 2 ml/minute. 5.0 ml of fractions were collected. The peak of synthetase activity corresponding to the major protein peak was eluted at 110 mM. Fractions showing the high enzymatic activity were carefully pooled, causing least disturbance to the solution. Mechanical disturbance of the eluent causes precipitation of denatured proteins.

3.10.5 Second ammonium sulphate fractionation:

The pooled fractions were again subjected to ammonium sulphate precipitation. The protein precipitating between 26 and 32% saturation was dissolved in 2-5 ml of .25 M phosphate buffer (pH 7) containing 1 mM EDTA, 1 mM DTE and 20% glycerol and was dialyzed against the same buffer for 2 hours. The dialysate was centrifuged at low speed (3000 x g) to remove any denatured protein and the contents was frozen.

3.10.6 Sephadex G-200 chromatography:

The procedure was carried out at 20°C. A column of

Sephadex G-200 (1 x 15cm) equilibrated with 0.25 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM EDTA, 1 mM DTT was prepared and enzyme solution was applied to it. Since the enzyme is relatively stable in high ionic strength buffer at 20°C, the Sephadex can be conveniently developed overnight with 0.25 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT. A flow rate of 5-8 ml per hour was maintained and fractions of 2-3 ml were collected. Fatty acid synthetase activity was eluted with a relative retention volume (elution volume/void volume) of 1.15 and a single protein peak was obtained. The active fractions from the leading edge and the main section of the peak were pooled. Fractions from the trailing edge of the peak sometimes contain fatty acid of lower specific activity and were therefore routinely discarded.

3.10.7 Polyacrylamide gel electrophoresis:

The polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (1969). Gel rods (60 x 4 mm) of 7% acrylamide were prepared in 30 mM Tris-glycine buffer (pH 8.4). The purified enzyme (100-200 µg) in saturated sucrose solution was applied to the cathode end of the gel rod. Bromophenol blue was used as a tracking dye. Electrophoresis was carried out at room temperature in 30 mM Tris-glycine buffer (pH 8.4) for 60-80 minutes with a current maintained at 2.5 mA per gel. After the electrophoresis, the gels were removed from the tubes and stained with Coomassie blue (0.1% in

isopropanol:acetic acid:water, 25:10:65) for 12-16 hrs. The gels were then destained with 10% acetic acid.

3.11 Purification of acetyl-CoA carboxylase

Acetyl-CoA carboxylase was purified according to the method of Miller and Levy (1975), which involved the following buffers:

(1) Buffer I contained 2-mercaptoethanol, 7 mM; EDTA 1.0 mM; glycerol (20%); and imidazole-HCl, 50 mM with final pH 6.5.

(2) Buffer II contained 2-mercaptoethanol, 7 mM; EDTA, 0.1 mM; glycerol, (20%); imadazole-HCl, 50 mM and potassium citrate, 20 mM with final pH 6.5.

(3) Buffer III contained 2 mercaptoethanol, 7 mM; EDTA 0.1 mM; Glycerol (20%); imidazole-HCl, 5 mM; and $MgSO_4$ 7 H_2O, 50 mM with final pH 6.5.

The purification procedure involved the following steps.

3.11.1 Preparation of extract:

Tissue was obtained from the lactating goat mammary gland from the local slaughter house. The mammary tissue was cut into small pieces and thoroughly washed with 0.15M KCl to make it milk free which was followed by fine mincing with scissors and mixed with 2 volumes of buffer I. The tissue was homogenized for 1 min. in Potter-Elvehjam homogenizer. The homogenate was filtered through a single layer of cheese cloth and subjected to centrifugation for 10 min. at 2000 x g. The fat layer and precipitate was discarded and

the supernatant solution was recentrifuged for 45 minutes at 10,5000 x g. The precipitate and the small amount of fat was discarded and the supernatant solution was filtered as above.

3.11.2 *Ammonium sulphate precipitation:*

The enzyme was precipitated by the addition of ammonium sulphate to 50% of saturation. The suspension was allowed to stand overnight and the precipitate was then sedimented by centrifugation for 30 mintues at 23,000xg. The precipitate was redissolved in buffer I. The solution was dialyzed against the buffer III for 18 hours with three changes of buffer. The enzyme solution was again centrifuged for 30 minutes at 23,000 x g to remove any denatured protein resulting from dialysis process.

3.11.3 Negative absorption with DE-52:

The supernatant solution was placed onto the column of DEAE-Cellulose (40 x 500 mm) equiliberated with buffer-III. The column was prepared and run at room temperature. Buffer III was added to wash through the enzyme, which did not adhere to the ion exchange resin. The enzyme was then concentrated to 11 to 12 mg of protein/ml in a Diaflo ultrafilteration device, model 400, containing a UM-10 membrane. This was essential in order to prevent undue loss as a result of dilution in the next step.

3.11.4 Ammonium sulphate fractionation:

An equal volume of neutralized saturated ammonium sulphate was added to the concentrated enzyme solution

and the suspension was allowed to stand overnight. The precipitate was sedimented at 23,000 x g for 30 minutes and the supernatant solution was discarded. The precipitate was dissolved in approximately 25 ml of buffer I containing 40% ammonium sulfate, stirred for 10 minutes and the contents centrifuged for 10 minutes at 23,000 x g. The supernatant stored for further enzyme assay. The procedure was repeated 4 times, each with 15-20 ml of butter-I containing 30, 20, 10 and 0% ammonium sulphate, respectively. The bulk of the enzyme activity was always found in the fractions eluted with the buffers containing 10 and 0% ammonium sulfate. These were pooled and the enzyme was precipitated by adding enzyme grade ammonium sulfate to 30% saturation. The content was kept for 1 hour and was sedimented for 30 minutes at 23,000 x g. The supernatant solution was discarded. The precipitate was redissolved in buffer II, to give a protein concentration of 20 - 30 mg/ml. There were always two visibly different components on redissolving the precipitate. One portion dissolved within a few hours whereas the other usually took 3 days to dissolve completely.

3.11.5 Gel filtration on Sepharose 2 B:

The enzyme solution containing both components of precipitate as obtained from step 3.11.4 was applied to Sepharose 2B column (25 x 450 mm) equilibrated with buffer II. The elution was done at room temperature (25°C) under a hydrostatic head of 50 cm of buffer, with a flow

with staining solution prepared by dissolving 1.25 gm of Coomassie brilliant blue in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid. Staining was done at room temperature. The time varied from 2 to 10 hours. The gels were removed from the staining solution, rinsed with distilled water and placed in destaining solution (75 ml of acetic acid, 50 ml of methanol and 875 ml of water). The gels were stored in 7.5% acetic acid solution.

3.12 Preparation of antisera:

Antisera were raised against the above mentioned purified enzymes viz. fatty acid synthetase and acetyl-CoA carboxylase. Antiserum was also developed against glucose-6-phosphate dehydrogenase, which was obtained from Sigma Chemical Company, MO, USA. Antiserum for each enzyme was prepared by the method of Buckner and Kolattakudy (1976) by immunizing different groups of rabbits for different enzymes. Approximately 5 mg of each of the purified enzyme was dissolved in 0.5 ml of 0.9% NaCl and was emulsified with 1.0 ml of complete Freund's adjuvant. The highly homogenized water-in-oil emulsion was prepared by repeatedly drawing in and injecting the emulsion. The thoroughly homogenized emulsion was colourless and remained unaltered when kept overnight. The emulsion was injected subcutaneously into rabbits at multiple sites. Two weeks after the first injection, 5 mg of each of purified enzyme protein, emulsified with incomplete Freund's adjuvant was injected

into the rabbits. Two weeks after the second injection, the rabbits were bled from the marginal ear vein. Blood was collected in perfectly clean and dry tubes. Maximum care was taken to avoid hemolysis. Tubes were kept undisturbed for 2 hours at room temperature and were kept at 4°C for 30 minutes for clot retraction. Later the tubes were centrifuged at 3000 rpm for 10 minutes and the antiserum was collected for each enzyme in 1 ml serum vials and stored at -20°C.

3.13 Immuno-diffusion studies:

Double diffusion analysis was done according to the method of Ouchterlony (1966). Microslides (75 x 25 mm) were coated with 3 mm thick 1% agarose in 0.9% sodium chloride solution. The agarose was heated to 95°C and was allowed to cool at room temperature. When the temperature reached 45°C, 3-4 ml of agarose was poured gently on the slide with the help of syringe so that the gel was layered evenly over the slide. The gels were allowed to solidify and the wells were cut using the gel punch. All the microslides were pre-coated with 0.1% agar solution to form a thin basal layer, prior to this procedure. For testing the antibodies formation against each above mentioned enzyme the central well was filled with the respective enzymes and the peripheral wells with the antisera raised against each enzyme. The charged slides were kept at 4°C for diffusion and the appearance of precipitin bands were observed after 24, 48, 72 and 96 hours. Nonagglutinated protein was removed from the

agarose by repeated washing with 0.9% NaCl and the NaCl was removed by washing with distilled water. The agglutinated protein bands were stained with solution of 0.5% amido black 5% mercuric chloride ($HgCl_2$) in 5% acetic acid for 2 to 5 minutes. Excess dye was removed from the agarose with 5% acetic acid.

3.14 Rocket immuno electrophoresis:

In order to estimate quantitatively the fatty acid synthetase, acetyl-CoA carboxylase and glucose-6-phosphate dehydrogenase from the explant cultured tissue, the rocket immunoelectrophoresis by the method of Laurell (1966) was employed. For this purpose 1% agarose was melted in a barbital buffer (ionic strength 0.07M, pH 8.6) on a boiling water bath and cooled to 45°C. The desired amount of antiserum was added and carefully mixed with the fluid agarose. The agarose antiserum mixture at a temperature of 40-45°C was poured gently on the small glass plates through a pipette so that a uniform layer of agarose antiserum was formed. The plates were left for atleast 30 minutes to cool down to room temperature. Holes (3-3.5mm diameter) were cut in the agarose gel along the line 2 cm from and parallel with one of the long edges. The distance between the centres of the adjacent holes was at least 8 mm. The holes were filled with antigen solution.

The glass plates were placed on the cooled surface (4°C) of the phaerograph and connected with the electrode vessels by agarose sheets, filter paper strips or linen bridges.

The electrophoresis was run with 10 volts/cm for 2 to 10 hours, depending upon the charge and amount of antigen applied in relation to antibody concentration and/or desired exactness of the estimation of peak height. The height of the various peaks formed could be measured directly under dark field illumination.

CHAPTER – 4

RESULTS

RESULTS

To investigate the effect of hormones on the lipid synthesizing capacity of the mid-pregnant goat mammary gland, a combination of optimal concentrations of insulin, prolactin and cortisol were used (i.e. each hormone 5 ug/ml of medium) during explant culture. The effect was seen on various lipogenic enzymes viz. acetyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthetase, medium-chain acylthioesterase, long-chain acylthioesterase, NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which play a major role in regulating the overall rate of lipogenesis in mammary gland. The effect of glucose alone and in combination with optimal concentration of the above hormones was also seen on lipogenesis in mid-pregnant goat mammary gland. Studies were further extended to observe the effect of combination of hormones, glucose and combination of hormones in addition with glucose on the rate of synthesis of some of the lipogenic enzymes viz. acetyl-CoA carboxylase (which is considered to be the rate limiting enzyme in fatty acid synthesis), fatty acid synthetase and glucose-6-phosphate dehydrogenase. In sequel to this study, the acetyl-CoA carboxylase and fatty acid synthetase were purified to homogeneity from the goat mammary gland tissue. Antibodies were raised against these enzymes in rabbits. Antibodies were also raised against standard glucose 6-phosphate dehydrogenase

procured in purified form. Immunological studies were conducted to investigate the effect of above mentioned experimental conditions on the amounts or rates of synthesis of these enzymes in explant cultured tissue of mid-pregnant goat mammary gland.

The results of the present study have been divided into the following four sections.

Section - A

4.1 Effect of hormones and glucose on the lipogenic activity of mid-pregnant goat mammary gland.

4.1.1 Acetyl-CoA synthetase:

4.1.1.1 Effect of combination of hormones:

The effect of combination of insulin, prolactin and cortisol was studied using 5 µg of each hormone/ml of medium-199 during explant culture. The Table-1 and Fig.1 show the effect of combination of hormones on the specific activity of acetyl-CoA synthetase at various intervals of incubation i.e. 12, 24 and 36 hrs of intubation at 37°C in a gaseous atmosphere of oxygen:carbon dioxide (95:5). The medium, in which hormones or glucose were not added during incubation period, served as control in the experiment. It is obvious from the results that the specific activity of acetyl-CoA synthetase increased markedly, about 2-fold (39.04 to 77.06) in the presence of insulin, prolactin and cortisol after 12 hours of incubation or explant culture. On prolonged incubation with hormones the specific activity of enzyme increased

Table - 1

Effect of incubation time on the activity of acetyl-CoA synthetase.

S.No.	Treatments	n moles of acetyl-CoA formed/ min/mg of protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	39.04	44.28	45.09
2.	Medium + hormones*	77.06	96.38	115.69
3.	Medium + glucose**	60.66	73.46	90.45
4.	Medium + hormones* + glucose**	87.95	136.89	175.55

* Concentration of insulin, prolactin and cortisol used was 5 ug/ml of culture medium each hormone.

** Concentration of glucose added to the medium was 2 mg/ml of culture medium.

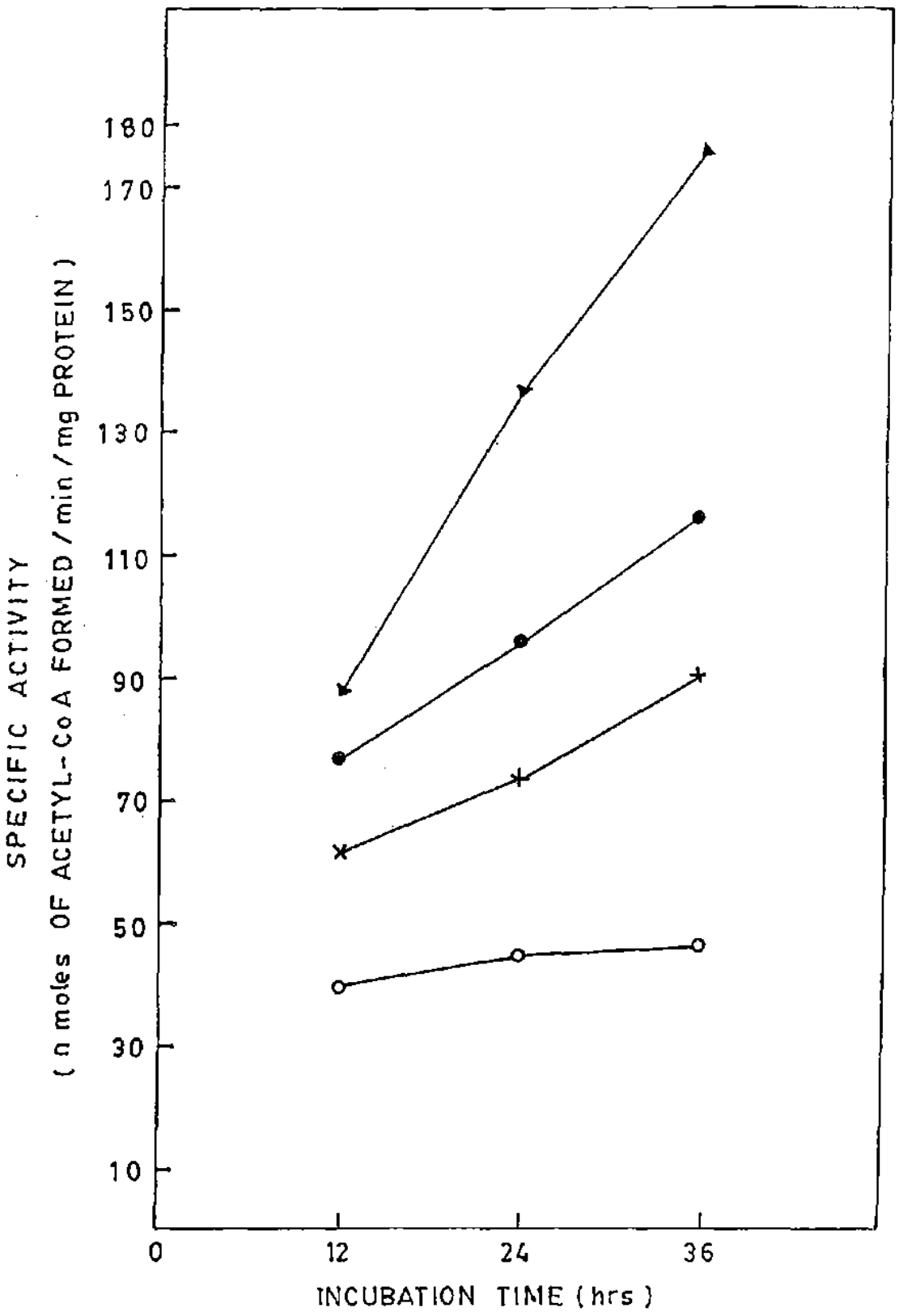
Fig. 1. Effect of combination of hormones and glucose on the specific activity of acetyl-CoA synthetase during different hours of explant culture:

(A) In the absence of hormones and glucose (□——□)

(B) In the presence of hormones (■——■)

(C) In the presence of glucose (x---x)

(D) In the presence of hormones and glucose (△——△)


SPECIFIC ACTIVITY
(n moles OF ACETYL-CoA FORMED / min / mg PROTEIN)
180
170
150
130
110
90
70
50
30
10
0
12
24
36
INCUBATION TIME (hrs)

or combination of hormones during the respective hours of incubation.

4.1.2 Acetyl-CoA carboxylase

4.1.2.1 Effect of Combination of hormones:

The combined effect of insulin, prolactin and cortisol was studied on the specific activity of acetyl-CoA carboxylase at different hours of incubation.

Table-2 and Fig-2 show the effect of insulin, prolactin and cortisol on the specific activity of acetyl-CoA carboxylase after 12, 24 and 36 hours of incubation. The specific activities of acetyl-CoA carboxylase increased significantly to 4.25, 5.26 and 5.97 after 12, 24 and 36 hours of incubation. This increase was about 2-fold as compared to the control experiment where medium-199 was used and the specific activities of enzyme after respective hours of incubation, were found to be 2.06, 2.58 and 2.68, respectively.

4.1.2.2 Effect of glucose:

The effect of glucose on the specific activity of acetyl-CoA carboxylase at varied hours of incubation has been shown in Table 2 and Fig.2. The specific activities of acetyl-CoA carboxylase were found to be 3.90, 4.22 and 5.02 after different hours of incubation (12, 24 and 36 hours) in comparison to that observed in the case of control experiments which were 2.06, 2.58 and 2.68 at the similar hours of explant culture incubation.

Table - 2

Effect of incubation time on the activity of acetyl-CoA carboxylase.

S.No.	Treatment	n moles of (C^{14}) bicarbonate incorporated into malonyl-CoA/ min/mg protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	2.06	2.58	2.68
2.	Medium + hormones*	4.25	5.26	5.97
3.	Medium + glucose**	3.90	4.22	5.02
4.	Medium + hormones* + glucose**	6.05	6.52	7.08

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2 mg/ml of culture medium.

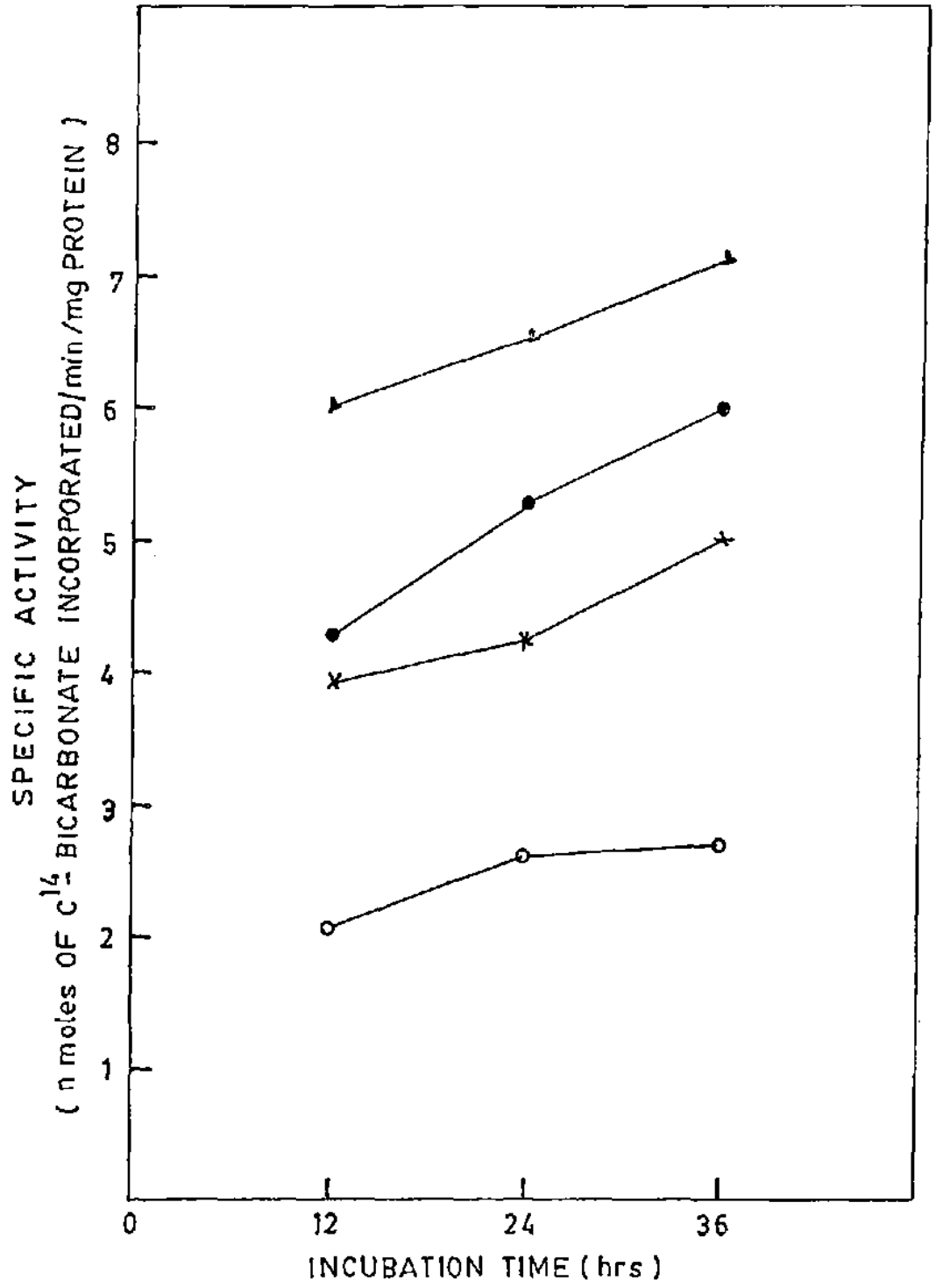
Fig. 2. Effect of combination of hormones and glucose on the specific activity of acetyl-CoA carboxylase during different hours of explant culture:

(A) In the absence of hormones and glucose (O——O)

(B) In the presence of hormones (●——●)

(C) In the presence of *glucose* (x---x)

(D) In the presence of hormones and glucose (△--△)


SPECIFIC ACTIVITY
(n moles OF C^{14} BICARBONATE INCORPORATED/min/mg PROTEIN)
8
7
6
5
4
3
2
1
0
12
24
36
INCUBATION TIME (hrs)

4.1.2.3 Effect of glucose with hormones combination

Table 2 and Fig.2 also show the effect of glucose in combination with insulin, prolactin and cortisol on the specific activity of acetyl-CoA carboxylase. The specific activities of acetyl-CoA carboxylase were increased considerably by 2-3-fold i.e 6.05, 6.52 and 7.08 from 2.06, 2.58 and 2.68 after 12, 24 and 36 hours of incubation. Thus, specific activities, so observed, when glucose in addition with combination of hormones was used in culture medium, were comparatively higher than those when either hormones or glucose was added to the culture medium separately.

4.1.3 Fatty acid synthetase

4.1.3.1 Effect of combination of hormones:

The effect of combination of hormones i.e. insulin, prolactin and cortisol was studied on the specific activities of fatty acid synthetase at different hours of incubation. The specific activities of the fatty acid synthetase increased markedly in the presence of insulin, prolactin and cortisol from 31.83, 36.10 and 37.92 to 50.31, 57.51 and 71.02 after 12, 24, and 36 hours of explant cultures, respectively (Table 3, Fig. 3).

4.1.3.2 Effect of glucose:

The results for the effect of glucose on the activities of fatty acid synthetase at different hours of incubation havebeen shown in Table 3 and Fig.3. When the culture medium was supplemented with glucose, it resulted in a substantial increase in the specific activities of

Table - 3

Effect of incubation time on the activity of fatty acid synthetase.

S.No.	Treatment	n moles of NADPH oxidised/ min/mg of protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	31.83	36.10	37.92
2.	Medium + hormones*	50.31	57.51	71.02
3.	Medium + glucose**	37.29	42.40	54.44
4.	Medium + hormones* + glucose**	64.71	79.45	76.93

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2mg/ml of culture medium.

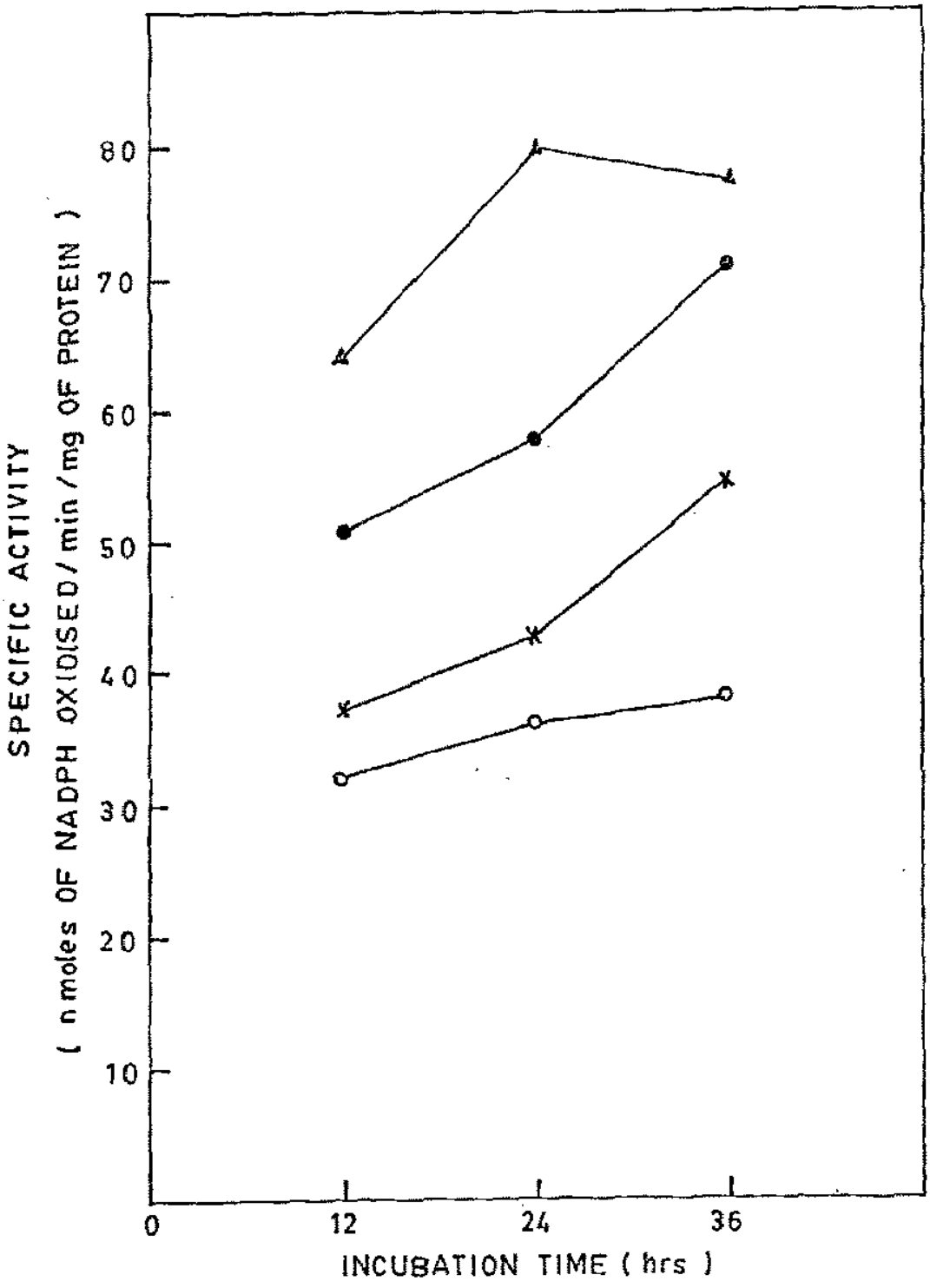
Fig. 3. Effect of combination of hormones and glucose on the specific activity of fatty acid synthetase during different hours of explant culture:

(A) In the absence of hormones and glucose (O——O)

(B) In the presence of hormones (●——●)

(C) In the presence of glucose (x--x)

(D) In the presence of hormones and glucose (▲--▲)


SPECIFIC ACTIVITY
(n moles OF NADPH OXIDISED / min / mg OF PROTEIN)
80
70
60
50
40
30
20
10
0
12
24
36
INCUBATION TIME (hrs)

fatty acid synthetase to 37.29, 42.40 and 54.44 after 12, 24 and 36 hours of incubation as compared to those in the control experiment, which were found to be 31.83, 36.10 and 37.92 after the same hours of incubation.

4.1.3.3 Effect of glucose in combination of hormones:

The Table 3 and Fig.3 also shows the effect of glucose in combination of hormones on the specific activities of fatty acid synthetase. It was observed that hormones combination and glucose together significantly increased the specific activities of fatty acid synthetase to approximately 2-fold (from 31.83, 36.1 and 37.92 to 64.71, 79.45 and 76.93) as observed in the case of control experiment at 12, 24 and 36 hours of incubation, respectively. The specific activities of the fatty acid synthetase were always higher as compared to the activities observed when the incubation was carried out in culture medium containing either glucose or combination of hormones.

4.1.4 Medium-chain acylthioesterase:

It is obvious from the Table 4 that the activity of medium-chain acylthioesterase could not be detected in any of the experimental conditions i.e. control, hormones combination, glucose and glucose in combination of hormones treated experiments carried out at different periods of incubation.

4.1.5 Long-chain acylthioesterase:

4.1.5.1 Effect of combination of hormones

The effect of combination of hormones (insulin,

Table - 4

Effect of incubation time on the activity of medium-chain acylthioesterase.

S.No.	Treatment	n moles of thiol group released/min/mg of protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	ND	ND	ND
2.	Medium + hormones*	ND	ND	ND
3.	Medium + glucose**	ND	ND	ND
4.	Medium + hormones* + glucose**	ND	ND	ND

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2 mg/ml of culture medium.

ND = Not detected.

Table - 5

Effect of incubation time on the activity of long-chain acylthioesterase.

S.No.	Treatments	n moles of thiol group released/min/mg of protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	4.51	4.95	4.10
2.	Medium + hormones*	6.21	7.27	4.75
3.	Medium + glucose**	5.36	6.05	4.20
4.	Medium + hormones* + glucose**	6.44	6.93	4.31

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2 mg/ml of culture medium.

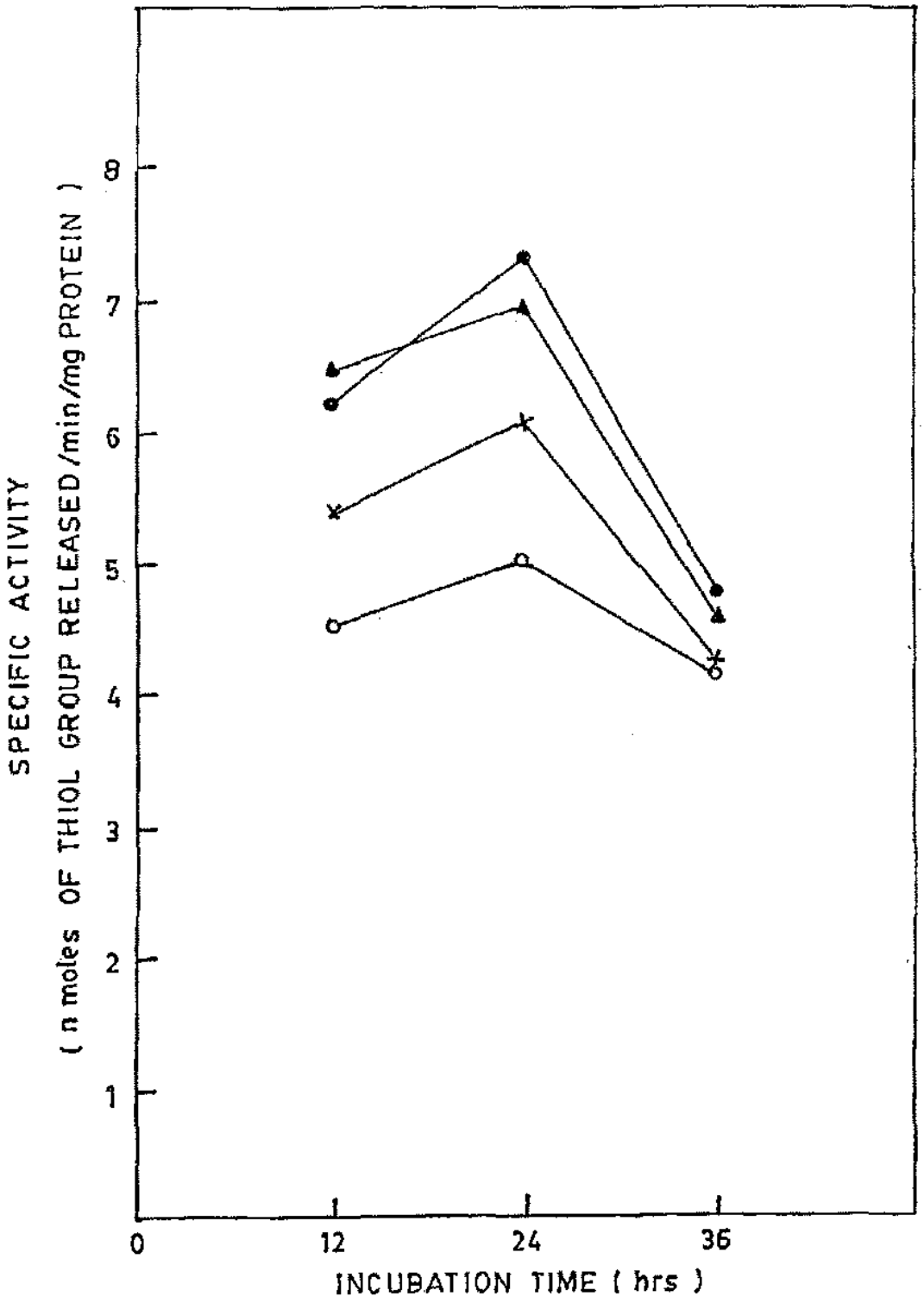
Fig. 4. Effect of combination of hormones and glucose on the specific activity of long-chain acyl thioesterase during different hours of explants culture:

(A) In the absence of hormones and glucose (O——O)

(B) In the presence of hormones (O——O)

(C) In the presence of glucose (x---x)

(D) In the presence of hormones and glucose (△--△)


SPECIFIC ACTIVITY
(n moles OF THIOL GROUP RELEASED/min/mg PROTEIN)
8
7
6
5
4
3
2
1
0
12
24
36
INCUBATION TIME (hrs)

observed as 5.36 and 6.05 at 12 and 24 hours of incubation. Whereas in the control experiment the specific activities were obtained as 4.51 and 4.95 after the corresponding hours of incubation. However, no change in the specific activity could be observed during 36 hours of incubation with glucose.

4.1.5.3 Effect of glucose in combination of hormones

The combined effect of combination of hormones and glucose was seen on the activities of long-chain acylthioesterase as given in Table 5 and Fig.4. The results show that the addition of hormones in combination with glucose in culture medium resulted in the increased specific activities of long-chain acylthioesterase, to 6.44, 6.93 and 4.31 from 4.51, 4.95 and 4.10 as observed in the case of control experiment at 12, 24 and 36 hours of incubation, respectively. However, the extent of increase in the specific activities of long-chain acylthioesterase was comparatively less when explant culture was carried out alone with combination of hormones (insulin, prolactin, and cortisol).

4.1.6 Glucose 6-phosphate dehydrogenase:

4.1.6.1 Effect of combination of hormones:

To observe the effect of insulin, prolactin and cortisol on the activity of glucose-6-phosphate dehydrogenase a combination of optimal concentration of each of these hormones was used in the medium. Table 6 and Fig.5 show the effect of combination of hormones on the activity of glucose-6-phosphate dehydrogenase at

varying periods of incubation at 12, 24 and 36 hours. The specific activity of glucose-6-phosphate dehydrogenase increased markedly, about 1.5-fold from 51.81 to 83.82 in the presence of insulin, prolactin and cortisol after 12 hours of incubation. On prolonged incubation with the same combination of hormones the specific activities of the enzyme increased to 86.75 and 91.25 after 24 and 36 hours of incubation, respectively. Whereas in the control experiment the specific activities of the enzyme were considerably lower (51.81, 53.58 and 54.25) at 12, 24 and 36 hours of incubation, respectively.

4.1.6.2 Effect of glucose

Table 6 and Fig.5 also show the effect of glucose on the specific activities of glucose-6-phosphate dehydrogenase at different hours of incubation. The addition of glucose in the culture medium markedly increased the specific activities of glucose-6-phosphate dehydrogenase to 61.45, 68.23 and 74.31 at 12, 24 and 36 hours of incubation, respectively from 51.81, 53.58 and 54.25 at the corresponding hours of incubation in the case of control.

4.1.6.3 Effect of glucose in combination of hormones:

The combined effect of hormones combination and glucose was observed on the specific activity of glucose-6-phosphate dehydrogenase (Table 6 and Fig.5). It is evident from the results that glucose in combination with insulin, prolactin and cortisol remarkably increased the specific activities of glucose-6-phosphate dehydrogenase

Table - 6

Effect of incubation time on the activity of glucose-6-phosphate dehydrogenase.

S.No.	Treatments	n moles of NADPH formed/min/mg of protein		
		Incuabtion Time (hrs)		
		12	24	36
1.	Medium	51.81	53.58	54.25
2.	Medium + hormones*	83.82	86.75	91.25
3.	Medium + glucose**	61.45	68.25	74.31
4.	Medium + hormones* + glucose**	90.82	102.85	107.95

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2 mg/ml of culture medium.

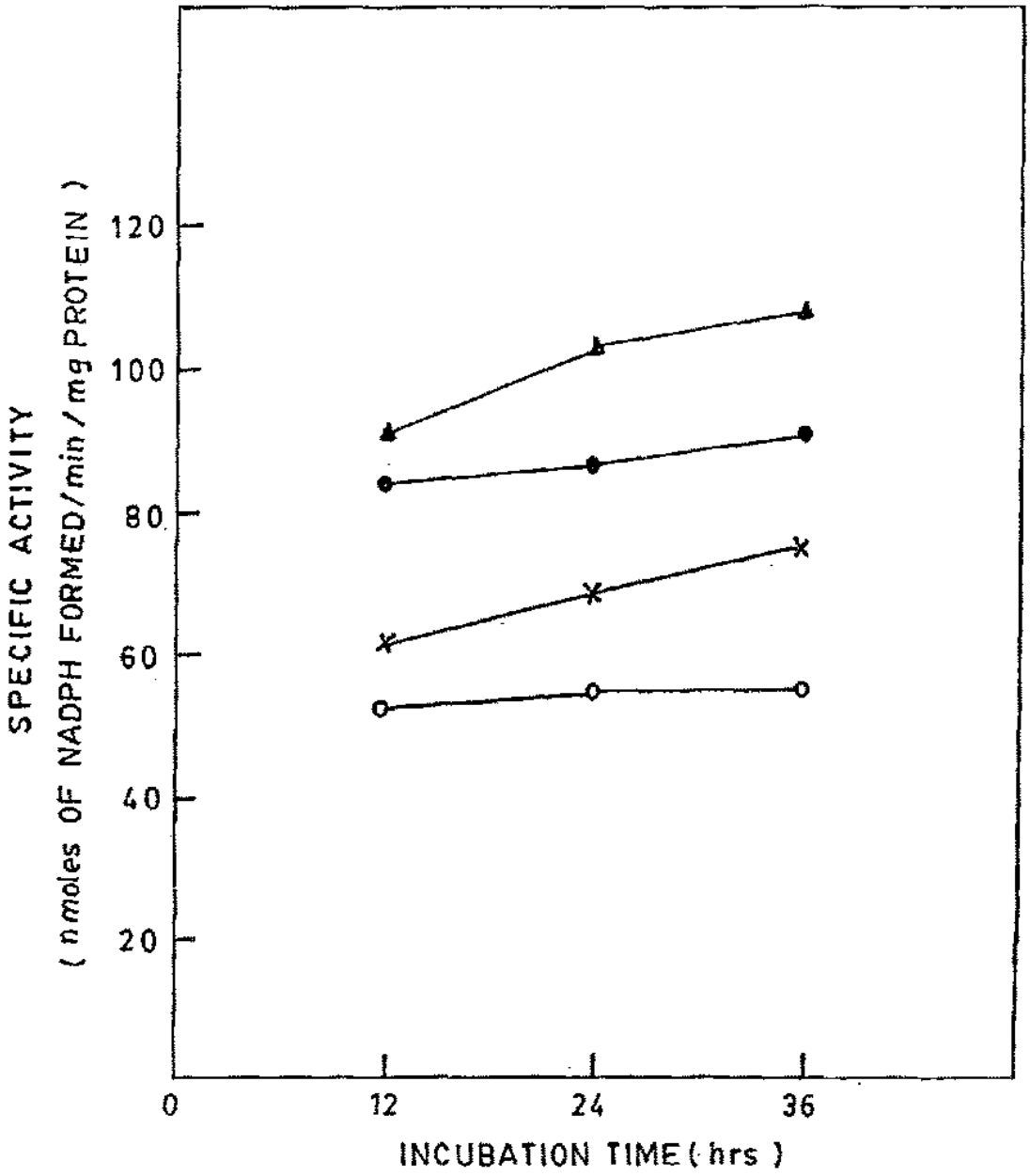
Fig. 5. Effect of combination of hormones and glucose on the specific activity of glucose-6-phosphate dehydrogenase during different hours of explant culture:

(A) In the absence of hormones and glucose (O——O)

(B) In the presence of hormones (●——●)

(C) In the presence of glucose (x--x)

(D) In the presence of hormones and glucose (▲--▲)


SPECIFIC ACTIVITY
(nmoles OF NADPH FORMED/min / mg PROTEIN)
120
100
80
60
40
20
0
12
24
36
INCUBATION TIME (hrs)

to almost 2-fold, from 51.81, 53.58 and 54.25 (in the case of control experiment) to 90.82, 102.85 and 107.95 after 12, 24 and 36 hours of incubation, respectively. These values were strikingly higher than those found in the cases when either glucose or hormones were added separately in the explant culture medium and the explant culture was carried out for the corresponding hours of incubation.

4.1.7 6-phosphogluconate dehydrogenase:

Similar experiments as mentioned in the case of glucose-6-phosphate dehydrogenase were conducted for 6-phosphogluconate dehydrogenase to see the effect of combination of hormones, glucose and glucose in addition with hormone combination, on the specific activity of 6-phosphogluconate dehydrogenase.

4.1.7.1 Effect of combination of hormones :

The effect of insulin, prolactin and cortisol in combination was studied on the specific activities of 6-phosphogluconate dehydrogenase at different hours of incubation. As it is obvious from the Table 7 and Fig.6 that the combination of hormones increased the specific activities of 6-phosphogluconate dehydrogenase at different hours of incubation. The specific activity of 6-phosphogluconate dehydrogenase increased significantly about 1.5-fold, from 54.56 to 82.52 in the presence of hormones combination in the culture medium after 12 hours of incubation. On prolonged incubation, the specific activities of 6-phosphogluconate dehydrogenase increased to 92.98 and 105.80at 24 and 36 hours of incubation

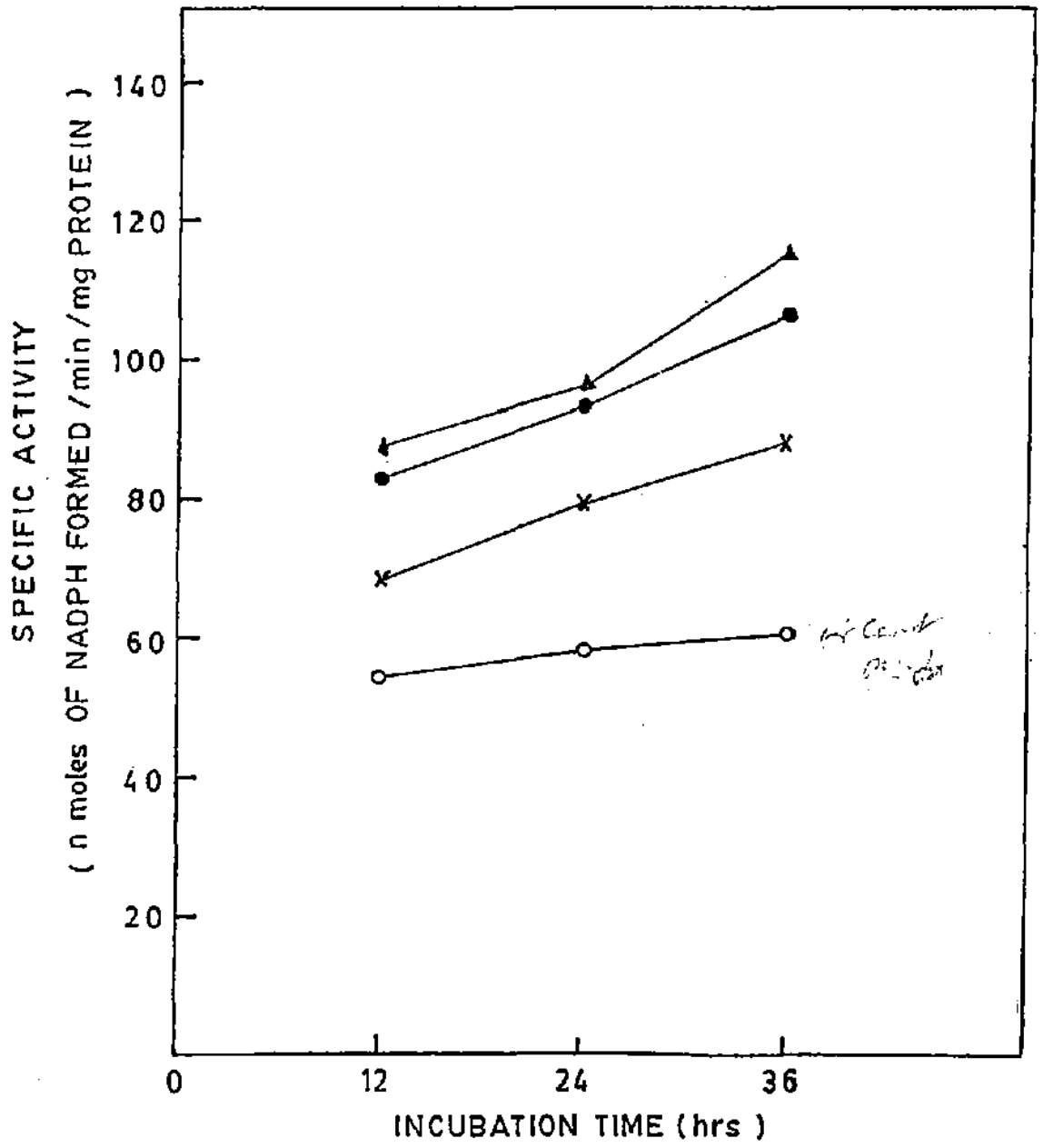
Table - 7

Effect of incubation time on the activity of 6-phosphogluconate dehydrogenase.

S.No.	Treatment	n moles of NADPH formed/ min/mg of protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	54.57	57.70	60.57
2.	Medium + hormones*	82.52	92.98	105.80
3.	Medium + glucose**	68.31	79.64	87.46
4.	Medium + hormones* + glucose**	86.22	95.92	113.76

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2 mg/ml of culture medium.


SPECIFIC ACTIVITY
(n moles OF NADPH FORMED /min /mg PROTEIN)
140
120
100
80
60
40
20
0
12
24
36
INCUBATION TIME (hrs)

period, respectively.

4.1.7.2 Effect of glucose:

Table 7 and Fig. 6 also depict the effect of glucose on the specific activities of 6-phosphogluconate dehydrogenases. The addition of glucose in the culture medium significantly enhanced the specific activities of 6-phosphogluconate dehydrogenase from 54.56, 57.70 and 60.5 to 68.31, 79.64 and 87.46 at 12, 24 and 36 hours of incubation, respectively. However, the increase in the specific activities in this case was comparatively less as compared to those observed during the incubation, carried out in the presence of hormones combination.

4.1.7.3 Effect of glucose in combination of hormones:

Table 7 and Fig. 6 show that glucose in combination with insulin, prolactin and cortisol, significantly increased the specific activities of 6-phosphogluconate dehydrogenase to 86.22, 95.92 and 113.76 at 12, 24 and 36 hours of incubation. Moreover these values were higher as compared to the specific activities found when only glucose was used in the culture medium during explant culture. The activities of this enzyme were also higher than those at different hours of incubation when the explant culture medium contained combination of hormones, only.

4.1.8 NADP-isocitrate dehydrogenase:

4.1.8.1 Effect of combination of hormones:

The combined effect of insulin, prolactin and cortisol

was studied on the specific activities of NADP-isocitrate dehydrogenase at different hours of incubation.

Table 8 and Fig.7 show the effect of combination of hormones, on the specific activities of NADP-isocitrate dehydrogenase at 12, 24 and 36 hours of incubation. The specific activities of NADP-isocitrate dehydrogenase increased, significantly, in the presence of insulin, prolactin and cortisol to 327.37, 368.57 and 315.61 after 12, 24 and 36 hours of incubation. Whereas the specific activities of the enzyme, observed in the case of control experiments, were 196.90, 224.5 and 209.50 after the corresponding hours of incubation.

4.1.8.2 Effect of glucose:

The effect of glucose on the specific activities of NADP-isocitrate dehydrogenase after different hours of incubation has been shown in Table 8 and Fig.7. The glucose addition to the culture medium led to the remarkable increase in the specific activities of NADP-isocitrate dehydrogenase to 287.57, 337.27 and 295.14 from 196.90, 214.50 and 209.50 after 12, 24 and 36 hours of incubation, respectively. However, this increase in the specific activities was comparatively less as compared to that observed when the explant culture was carried out in the presence of hormones combination for the similar hours of incubation.

4.1.8.3 Effect of glucose in combination of hormones

It is observed from the Table 8 and Fig.7 that glucose in combination with insulin, prolactin and cortisol

Table - 8

Effect of incubation time on the activity of NADP-isocitrate dehydrogenase.

S.No.	Treatment	n moles of NADPH formed/ min/mg of protein		
		Incubation Time (hrs)		
		12	24	36
1.	Medium	196.90	214.50	209.50
2.	Medium + hormones*	327.37	368.57	315.61
3.	Medium + glucose**	287.57	337.27	295.14
4.	Medium + hormones*+ glucose**	354.35	382.72	317.63

* The concentration of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** The concentration of glucose used was 2 mg/ml of culture medium.

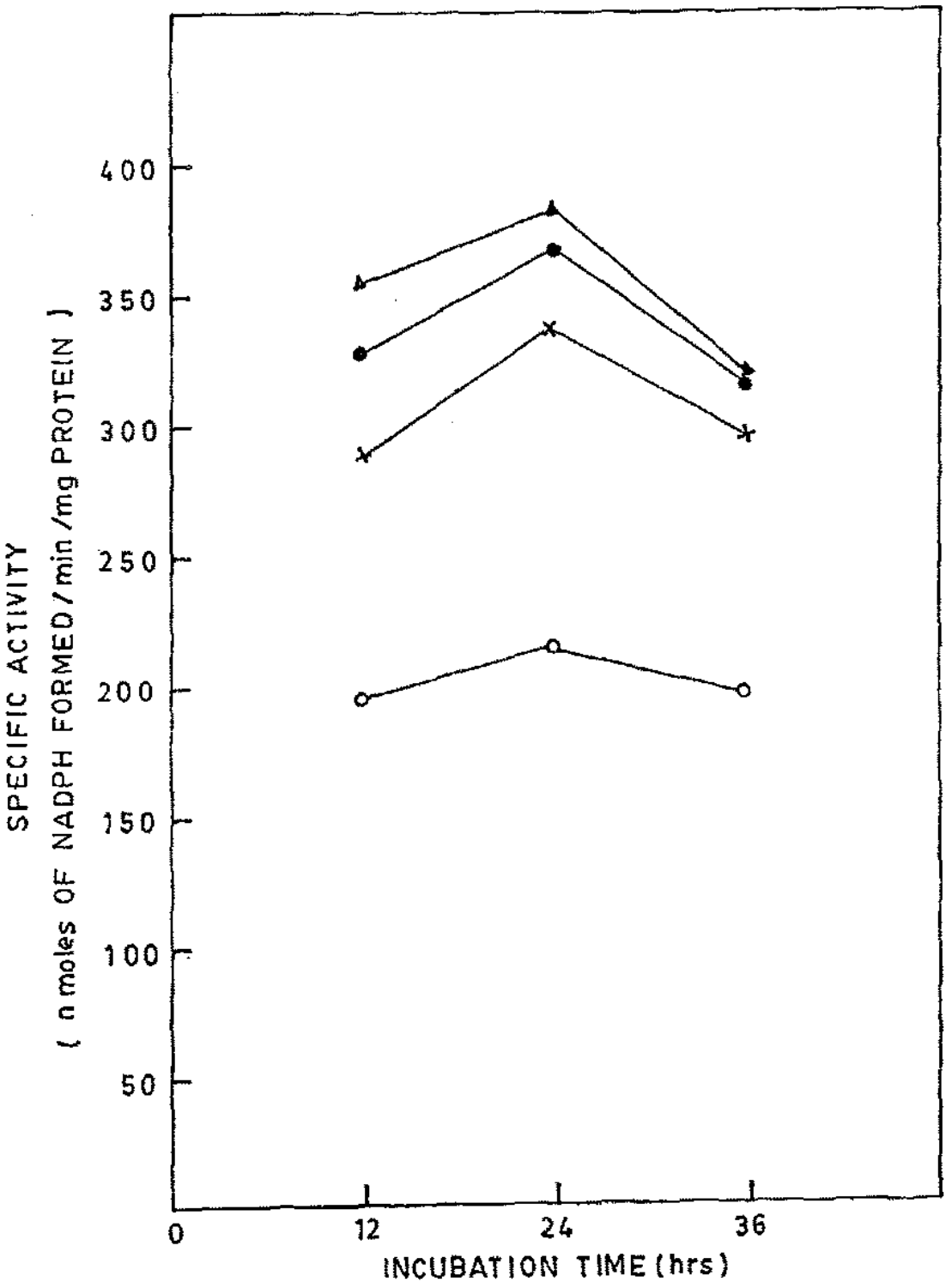
Fig. 7. Effect of combination of hormones and glucose on the specific activity of NADP-isocitrate dehydrogenase during different hours of explant culture:

(A) In the absence of hormones and glucose (O——O)

(B) In the presence of hormones (■——■)

(C) In the presence of glucose (x---x)

(D) In the presence of hormones and glucose (▲--▲)


SPECIFIC ACTIVITY
(n moles OF NADPH FORMED / min /mg PROTEIN)
400
350
300
250
200
150
100
50
0
12
24
36
INCUBATION TIME (hrs)

increased the specific activities of NADP-isocitrate dehydrogenase markedly to 354.35, 382.72 and 317.63 at 12, 24 and 36 hours of incubation. These values were strikingly higher in comparison to the values observed in the case of experiment where either glucose or combination of hormones was used during explant culture.

It was also observed that the prolonged incubation for 36 hours resulted in the decrease in the specific activities of NADP-isocitrate dehydrogenase in comparison to the specific activities obtained after 12 or 24 hours of explant cultures in all the three experimental conditions.

Section - B

4.2 Purification of acetyl-CoA carboxylase and fatty acid synthetase.

4.2.1 Purification of acetyl-CoA carboxylase:

The acetyl-CoA carboxylase was purified from the goat mammary gland. The purification Chart of this enzyme is given in Table 9, which depicts various steps undertaken during the purification process of this enzyme. The starting point for the purification of acetyl-CoA carboxylase was cytosol obtained from goat mammary gland, which contained 13,860 mg protein and 255.4 units of enzyme with the specific activity of 0.018. The first ammonium sulphate fractionation of the cytosol resulted in increase in the specific activity of the enzyme to 0.045 (2.5 fold-purification) and a yield of 89%, was

Table - 9

Purification chart of acetyl-CoA carboxylase. One unit is defined as the quantity which catelyzes the incorporation of 1 u mole of $HC^{14}O_3$ into malonyl-CoA per minute at 37°C. Specific activity is defined as the units per mg of protein.

S.No.	Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Total units	Specific activity (Units/mg)	Yield (%)	Fold purification
1.	Crude extract	1050	13.20	13,860.00	255.40	0.018	100.00	0.0
2.	Ammonium sulphate precipitation 0-50%	200	25.20	5,040.00	227.00	0.045	89.00	2.5
3.	Post-dialysis	212	19.57	4,148.00	218.00	0.052	85.40	2.9
4.	Negative adsorption (DE-52)	305	7.04	2,147.00	195.00	0.090	76.29	5.0
5.	Ammonium sulphate fractionation	22	10.62	233.64	95.75	0.409	37.50	23.0
6.	Sepharose 2B	48	1.26	60.48	49.50	0.818	19.2	45.5

Fig. 8. Sepharose 2B column chromatography of acetyl-CoA carboxylase

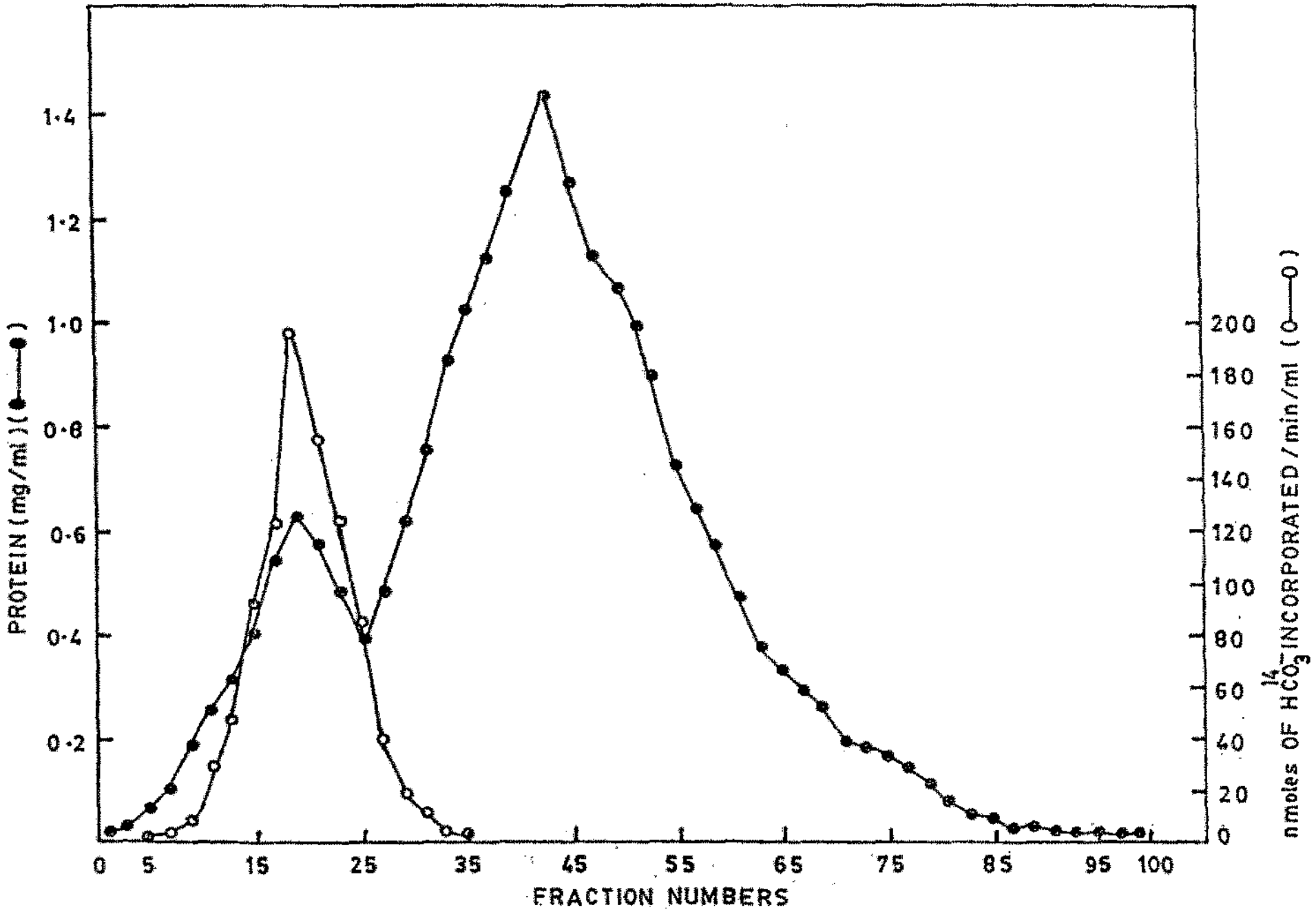
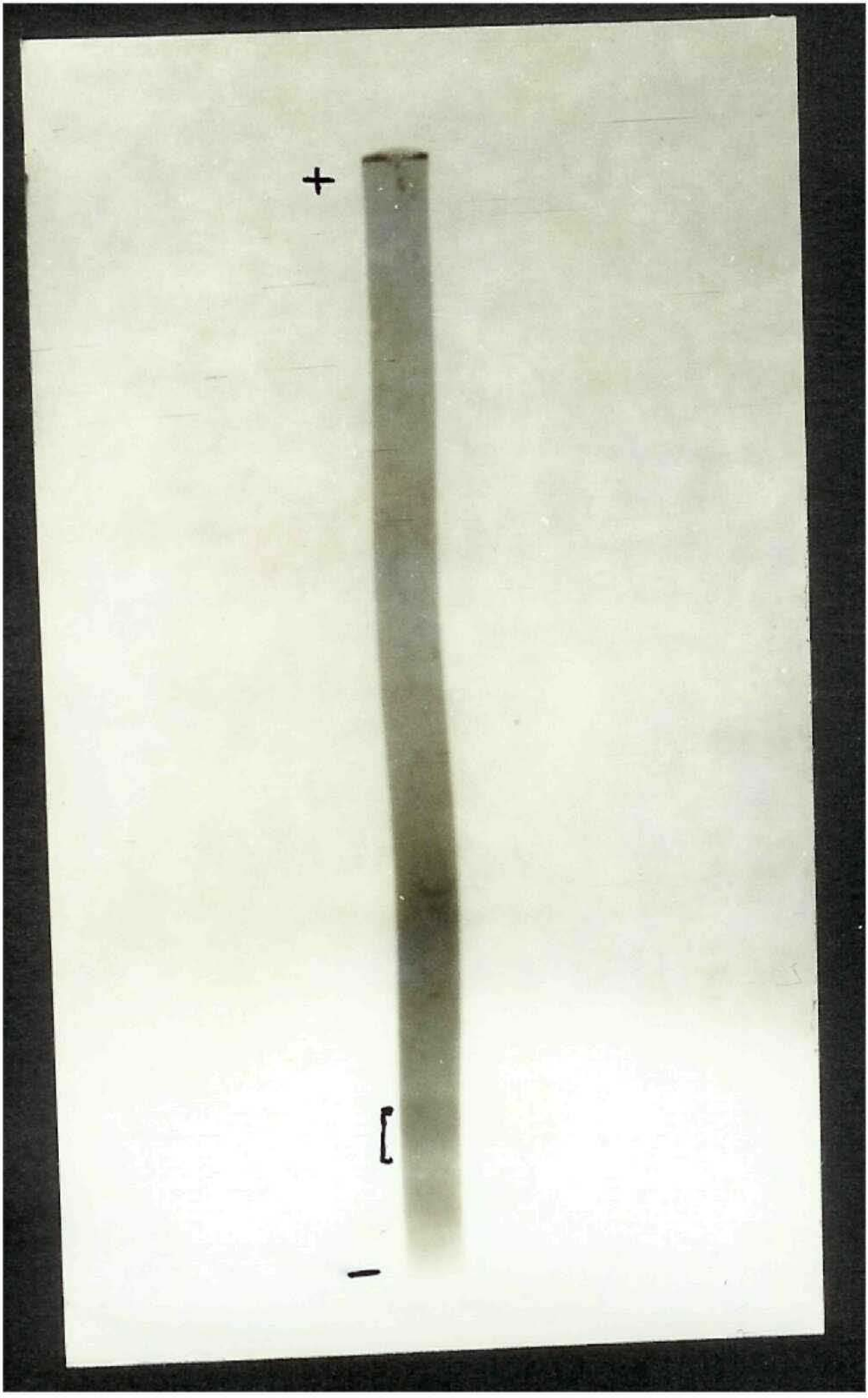

PROTEIN (mg/ml) (●—●)
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
5
15
25
35
45
55
65
75
85
95
100
FRACTION NUMBERS
200
180
160
140
120
100
80
60
40
20
0
nmoles OF $H^{14}CO_3^-$ INCORPORATED / min / ml (○—○)

Plate 3. Polyacrylamide gel electrophoresis of purified acetyl-CoA carboxylase.



obtained. The overnight dialysis of this fraction increased the specific activity to 0.052. The enzyme was purified to 2.9-fold and the total units of enzyme left were 218.00, which represented a yield of 85.4%. The negative adsorption on DEAE-Cellulose column resulted in further enhanced specific activity (0.090) of the enzyme. The enzyme was purified to 5-fold and a yield of 76.29% was obtained. Next step in the purification, which involved the second ammonium sulphate fractionation, led to a 23.0 fold purification of the enzyme with specific activity of 0.409. Total units of the enzyme left in this step were 95.75 and the yield achieved was 37.5%. The last step involving the elution of enzyme through Sepharose-2B column and pooling of the fractions having highest enzyme activity resulted in a 45.5-fold purification of the enzyme, with the specific activity of 0.818. Total units of the enzyme left at the end of the whole procedure were 49.05, which represented a 19.2% yield of the enzyme. The elution profile is given in Fig.8.

This purified enzyme so obtained, when subjected to polyacrylamide gel electrophoresis gave only one band as shown in Plate 3. This confirmed that the enzyme acetyl-CoA carboxylase was purified to homogeneity.

4.2.2 Purification of fatty acid synthetase:

This enzyme was also purified from the goat mammary gland. The results of purification have been shown in Table 10. The starting point for the purification of fatty acid synthetase was cytosol, which contained 1900 mg

protein and 25225.50 units with a specific activity of 13.22. After the first ammonium sulphate fractionation of the cytosol, the specific activity of the enzyme was increased to 47.48 which resulted in 3.6-fold purification and a yield of 62.15%. The total units of the fatty acid synthetase left were 15678.00. Treatment of this fraction with calcium phosphate gel led to the increase in the specific activity to 53.73. A 4.06-fold purification was obtained with a yield of 60.93%. Total units of the enzyme obtained in this case were 15372.00. Elution of the calcium phosphate gel treated fraction through DEAE-Cellulose column, with a linear gradient of 50 to 250 mM phosphate buffer (pH 7.0) and pooling together of the fractions having highest activity resulted in further increase in the specific activity of the enzyme to 192.76. A 14.6-fold purification with a yield of 39.33% was obtained. The total units of the enzyme obtained at the end of this step were 9923.40. The elution profile of the enzyme through DEAE-Cellulose column is given in Fig.9. The last step in the purification sequence which involved the second ammonium sulphate fractionation, yielded a purified enzyme with a specific activity of 294.37. This represented the purification of the enzyme to be 22.3-fold over that of cytosol. The yield was 11.95%. Total units of the enzyme fatty acid synthetase left at the end of all the purification steps were 3014.40.

When this purified enzyme so obtained was subjected to Sephadex G-200 column chromatography, it emerged as a

Table - 10

Purification chart of fatty acid synthetase. One unit is defined as n moles of NADPH oxidised per minute.

S.No.	Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Units (ml)	Total units	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
1.	Cytosol	250	7.6	1900.00	100.50	25225.50	13.22	100.00	0.0
2.	First ammonium sulphate fractionation (25-40% saturation)	26	12.7	330.20	603.00	15678.00	47.48	62.15	3.6
3.	Calcium phosphate gel-treatment	48	5.96	286.00	320.25	15372.00	53.73	60.93	4.06
4.	DEAE-Cellulose	180	0.286	51.48	55.13	9923.40	192.76	39.33	14.6
5.	Second ammonium sulphate fractionation (26-32%) saturation	8	1.28	10.24	376.8	3014.40	294.37	11.95	22.3

Fig. 9. Elution profile of DEAE-Cellulose chromatography of fatty acid synthetase.

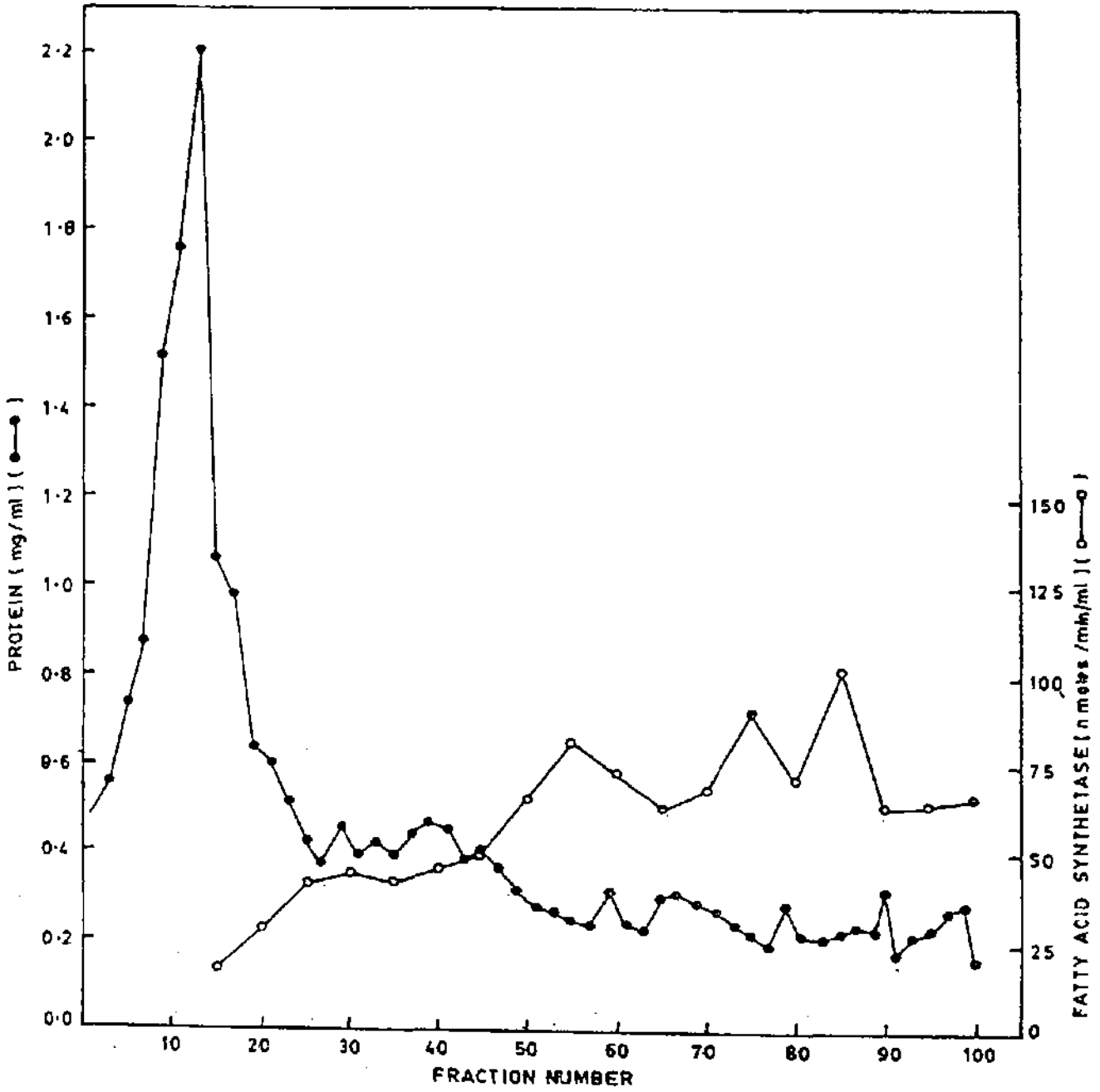

PROTEIN (mg/ml) (● — ●)
FATTY ACID SYNTHETASE (n moles /mh/ml) (○ — ○)
FRACTION NUMBER
2·2
2·0
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
150
125
100
75
50
25
0
10
20
30
40
50
60
70
80
90
100

Fig. 10. Sephadex-G 200 column chromatography of fatty acid synthetase, ascertaining the homogeneity of enzyme protein.

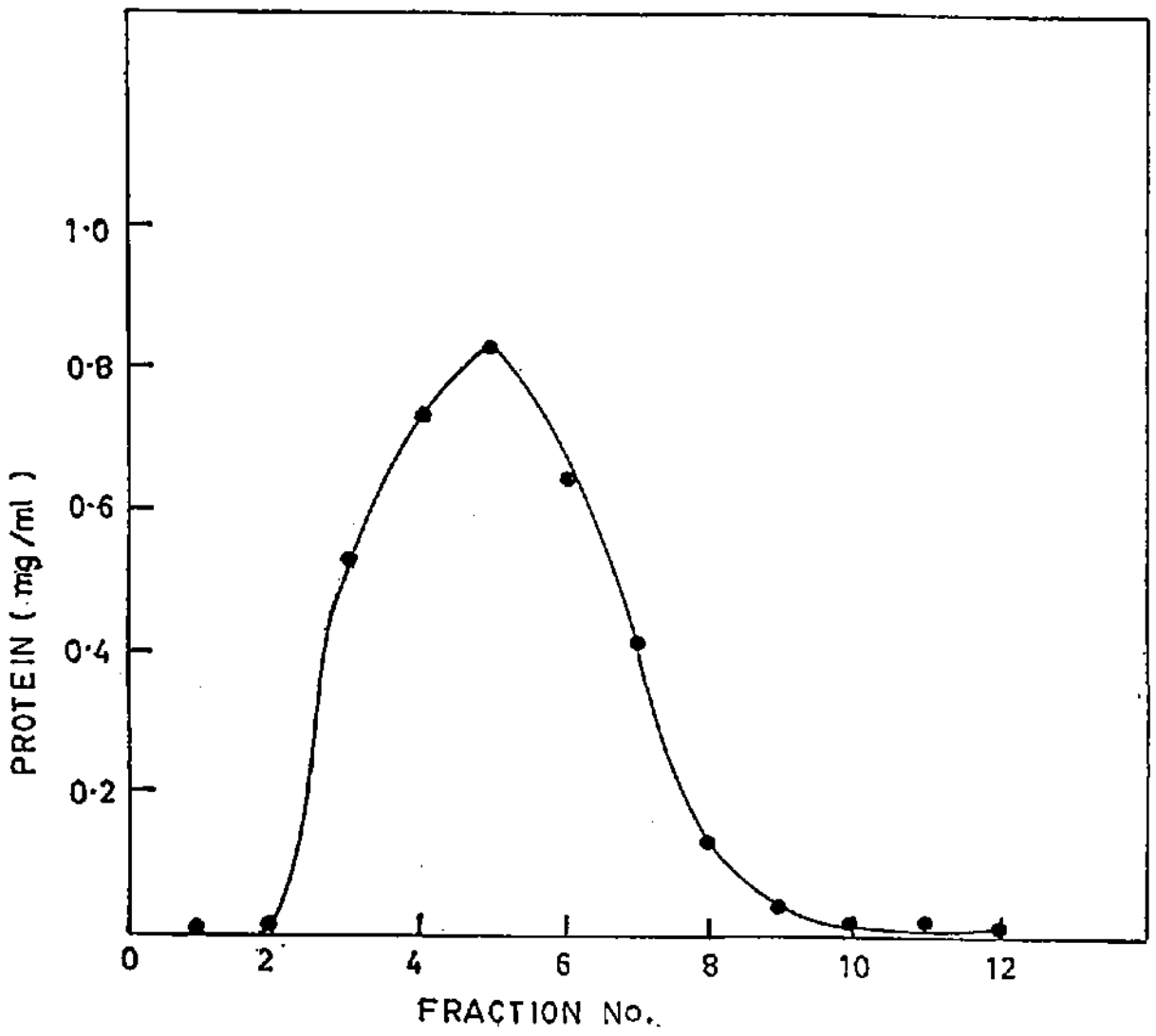

1·0
0·8
0·6
0·4
0·2
PROTEIN (mg/ml)
0
2
4
6
8
10
12
FRACTION No.

Plate 4. Polyacrylamide gel electrophoresis of purified fatty acid synthetase.

single enzyme protein peak which shows homogeneity of the purified enzyme (Fig.10). This was further ascertained, when the purified enzyme was subjected to polyacrylamide gel electrophoresis. This gave a single band as shown in Plate 4, which revealed that the enzyme, fatty acid synthetase was purified to homogeneity.

Section - C

4.3 Development and assessment of the specificity of the antibodies:

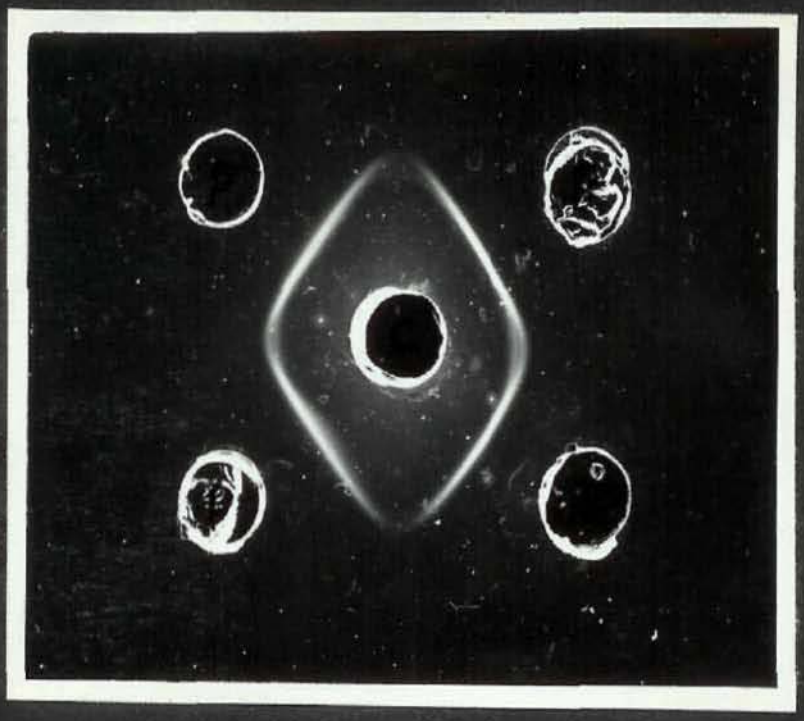
The antibodies were developed against acetyl-CoA carboxylase and fatty acid synthetase, which were purified to homogeneity as mentioned in the Section-B. The antibodies were also developed against the purified enzyme glucose-6-phosphate dehydrogenase obtained as standard. The specificity of the antibodies, thus developed in rabbits against the different enzymes was checked by Ouchterlony double immunodiffusion technique (1966).

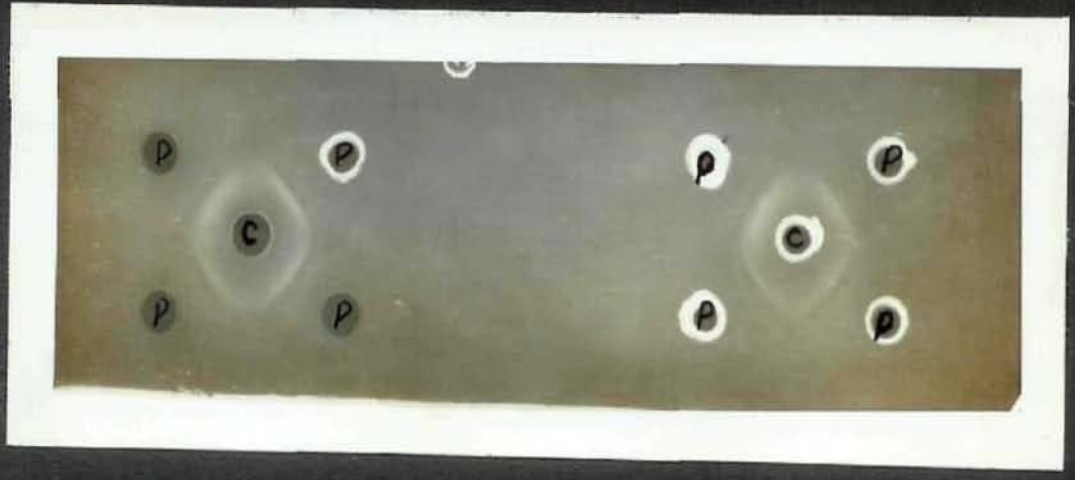
4.3.1 Assessment of the immunospecificity of antibodies developed against acetyl-CoA carboxylase:

The immunospecificity of the antibodies developed against acetyl-CoA carboxylase was assessed by Ouchterlony (1966) double immunodiffusion technique. It is obvious from the Plate 5 that the antigen (acetyl-CoA carboxylase) and antibodies on diffusion through the agarose gel, agglutinated to form only a single precipitin line. The appearance of single precipitin line signified the homogeneity as well as immunospecificity of the antibodies

Plate 5. Immunodiffusion analysis of goat mammary gland acetyl-CoA carboxylase with anti-acetyl-CoA carboxylase (Lactating goat mammary gland). Central (C) well containing purified acetyl-CoA carboxylase and the peripheral (P) wells containing anti-acetyl-CoA carboxylase.

Plate 6. Immunodiffusion analysis of goat mammary gland fatty acid synthetase with anti-fatty acid synthetase (Lactating goat mammary gland). Central (C) well containing purified fatty acid synthetase and the peripheral (P) wells containing anti-fatty acid synthetase.




P
P
P
P
C
C
P
P
P
P

so developed against acetyl-CoA carboxylase.

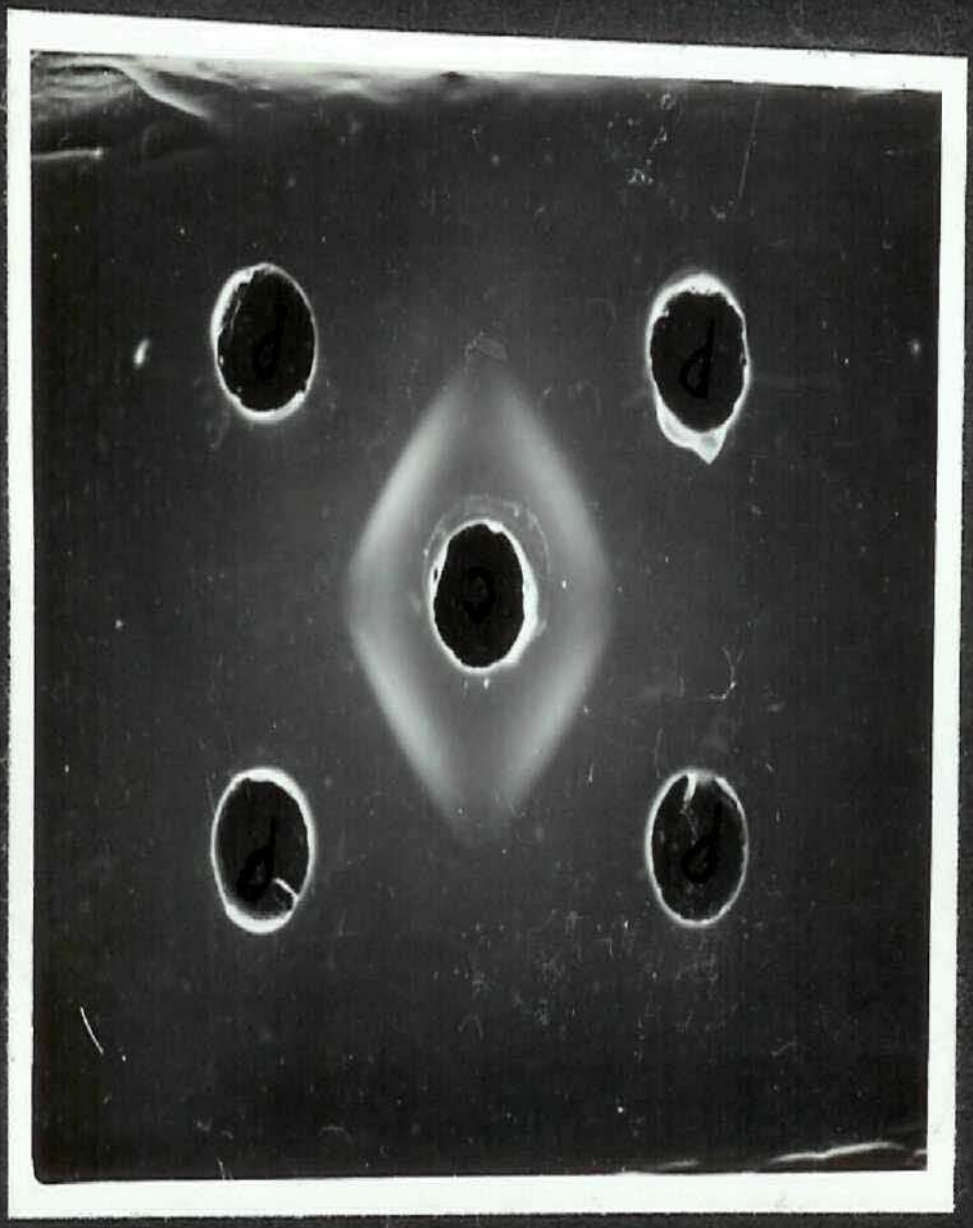
4.3.2 Assessment of the immunospecificity of the antibodies developed against fatty acid synthetase:

The immunospecificity or the immunogenecity of the antibodies developed against fatty acid synthetase was seen by Ouchterlony (1966) double immuno diffusion technique. On immunodiffusion,the antibodies against fatty acid synthetase gave one precipitin band as shown in Plate 6. This single precipitin line indicated the homogeneity of the antiserum as well as its immuno-specificity against its antigen (fatty acid synthetase) which has been purified as a homogenous protein.

4.3.3 Assessment of the immunospecificity of the antibodies against glucose 6-phosphate dehydrogenase

Similarly the antibodies were raised against the purified glucose-6-phosphate dehydrogenase and their immunospecificity against glucose-6-phosphate dehydrogenase was checked by Ouchterlony's (1966) double immunodiffusion technique. Like the other two antibodies against two different enzymes, respectively, as mentioned above, the antibodies against this enzyme also on immunodiffusion gave only one precipitin band as shown in Plate 7. The appearance of single precipitin line ascertained the homogeneity as well as the immunospecificity of the antibodies developed against the purified enzyme.

Plate 7. Immunodiffusion analysis of goat mammary gland glucose-6-phosphate dehydrogenase (Lactating goat mammary gland), with anti-glucose-6-phosphate dehydrogenase. Central (C) well containing purified glucose-6-phosphate dehydrogenase and the peripheral wells (P) containing anti-glucose-6-phosphate dehydrogenase.



Section - D

4.4 Effect of combination of hormones (insulin, prolactin and cortisol), glucose and glucose in addition with combination of hormones on the synthesis of some of the lipogenic enzymes.

Insulin, prolactin and cortisol were used in combination (5 µg/ml of medium each) to see their effect on the synthesis of some of the lipogenic enzymes viz. acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase in mid-pregnant goat mammary gland using explant culture system. Similar experiments were also conducted to observe the effect of glucose and glucose with the combination of hormones (insulin, prolactin and cortisol) on the synthesis of the above mentioned enzymes.

The results obtained for the influence of the various treatments, as mentioned above, on the synthesis of lipogenic enzymes have been described as below.

4.4.1 Quantitative estimation of acetyl-CoA carboxylase

For quantitation of acetyl-CoA carboxylase enzyme in the explant cultured tissue, the immunological measurements, employing rocket immunoelectrophoretic technique were used. The increasing amount of antigen (acetyl-CoA carboxylase) ranging from 20 to 80 µg were filled into the wells made on the agarose anti-acetyl-CoA carboxylase, gel slab. The desired dilution of antiserum was used for this purpose. On subjecting the slab to rocket immunoelectrophoresis, it was observed that rocket shaped areas formed after reaction of antigen with anti-acetyl-CoA

Plate 8 Rocket immunoelectrophoresis of antigen (acetyl-CoA carboxylase) on agarose containing anti-acetyl-CoA carboxylase, using varying amounts of antigen from well No. 1 to 4.

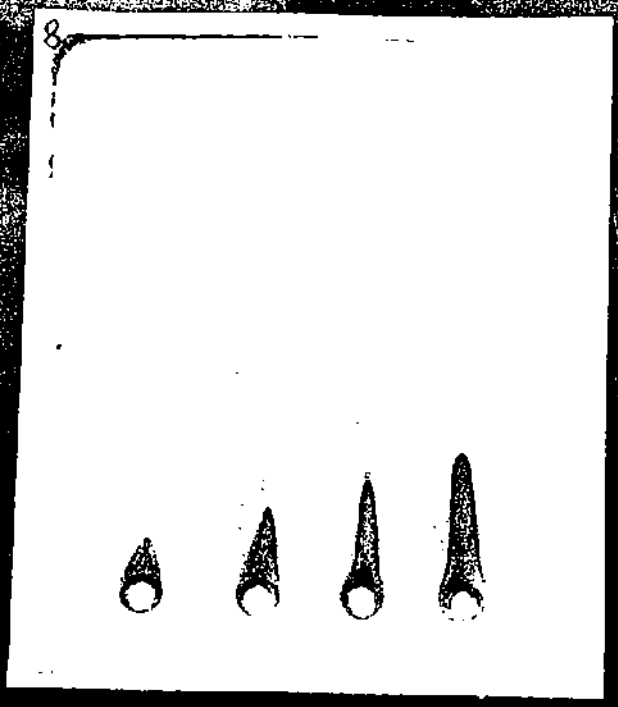
Plate 10. Rocket immunoelectrophoresis of unknown samples of acetyl-CoA-carboxylase obtained from:

(A) Explants cultured in the absence of hormones and glucose.

(B) Explants cultured in the presence of hormones.

(C) Explants cultured in the presence of glucose.

(D) Explants cultured in the presence of hormones and glucose.



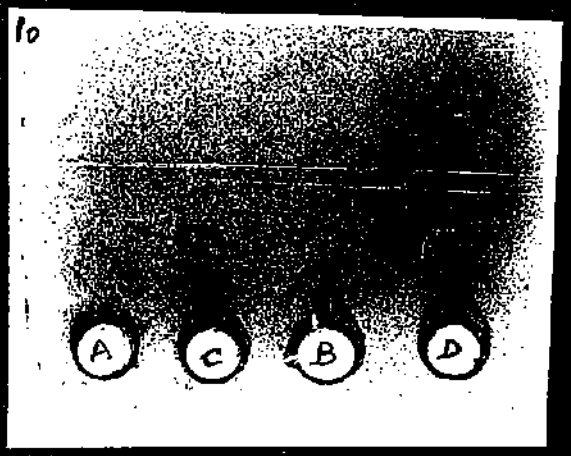

A
C
B
D

Plate 9. Preparation of Standard curve for acetyl-CoA carboxylase (antigen) using rocket immunoelectrophoretic technique.

carboxylase, increased proportionately with the increase in the amount of the antigen applied (Plate 8). The results shown in Plate 9 reveal that the areas occupied by the different amounts of antigen on the gel slab were linear function of the increasing amount of antigen. For estimating the amount of antigen (acetyl-CoA carboxylase) present in cytosolic fractions obtained from the cultured tissues under the effect of various conditions (Control, hormone treated, glucose treated and glucose in combination with hormones treated experiments), rocket immunoelectrophoresis was carried out. The rocket shaped areas so formed as given in Plate 10 were correlated with the standard curve (Plate 9). It was observed that the synthesis of acetyl-CoA carboxylase was increased to 216% of the control in the case when insulin, prolactin and cortisol were added together in explant culture medium, during 24 hours of incubation or explant culture. When only glucose was added to the culture medium, after 24 hours of incubation it was observed that the synthesis of acetyl-CoA carboxylase was increased to 169% of the control. However, in the case, when glucose in combination with hormones was used in culture medium, the acetyl-CoA carboxylase was synthesized to a greater extent. It's synthesis was increased by 2.6-fold i.e. 260% of the control as given in Table 11.

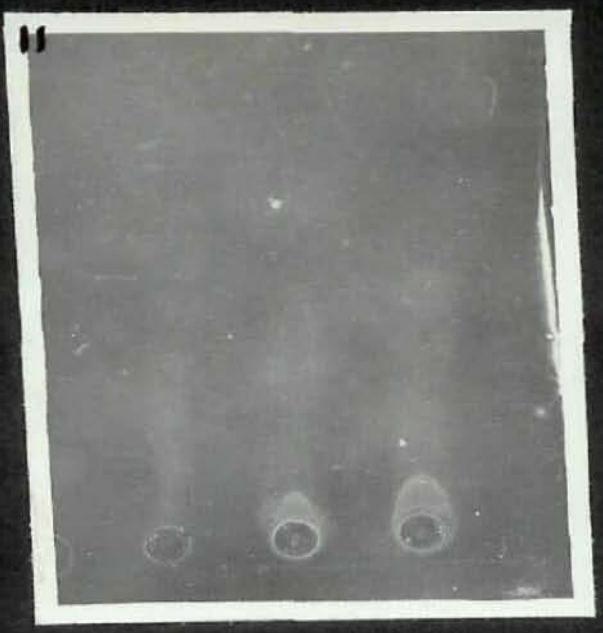
4.4.2 Quantitative estimation of fatty acid synthetase:

For the quantitative estimation of fatty acid synthetase, similar technique was used as mentioned in section 4.4.1, however, the amount of the enzyme (antigen)

Plate 11. Rocket immunoelectrophoresis of antigen (fatty acid synthetase) on agarose conteining anti-fatty acid synthetase, using varying amounts of antigen from well 1 to 3.

Plate 12. Preparation of Standard curve for fatty acid synthetase (antigen) using rocketimmunoelectrophoresis.

11



12

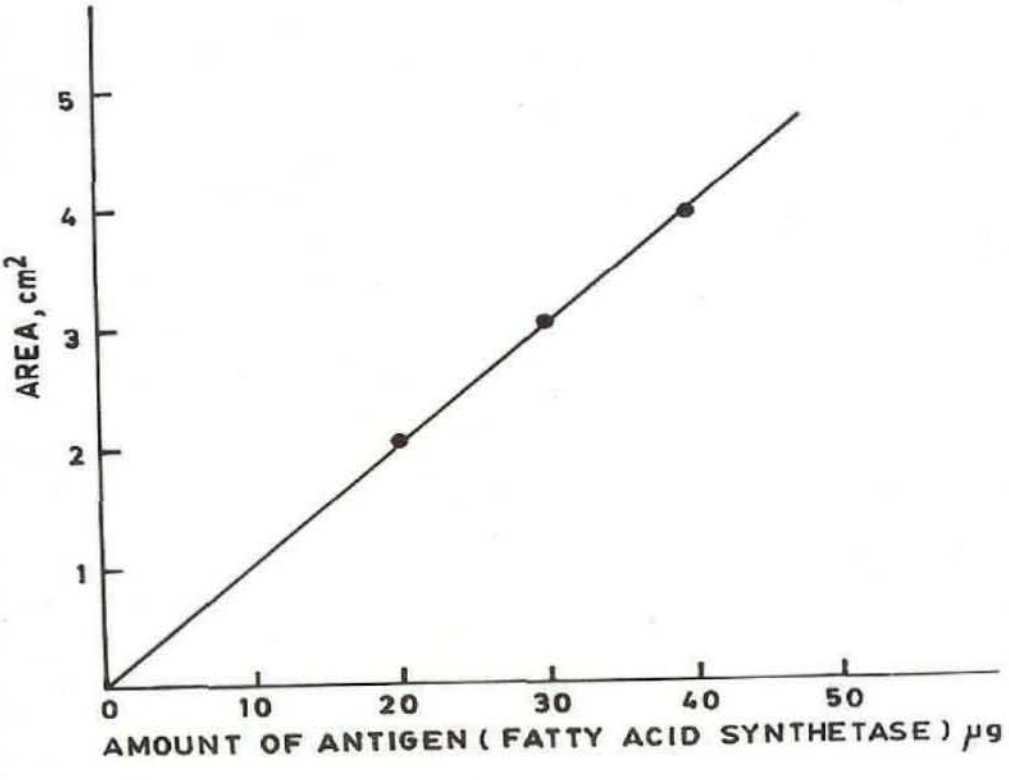

AREA, cm²
1
2
3
4
5
0
10
20
30
40
50
AMOUNT OF ANTIGEN (FATTY ACID SYNTHETASE) µg

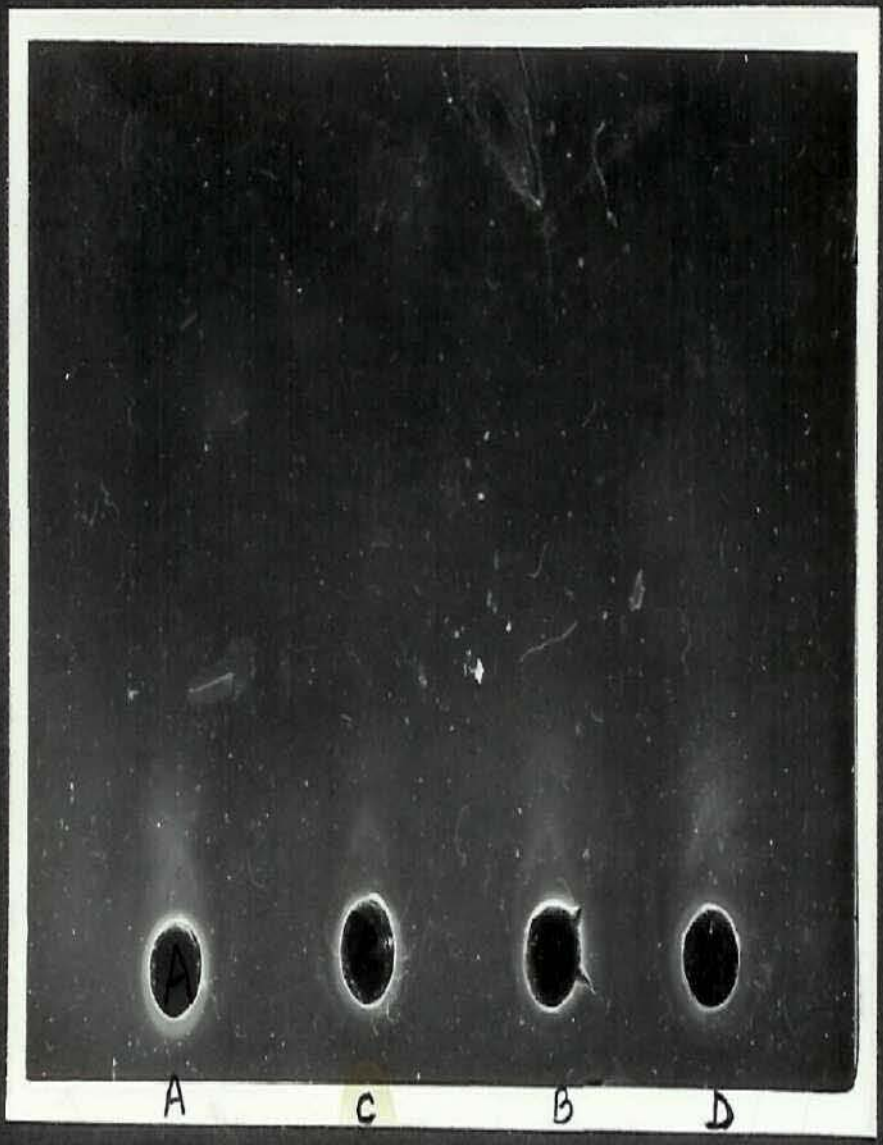
Plate 13. Rocket immunoelectrophoresis of unknown samples of fatty acid synthetase obtained from:

(A) Explants cultured in the absence of hormones and glucose.

(B) Explants cultured in the presence of hormones.

(C) Explants cultured in the presence of glucose.

(D) Explants cultured in the presence of hormones and glucose.


A
A
C
B
D

was taken in the range of 10 to 40 µg for the purpose. A standard graph, as shown in Plate 12 was obtained with the help of the rocket shaped areas obtained during rocket immunoelectrophoresis using different amounts of antigen (Plate 11) and these were linear function of the amount of the antigen. When the areas, occupied by the cytosolic fractions obtained from control experiments, combination of hormones treated experiment, glucose treated and glucose plus hormones treated explant cultured tissues from mid-pregnant goat mammary gland as given in Plate 13, were correlated with the standard curve (Plate 12), it was observed that the synthesis of fatty acid synthetase was increased to 167% of the control when combination of hormones (insulin, prolactin and cortisol) was added in the culture medium during 24 hours of explant culture. The synthesis was increased to 127% of the control when glucose alone was added to the culture medium and the explant culture was carried out for the similar hours of incubation (24 hours). However, the addition of glucose plus combination of hormones to the culture medium during 24 hours of incubation caused the synthesis of fatty acid synthetase to increase to 241% of the control as shown in Table 11.

4.4.3 Quantitative estimation of glucose-6-phosphate dehydrogenase:

The effect of combination of hormones, glucose and glucose plus combination of hormones on the synthesis of glucose-6-phosphate dehydrogenase in mid-pregnant goat

Plate 14. Preparation of Standard curve for glucose-6-phosphate dehydrogenase (antigen) using rocket immunoelectrophoretic technique.

mammary gland, using explant culture system was also studied. Plate 14 depicts that rocket shaped areas occupied by the increasing amount of antigen(glucose-6-phosphate dehydrogenase) on the gel slabs consisting of agarose and desired dilution of antiserum, so developed against purified glucose-6-phosphate dehydrogenase, were linear function of the increasing amount of antigen. When the cytosolic fractions from the control experiment, hormones treated, glucose treated and glucose plus combination of hormones treated explant cultured tissues were applied on the agarose gel slab containing desired dilution of antiserum, the areas so obtained after rocket immunoelectrophoresis were extrapolated from standard graph (Plate 14) to find out the total amount of antigen present. It was observed that the addition of combination of hormones caused the increase in the synthesis of glucose-6-phosphate dehydrogenase to 166% of the control after 24 hours of incubation or explant culture. When only glucose (2 mg/ml of culture medium) was added to the culture medium, the synthesis of glucose-6-phosphate dehydrogenase was increased to 129% of the control. However, by adding hormones in combination with glucose to the explant culture medium, the synthesis of glucose-6-phosphate dehydrogenase enhanced to 185% of the control after 24 hours of incubation (Table 11).

On comparing the relative percent increase in the synthesis of the lipogenic enzymes (acetyl-CoA carboxylase,

Table-11

Effect of combination of hormones (insulin, prolactin and cortisol), glucose and combination of hormones plus glucose on the synthesis of lipogenic enzymes, during explant culture for 24 hours. Values are expressed as percentages of the controls.

Treatments	Acetyl-CoA carboxylase	Fatty acid synthetase	Glucose-6-phosphate dehydrogenase
Medium (control)	100	100	100
Medium + hormones*	216	167	166
Medium + glucose**	169	127	129
Medium + hormones* + glucose	260	241	185

* Concentrations of insulin, prolactin and cortisol used were 5 ug/ml of culture medium each.

** Concentration of the glucose added to the culture medium was 2 mg/ml of medium.

Table - 12

Effect of combination of hormones (insulin, prolactin and cortisol), glucose and combination of hormones plus glucose on the specific activities of the lipogenic enzymes, during explant culture for 24 hours. Enzyme specific activities are expressed as percentages of the controls.

Treatments	Acetyl-CoA carboxylase	Fatty acid synthetase	Glucose-6-phosphate dehydrogenase
Medium (control)	100	100	100
Medium + hormones*	204	160	162
Medium + glucose**	164	118	127
Medium + hormones* +glucose**	252	220	192

* Concentrations of insulin, prolactin and cortisol used were, 5 ug/ml of culture medium each.

** Concentration of the glucose added to the culture medium was 2 mg/ml of medium.

Plate 15. Effect of various combinations of hormones and glucose on the synthesis and specific activity of acetyl-CoA carboxylase.

Synthesis ()

Specific activity ()

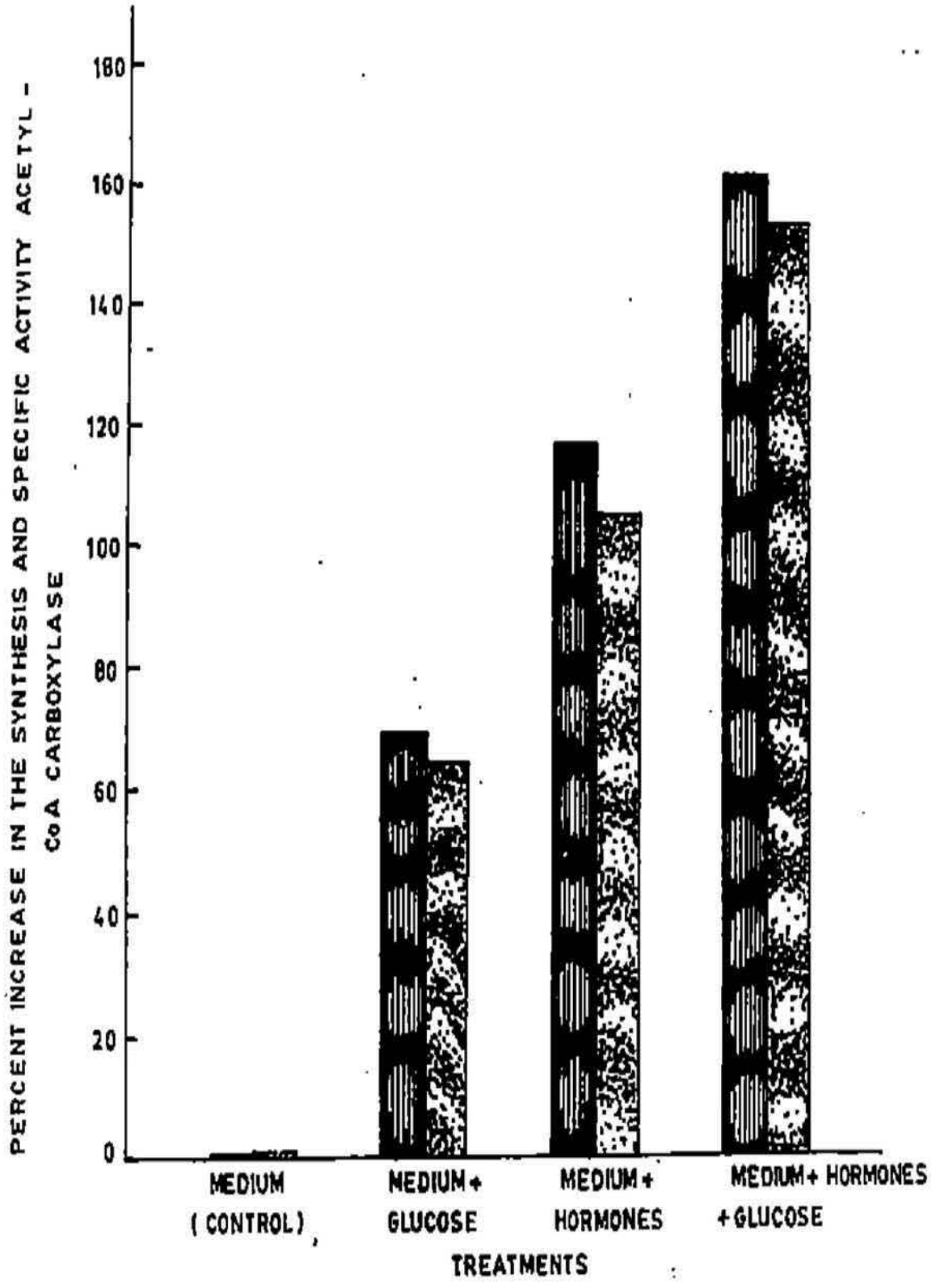
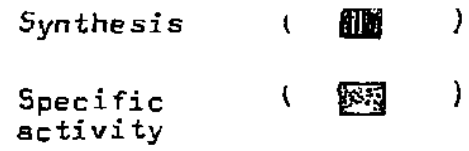

PERCENT INCREASE IN THE SYNTHESIS AND SPECIFIC ACTIVITY ACETYL - CoA CARBOXYLASE
180
160
140
120
100
80
60
40
20
0
MEDIUM (CONTROL)
MEDIUM + GLUCOSE
MEDIUM + HORMONES
MEDIUM + HORMONES + GLUCOSE
TREATMENTS

Plate 16. Effect of various combinations of hormones and glucose on the synthesis and specific activity of fatty acid synthetase.



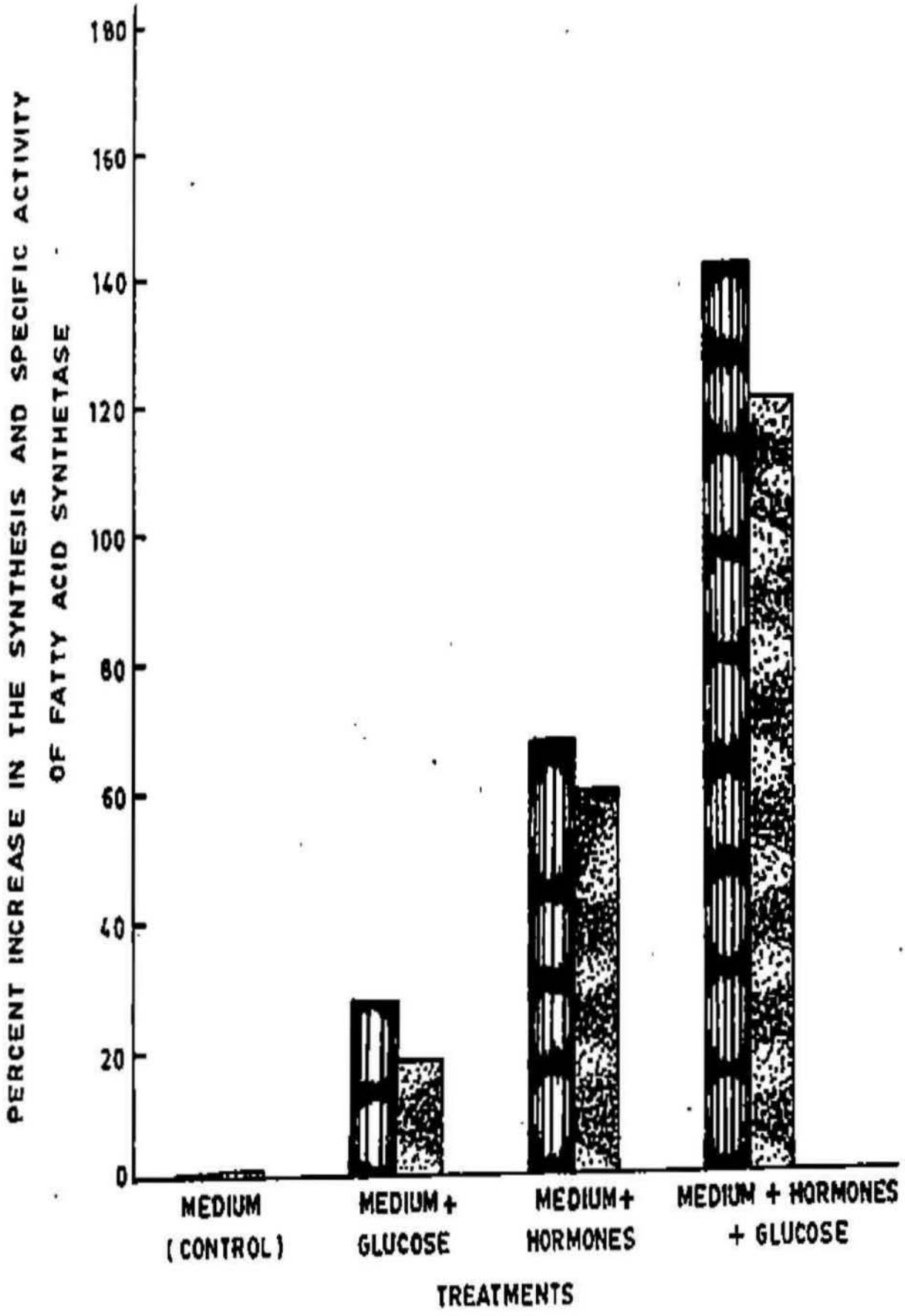
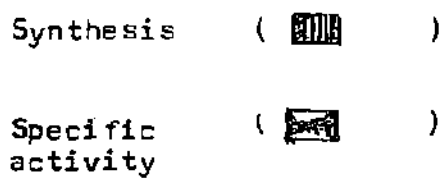
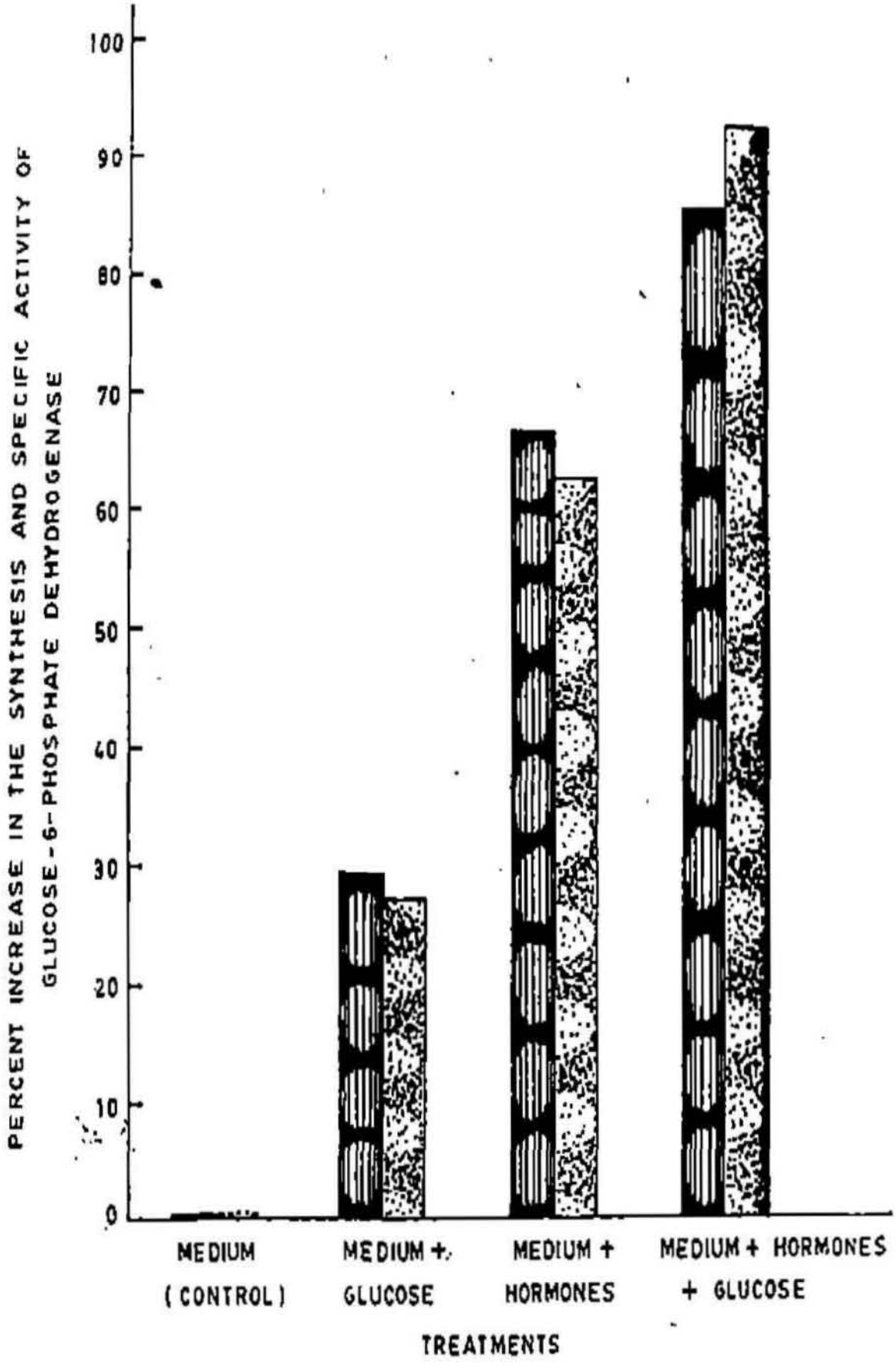

PERCENT INCREASE IN THE SYNTHESIS AND SPECIFIC ACTIVITY OF FATTY ACID SYNTHETASE
180
160
140
120
100
80
60
40
20
0
MEDIUM (CONTROL)
MEDIUM + GLUCOSE
MEDIUM + HORMONES
MEDIUM + HORMONES + GLUCOSE
TREATMENTS

Plate 17. Effect of various combinations of hormones and glucose on the synthesis and specific activity of glucose-6-phosphate dehydrogenase.




PERCENT INCREASE IN THE SYNTHESIS AND SPECIFIC ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE
100
90
80
70
60
50
40
30
20
10
0
MEDIUM (CONTROL)
MEDIUM + GLUCOSE
MEDIUM + HORMONES
MEDIUM + HORMONES + GLUCOSE
TREATMENTS

fatty acid synthetase and glucose-6-phosphate dehydrogenase) shown in Table 11, with the relative percent increase in the specific activities of the respective enzymes (Table 12), it was found that the apparently observed increase in the specific activities of these enzymes were due to the increases in their synthesis as shown in Plates 15, 16 and 17.

CHAPTER - 5

D I S C U S S I O N

DISCUSSION

The present study was undertaken to elucidate the effect of combination of hormones (insulin, prolactin and cortisol) on the lipogenic capacity of mid-pregnant goat mammary gland using explant culture technique. The above hormones, in combination, were used to observe their effect on various enzymes closely related with fatty acid synthesis viz. acetyl-CoA synthetase, acetyl-CoA carboxylase fatty acid synthetase, and the enzymes which govern the chain-length of the fatty acids so synthesized by fatty acid synthetase (medium-chain acylthioesterase and long-chain acyl thioesterase). The effect of these hormones was also seen on various reducing equivalents (NADPH) generating enzymes viz. glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-isocitrate dehydrogenase. These enzymes are considered as important lipogenic enzymes since they generate reducing equivalents in the form of NADPH, which are essentially required for the effective fatty acid biosynthesis. The effect of glucose alone and in addition with the combination of hormones (insulin, prolactin and cortisol) was also seen on above mentioned enzymes. The experiments were further extended to identify the mechanism of the regulation of the activities of the above mentioned lipogenic enzymes i.e. whether the changes in the activities of the enzymes under the influence of the above treatments were due to the activation of the pre-existing enzymes or due to

the changes in the synthesis of the enzymes in the explants.

5.1 Effect of combination of hormones and glucose on the lipogenic activity of mid-pregnant goat mammary gland

During the present study, the effect of combination of hormones (insulin, prolactin and cortisol), glucose and glucose in addition with the combination of hormones was seen on the following enzymes.

5.1.1 Acetyl-CoA synthetase:

The enzyme, responsible for the formation of extra-mitochondrial acetyl-CoA, exists in cytosolic fraction. This acetyl-CoA so formed functions as a basic precursor for the fatty acid synthesis. A perusal of the literature showed that almost no significant studies on this enzyme in the mammary gland of ruminants have been made. During the present studies, it has been observed that the specific activity of acetyl-CoA synthetase was markedly increased during the explant culture of the tissue from mid-pregnant goat mammary gland, when hormones (insulin, prolactin and cortisol), glucose and glucose in combination of hormones were added to the culture medium, as shown in Table 1 and Fig.1. The steady increase in specific activity of acetyl-CoA synthetase at the rate of 2- to 4-fold was observed upto 36 hours of explant culture. Merineze (1971) had shown the sensitivity of this enzyme to the hormones involved in mammogenesis and lactogenesis. He found that the specific activity of acetyl-CoA synthetase increased in bovine mammary gland as the animal passed to lactation stage from non-lactation. This increase in the specific

activity of acetyl-CoA synthetase as reported by Mereneze (1971) may be attributed to the influence of lactogenic hormone complex (insulin, prolactin and cortisol), which governs the overall rate of lipogenesis or the rate of fatty acid synthesis in the mammary gland. Acetyl-CoA synthetase, being one of the lipogenic enzymes, may be under the influence of these hormones for maximal rate of lipogenesis. Mallenberger and Bauman (1974) had observed major increase in the specific activity of acetyl-CoA synthetase alongwith the other lipogenic enzymes, between mid-pregnancy and lactation, while conducting tissue incubation studies on rabbit mammary gland. These results obviously reflect the interaction of the lactogenic hormone complex (insulin, prolactin and cortisol), which exhibits its action after mid-pregnancy only, with the lipogenic capacity of the mammary gland. This phenomena substantiates the present findings. The increase in the specific activity of acetyl-CoA synthetase, when explants from mid-pregnant goat mammary gland were cultured in the medium containing glucose only, may be attributed to the increased availability of the certain metabolites of glucose oxidation, to be responsible for the activation of acetyl-CoA synthetase activity. The similar reports were put forward by Volpe and Vagelos (1974). They suggested the increase in the specific activity of fatty acid synthetase due to the increase in the concentration of certain intermediates of glycolytic pathway. It is well known that ATP is required as a cofactor in the

reaction catalyzed by acetyl-CoA synthetase. Thus, the addition of glucose to the culture media during explant culture may be leading to the enhanced production of ATP which in turn results in the increase in the specific activity of acetyl-CoA synthetase.

The combined effect of combination of hormones and glucose resulted in a maximal increase (2- to 4-fold) in the specific activity of acetyl-CoA synthetase in present studies, upto 36 hours of explant culture. It may be due to the potentiating effect of the combination of hormones on the glucose mediated increase in the specific activity of acetyl-CoA synthetase as shown by different workers with regard to different lipogenic enzymes, namely acetyl-CoA carboxylase (Mayer, 1978), fatty acid synthetase (Speake et al., 1975, 1976a,b), glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Betts and Mayer, 1977 and Rivera and Cummins, 1971).

5.1.2 Acetyl-CoA carboxylase:

Acetyl-CoA carboxylase catalyzes the carboxylation of acetyl-CoA to malonyl-CoA in the biosynthesis of fatty acids. It is recognized as a rate limiting enzyme in the process of overall synthesis of fatty acids. It has been observed that the specific activity of acetyl-CoA carboxylase was increased substantially, when explants from mid-pregnant goat mammary tissue were cultured in the medium containing combination of hormones (Table 2 and fig. 2). It has long been recognized that the specific activity of acetyl-CoA carboxylase is influenced by

hormones (Mellenberger and Bauman, 1974; Witter et al., 1979; Denton, 1974; Goodridge, 1973, 1975; Volpe and Vagelos, 1976 and Lemmen, 1983). Manning et al. (1976a) have also reported that the specific activity of acetyl-CoA carboxylase was increased, when the explants from mid-pregnant rabbit mammary gland were cultured in the presence of combination of hormones (insulin, prolactin and cortisol) and no increase in the specific activity was observed by them in the absence of this combination of hormones. Thus the results in terms of increase in the specific activity of acetyl-CoA carboxylase, in the explants from mid-pregnant goat mammary gland, are in accordance with the results reported by Manning et al. (1976a). Manning et al. (1976a) had observed the increase in the specific activity of acetyl-CoA carboxylase in the explants of mid-pregnant rabbit mammary gland upto 60 hrs of incubation. On the contrary, the increase in the specific activity of acetyl-CoA carboxylase upto 36 hrs of explant culture has been observed in the present study.

It has been found that the specific activity of acetyl-CoA carboxylase was increased to lesser extent when glucose alone was added to the culture medium (Table 2 and Fig.2) than that when combination of hormones was used in the culture medium. The increase in the specific activity of acetyl-CoA carboxylase on addition of glucose to the culture media, can be substantiated on the basis of the reports put forward by Goodridge (1975), who had reported that the total activity of acetyl-CoA carboxylase as well

as fatty acid synthetase were stimulated in isolated liver cells from neonatal chicks by glucose at 6 hours of incubation. He had reported that fatty acid synthesis in these cells was markedly stimulated by fructose and other components that could enter glycolytic pathways. These components caused an increase in the cellular concentration of citrate which in turn activated acetyl-CoA carboxylase. These experiments demonstrated that citrate could regulate the activity of acetyl-CoA carboxylase in intact cells. Thus, these reports substantiate the present findings that the increase in the specific activity of acetyl-CoA carboxylase in the explants from mid-pregnant goat mammary tissue, when glucose was added to the medium may be due to an increase in the intracellular concentration of citrate, which in turn stimulates the over all activity of acetyl-CoA carboxylase. During the present studies it has also been observed that the combined action of combination of hormones and glucose have resulted in maximal (2- to 3-fold) increase in the specific activity of acetyl-CoA carboxylase when explant culture was carried out upto 24 and 36 hours, respectively. This increase could be explained on the basis of the fact that the combination of hormones had a potentiating effect on the glucose triggered increase in the specific activity of acetyl-CoA carboxylase.

5.1.3 Fatty acid synthetase

When insulin, prolactin and cortisol were used together in combination during the culturing of the explants from mid-pregnant goat mammary gland it resulted in

significant increase in the specific activity of fatty acid synthetase after 12, 24 and 36 hours of incubation, respectively (Table 3, fig.3).

Speake et al (1975, 1976a,b) have reported that when explants of mammary gland from mid-pregnant rabbits were cultured with insulin, prolactin and cortisol, cytodifferentiation occured, which was measured by the increased synthesis of medium-chain fatty acids, the characteristics of the rabbit milk. This was accompanied by an increase in the apparent specific activity of fatty acid synthetase and also an increase in the apparent rate of synthesis of fatty acid synthetase in the explants. Similar results have been reported by Forsyth et al. (1972), who had reported that on culturing the mammary explants from the rabbit (23 days pregnancy) for 2 days with insulin, prolactin and cortisol, the rate of fatty acid synthesis was increased to 5-fold. However, no such information is available on ruminant mammary explants. The extent of the increase in the specific activity of fatty acid synthetase under the influence of insulin, prolactin and cortisol observed in present studies were slightly lesser than that reported by Speake et al. (1975, 1976a,b).

Dils et al. (1974) had also reported that there was a marked increase in the specific activity of fatty acid synthetase after 40 hr in culture of the explants from mid-pregnant rabbit mammary gland when insulin, prolactin and cortisol were added to the medium. After having maximum stimulation of over all lipogenesis, the explants

were transferred to hormone free medium. This resulted in a dramatic decline in the specific activity of fatty acid synthetase. Thus, these results are in agreement with the previous findings which explain that insulin, prolactin and cortisol used in combination, have got a stimulatory effect on the specific activity of fatty acid synthetase.

A steady increase in the specific activity of fatty acid synthetase has been observed up to 36 hrs of explant culture, which is equivocal to the increase in the specific activity of fatty acid synthetase observed upto 40 hrs of incubation as reported by Dils et al. (1974).

In present studies an increase in the specific activity of fatty acid synthetase has also been observed when explants were cultured in the presence of glucose alone (Table 3 and Fig. 3). This may be due to the concentration of certain intermediates of glycolytic pathway or beyond, which affect the specific activity of fatty acid synthetase by causing an increase in the synthesis of fatty acid synthetase as reported by Volpe and Vagelos (1974).

It was further observed that combination of hormones and glucose resulted in a maximal increase (approximately 2.25-fold) in specific activity of fatty acid synthetase which could be attributed to the combined action of glucose and hormones in potentiating each others effect as reported in the literature for other lipogenic enzymes like glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Leader and Barry, 1969;

Rivera and Cummins, 1971; Betts and Mayer, 1977).

5.1.4 Medium-chain acylthioesterase:

No activity of medium-chain acylthioesterase could be detected in the particle free supernatant from the explants from mid-pregnant goat mammary tissue, cultured in the media containing combination of hormones, glucose and glucose plus combination of hormones (Table 4).

However, it has been established that the milk fat from the goat contains short, medium and long-chain fatty acids (Marei et al., 1969). Grunnet and Knudsen (1979a) have also reported that the tissue slices from lactating goat mammary gland synthesized short ($C_{4:0}$ to $C_{6:0}$), medium ($C_{8:0}$ to $C_{10:0}$) and long-chain ($C_{16:0}$ to $C_{18:0}$) fatty acids in proportions similar to that found in goat milk fat. But in contrast, the particle free supernatant fraction and the purified fatty acid synthetase from this tissue, synthesized predominantly short-chain and long-chain fatty acids. The enzyme terminating acyl-thioesters of low molecular weight (medium-chain acyl-thioesterase) could not be detected in the particle free supernatant, like the one found in rabbit and rat mammary gland (Knudsen et al., 1975; Libertini and Smith, 1978; Smith and Stern, 1981). Thus the present observations reflecting the absence of the medium-chain acyl-thioesterase in the particle free supernatant obtained from the explants from the mid-pregnant goat mammary tissue, are in accordance with the reports put forward by Grunnet and Knudsen (1979 a,b).

5.1.5 Long-chain acyl thioesterase:

The present study has shown that the specific activity of long-chain acyl thioesterase in the particle free supernatant, responsible for the synthesis of long-chain fatty acids ($C_{16:0}$ to $C_{18:0}$), increased considerably in the explants from mid-pregnant goat mammary tissue, cultured in the presence of combination of hormones, glucose and glucose plus combination of hormones (Table 5 and Fig. 4). However, this increase in the specific activity was found maximum upto 24 hours of culture of the explants under all the three treatments.

However, very scanty, or rather, negligible information is available on the stimulatory effect of this combination of hormones and glucose on the specific activity of long-chain acyl thioesterase employing explant culture techniques. But it has been established that the long-chain fatty acids constitute a significant portion of the fatty acids so synthesized in the goat mammary gland, as found in the goat milk fat (Marai et al., 1969). Skarda et al. (1978, 1982a) have reported that the explants from mid-pregnant goat mammary tissue respond well and a maximum stimulation of the over all lipogenesis is achieved when the explants from the mid-pregnant goat mammary tissue are exposed to insulin, prolactin and cortisol together in the culture medium.

Hallowes et al. (1973) have demonstrated that insulin, prolactin and cortisol are necessary for the maximal fatty acid synthesis in mammary explants from

mid-pregnant rat. The above informations reflect that insulin, prolactin and cortisol are needed for the enhanced capacity of over all lipogenesis in explants from mid-pregnant animals and the increase in the specific activity of long-chain acyl thioesterase observed in present study, contributes a part of it to the over all increase in lipogenesis. This is further substantiated by the present observations with other lipogenic enzymes.

5.1.6 Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are the enzymes involved in the generation of NADPH for the effective synthesis of fatty acids. Mammary explants from the mid-pregnant goat mammary gland when cultured in the presence of a combination of hormones (insulin, prolactin and cortisol), glucose and glucose with combination of hormones, led to the increase in the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Table 6,7 and Fig. 5,6). The increase in the specific activities of both the enzymes was studied upto 36 hours of explant culture under the effect of all the above mentioned three treatments. The significant increase in the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in present studies reflects the enhanced lipogenic activity of the mammary explants from mid-pregnant goat.

Studies conducted by Betts and Mayer (1977) on

6-phosphogluconate dehydrogenase in explants from mid-pregnant rabbit mammary gland and by Leader and Barry (1969) and Oka and Perry (1974) on glucose-6-phosphate dehydrogenase in organ culture of mammary tissue from mice mammary epithelial cells, respectively revealed that the increases in the specific activities were markedly influenced when explants or cells were cultured in the presence of insulin, prolactin and cortisol together. This observation is in agreement with the present findings.

The present results are further supported by the findings of Rivera and Cummins (1971) who showed that the maximal increase in the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were obtained when mammary explants from mid-pregnant mice were cultured in the presence of insulin, prolactin and cortisol together in the culture medium. The necessity of this combination of hormones is justified by by the fact that insulin itself is a potent stimulus, whereas both hydrocortisol and prolactin require insulin for manifestation of their effects. According to Oka and Perry (1974) the mouse mammary epithelial cells on culturing in the presence of hydrocortisone and insulin, increased the specific activity of glucose-6-phosphate dehydrogenase by about 250% of the control. The effect of hydrocortisone was also manifested in the presence of insulin and prolactin and resulted in a maximal increase in the enzyme activity. In the present experiments the specific activities of glucose-6-phosphate dehydrogenase

and 6-phosphogluconate dehydrogenase were increased to 91.25 and 105.80 from 54.25 and 60.57, respectively. In other words the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were increased to 160% and 175% of the control, respectively, after 36 hours of incubation, insulin, prolactin and cortisol were added together to the culture medium. Though this increase in the activity of glucose-6-phosphate dehydrogenase observed in the present studies is comparatively lower, yet it is in agreement with the results reported by Oka and Perry (1974), because of the action of the similar combination of hormones.

Rivera and Cummins (1971) showed that continuous maximal increase in the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were obtained when mammary explants from mid-pregnant mice were cultured with insulin, prolactin and cortisol. Hormones were required in the medium for several hours in order to produce the maximal increases in the enzyme activities which were measured after 48 hours in culture. After this time the continued presence of hormones was not required to maintain enzyme activity over the following 24 hours. The present studies showed that the sustained increase in the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was seen upto 36 hours of explant culture which appears to be equivocal to the previous studies.

It has been observed in present studies that the glucose alone and glucose plus the combination of hormones (insulin, prolactin and cortisol) markedly increased the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. However, the maximum increase in the activities was observed when glucose and combination of hormones were added together to the culture media. The activities of both the enzymes under these conditions were found increasing upto 36 hours in culture. Similar reports have been put forward by Green et al (1971) who have studied the hormonal regulation of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in mammary explants from mid-pregnant mice. They showed that insulin, glucose, amino acid and inorganic salts were the minimal requirements needed to increase the enzyme activities in the explants from mice. Replacement of insulin in the incubation medium by cysteine resulted in the increased uptake of glucose into the alveolar cells. Glucose could be replaced by other monosaccharides like mannose and fructose (Green et al., 1971) to get the similar results. With higher glucose concentration in the culture medium, insulin did not potentiate the glucose-mediated increase in the enzyme activity (Green et al., 1971). Leader and Barry (1969) showed that glucose was necessary, initially, in order to trigger the increase in enzyme activity which occured after 12 hours in culture. Green et al. (1971) suggested that increase in the activities of glucose-6-phosphate dehydrogenase and

6-phosphogluconate dehydrogenase resulted from an increased uptake of glucose by the mammary tissue and that the increase in the activities were caused by a metabolic product of glucose, such as glucose-6-phosphate that stimulated the formation of mRNA during initial hours after glucose was added to the culture medium. Betts and Mayer (1977) showed the obligatory requirement of the glucose to produce an increase in the enzyme activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and the potentiating effect of insulin, prolactin and cortisol on the glucose triggered increases in enzyme activities as well as amounts.

Therefore, the present findings are in accordance with the previous results which indicated that glucose as such and in combination with hormones (insulin, prolactin and cortisol) leads to the enhanced specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

5.1.7 NADP-isocitrate dehydrogenase

These studies indicate that the specific activity of NADP-isocitrate dehydrogenase increased markedly under the influence of combination of hormones (insulin, prolactin and cortisol), glucose and glucose in addition with combination of hormones (Table 8 and Fig. 7). It has been found that the specific activity of NADP-isocitrate dehydrogenase increased upto 24 hours of the incubation of the explant from the mid-pregnant goat mammary tissue.

This increase in the specific activity of NADP-isocitrate dehydrogenase in all the three treatments, was at the rate of 150% to 180% of the control upto 24 hours of incubation which was 8-10 times more than that reported in the rat mammary gland by Bauman et al. (1970).

The higher specific activity of NADP-isocitrate dehydrogenase obtained in present studies may be explained in the light of the fact that the possible alternate source of NADPH is the NADP-isocitrate dehydrogenase in the ruminants. The role of the NADP-isocitrate dehydrogenase pathway in producing NADPH in ruminant fatty acid synthesis, is consistant with the availability of citrate in the cytosol of the ruminant mammary tissue, due to the absence of citrate clevage pathway and the ability of ruminant mammary tissue to synthesize fatty acids in the absence of glucose. The high specific activity of NADP-isocitrate dehydrogenase coupled with high levels of citrate or isocitrate (Baldwin and Yang, 1974) in the ruminant mammary tissue may presumably reflect its role in NADPH formation for fatty acid synthesis. Whereas in non-ruminants NADP-isocitrate dehydrogenase does not appear to be of major importance in generation of reducing equivalents because of its low activity (Leveille and Hanson, 1966; Wise and Ball, 1964).

The adaptation of NADP-isocitrate dehydrogenase to the initiation of lactation has been studied in rats, rabbits and guinea pigs and shown to increase 3- to 8-folds in activity from pregnancy to mid-lactation in the

attributed to the increased availability of citrate or isocitrate in the cytosol of the mammary tissue due to the absence of citrate cleavage pathway in goat mammary gland. This presumably reflects the high generation of reducing equivalents by NADP-isocitrate dehydrogenase as reported by Baldwin and Yang (1974). The maximal increase in the specific activity of NADP-isocitrate dehydrogenase was observed in present studies when glucose and combination of hormones were added together to the culture medium. This may be attributed to the fact that combination of hormones (insulin, prolactin and cortisol) may be having a potentiating effects on the action of glucose on the activity of NADP-isocitrate dehydrogenase, as explained in the case of other dehydrogenases like glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Leader and Barry, 1969; Rivera and Cummins, 1971; Betts and Mayer, 1977).

5.2 Purification of some of the lipogenic enzymes:

5.2.1 Acetyl-CoA carboxylase:

Acetyl-CoA carboxylase has been purified from different species. However, fewer information is available on the purification of acetyl-CoA carboxylase from goat mammary gland. During present studies the purification of acetyl-CoA carboxylase was carried out by the method of Miller and Levy (1975). A 45.5-fold purification and a 19.2% yield of acetyl-CoA carboxylase have been obtained (Table 9), which are comparatively lower than that obtained by Miller and Levy (1975). They

obtained 65- to 67- fold purification and a 24% yield of acetyl-CoA carboxylase. Some enzyme protein as well as enzyme activity might have been lost during purification steps in present study. The specific activity of the purified enzyme has been found to be 0.818, which is in agreement with that reported by Miller and Levy (1975). The differences, so observed in present studies may be attributed to the species difference. Polyacrylamide gel electrophoresis of the purified enzyme was carried out according to the method of Betts and Mayer (1975) with some modifications. A single protein band (Plate 3) was obtained on polyacrylamide gel, essentially in accordance with the results reported by Ahmad et al. (1978) for this enzyme from rat mammary gland.

5.2.2 Fatty acid synthetase:

The purification of fatty acid synthetase was carried out by the method of Dils and Carey (1975) with some modifications. A 22-fold purification and 12% yield of fatty acid synthetase was obtained (Table 10) which compared favourably with the 18-fold purification and a 16% yield of fatty acid synthetase obtained by Dils and Carey (1975). Elution of this purified enzyme through Sephadex G-200 column has given a single protein peak (Fig.10) which is in agreement with the results reported by Dils and Carey (1975).

No adequate data was available on the purification of fatty acid synthetase from goat mammary gland, however, reports are available on the purification of fatty acid

synthetase from cow mammary gland (Knudsen, 1972; Kinsella et al., 1975). Kinsella et al. (1975) purified fatty acid synthetase from bovine mammary gland and obtained a 20-fold purification and a 9% yield of the fatty acid synthetase similar to the present findings.

Polyacrylamide gel electrophoresis of the purified enzyme was carried out according to the method suggested by Dils and Carey (1975). The appearance of only single protein band on 7% polyacrylamide gel (Plate-4) during present studies is in accordance with the polyacrylamide gel electrophoresis pattern of the purified fatty acid synthetase as reported by Dils and Carey (1975).

5.3 Preparation of antibodies and assessment of their specificity:

Antibodies were raised against the purified enzymes mentioned in the Section 5.2. Similarly, antibodies were also prepared against glucose-6-phosphate dehydrogenase which was obtained purified as such.

5.3.1 Preparation of the antibodies against acetyl-CoA carboxylase and their assessment

Antibodies against purified acetyl-CoA carboxylase were prepared according to the method of Buckner and Kolattukudy (1976). The immunodiffusion studies were performed using the method of Ouchterlony (1966), double immunodiffusion technique with the purified acetyl-CoA carboxylase from goat mammary gland and the antisera obtained from rabbits. A single precipitin line was obtained on the agarose gel (Plate 5) which compared

fairly with the single precipitin line obtained by Ahmad et al. (1978). They also reported the formation of single precipitin line when purified acetyl-CoA carboxylase from rat mammary gland was allowed to diffuse with the antibodies developed against it in rabbits.

5.3.2 Preparation of antibodies against fatty acid synthetase and their assessment

Antibodies against purified fatty acid synthetase were developed in rabbit according to the method of Buckner and Kolattukudy (1976). When immunodiffusion assays were performed with the purified fatty acid synthetase from goat mammary gland and the antisera obtained from the rabbits, using the method of Ouchterlony (1966) double immunodiffusion technique. A single precipitin line (Plate 6) was obtained, which was similar to the single precipitin line obtained by Buckner and Kolattukudy (1976). They further, obtained one precipitin line on performing the immunodiffusion assay with purified fatty acid synthetase from goose uropygial glands and the antisera, obtained from the rabbits. The present results are further substantiated by the results reported by the different workers who have purified the fatty acid synthetase from different sources like liver cells of developing chick (Fischer and Goodridge, 1978), rat mammary gland (Smith, 1973) and rat liver and brain (Volpe et al., 1973 and Gharbi-Chihi et al., 1983) and have reported the formation of a single precipitin line

on immunodiffusion of the purified fatty acid synthetase from different sources with the corresponding antibodies developed in rabbits.

5.3.3 Preparation of antibodies against glucose-6-phosphate dehydrogenase and their assessment.

Antibodies against glucose-6-phosphate dehydrogenase were developed in rabbits according to the method of Betts and Mayer (1977). On performing immunodiffusion assay with the purified glucose-6-phosphate dehydrogenase and the antisera obtained from rabbit, by the method of Ouchterlony (1966) double immunodiffusion technique a single precipitin line was obtained (Plate 7). This is in agreement with the results reported by Betts and Mayer (1977) for 6-phosphogluconate dehydrogenase.

5.4 Effect of combination of hormones, glucose and glucose in addition with combination of hormones on the synthesis of some of the lipogenic enzymes

Studies were carried out to elucidate the fact whether the increasesin the specific activities of the lipogenic enzymes (as observed in present study) were due to the activation of the pre-existing less active enzyme molecules or due to increases in the concentration of the enzymes in the tissue explants, cultured under the effect of different treatments as mentioned above. To investigate the latter part of the above mentioned problem, quantitative estimation of the lipogenic enzymes was done using immunological technique. It was observed that when combination of hormones, glucose and glucose in

addition with combination of hormones were added seperately to the culture media, while culturing the explants from mid-pregnant goat mammary gland for 24 hours, there was an increase in the synthesis or amounts of the lipogenic enzymes under study (acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase). The maximum increase in the synthesis was observed when glucose and combination of hormones were used together. It was increased to 216%, 167% and 166% of the control in the case of acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase, respectively, when hormones combination was added to the culture medium. Whereas, when glucose and hormones combination were put together to the culture medium, the values increased to 260%, 241% and 185% of the control in the case of acetyl-CoA carboxylase fatty acid synthetase and glucose-6-phosphate dehydrogenase, respectively. However, the increase in synthesis of these lipogenic enzymes was comparatively less when glucose alone was added to the culture medium. The results for increase in the synthesis of these enzymes were found parallel with the increases in the specific activities of these enzymes under study (Tables 11, 12 and Plates 15, 16 and 17).

Mayer (1978) reported that the increase in the activity of acetyl-CoA carboxylase in the explant culture tissue was due to the increase in the synthesis of the enzyme. After conducting immunological titrations with antibodies against fatty acid synthetase (Dils et al.,1974)

it was found that the changes in the specific activity of fatty acid synthetase, when mammary explants from 16 day pregnant rabbits were cultured with insulin, prolactin and cortisol, were due to the changes in the amount of the fatty acid synthetase in the explants rather than by changes in the activity of the constant amount of the pre-existing enzyme. Similar reports regarding fatty acid synthetase have been given by Speake et al. (1975, 1976a,b), who have used antibodies raised against fatty acid synthetase to measure the rate of synthesis or amount of the fatty acid synthetase in the explant cultured tissue. They have reported that the changes which occured in the activity of fatty acid synthetase in the mammary explants from mid-pregnant rabbit tissue, were due to the increases in the amount of the enzyme. Betts and Mayer (1977) have also reported that changes in the enzyme activity of 6-phospho-gluconate dehydrogenase in the explants of mammary gland from mid-pregnant rabbits cultured in the presence of insulin, prolactin and cortisol, were found to be due to corresponding changes in the synthesis of the enzyme (6-phosphogluconate dehydrogenase). Similar results have been presumed regarding glucose-6-phosphate dehydrogenase by Leader and Barry (1969).

The increase in the synthesis of the enzymes, when glucose alone was added to the culture medium, may be attributed to the increased uptake of glucose by the mammary explants. The increases in the synthesis of the lipogenic enzymes are caused by a metabolic product of

glucose that stimulates the formation of mRNAs required for the increases in the synthesis and in turn increases in the activities of the respective enzymes as suggested by Leader and Barry (1969) and subsequently by other research workers (Rivera and Cummins, 1971 and Betts and Mayer, 1977). Their reports are confined to lipogenic enzymes like glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

The combined effect of glucose and combination of hormones leading to maximal increase in the synthesis of acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase, as observed in present studies, may be substantiated by the fact that the hormones (insulin, prolactin and cortisol) do show the obligatory requirement for the increase in the enzyme synthesis and the potentiating effect of glucose on the hormones triggered increase in the synthesis of the respective enzymes under study.

On perusal of the present findings it can be put forward that the increases in the specific activities of the lipogenic enzymes in the explants from the mid-pregnant goat mammary tissue under the influence of hormones, glucose and glucose in combination of hormones (as observed in present studies) were due to the increases in the synthesis of the enzymes.

CHAPTER – 6

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSION

The effect of combination of hormones (insulin, prolactin and cortisol, each at the rate of 5 µg/ml of media), glucose and combination of hormones in addition to glucose was seen on the activities of enzymes associated with fatty acid synthesis viz. acetyl-CoA synthetase, acetyl-CoA carboxylase, fatty acid synthetase, medium-chain acyl-thioesterase and long-chain acyl thioesterase in mammary explants from mid-pregnant goat. The explant culture was carried out for 12, 24 and 36 hours. The specific activities of acetyl-CoA synthetase, acetyl-CoA carboxylase and fatty acid synthetase were found increasing steadily upto 36 hours of incubation. Whereas the increase in the specific activity of long-chain acyl-thioesterase was observed only upto 24 hours of incubation under the effect of all the three treatments mentioned above. The presence of combination of hormones (insulin, prolactin and cortisol) in the culture medium, resulted in significant increase in the specific activities of acetyl-CoA-synthetase (from 39.04, 44.28 and 45.09 to 77.06, 96.38 and 115.69), acetyl-CoA carboxylase (from 2.06, 2.58 and 2.68 to 4.25, 5.26 and 5.97) and fatty acid synthetase (from 31.83, 36.10 and 37.92 to 50.31, 57.51 and 71.02), after 12, 24 and 36 hours of incubation, respectively. When glucose alone was added to the culture medium, it led to the increase in the specific activities of acetyl-CoA synthetase (60.66, 73.46 and 90.45), acetyl-CoA carboxylase

(3.90, 4.22 and 5.02) and fatty acid synthetase (37.29, 42.40 and 54.44), respectively, after 12, 24 and 36 hours of incubation. The extent of increase in the activities of all the three enzymes under the effect of combination of hormones was comparatively higher than that observed when only glucose was added to the culture medium. The maximal increase (2-3 fold) in the activities of these enzymes was observed when combination of hormones and glucose were added together to the culture medium. The specific activity of long-chain acyl thioesterase was also increased due to the addition of combination of hormones to the culture medium from 4.51 to 6.21, 4.95 to 7.27 and 4.1 to 4.75, after 12, 24 and 36 hours of incubation, respectively. On addition of glucose alone to the culture medium, the specific activity of long-chain acyl thioesterase was increased to 5.36, 6.05 and 4.75 from control values. However, it was not possible to detect the activity of medium-chain acyl thioesterase under the influence of any of the three treatments.

Similarly the effect of these treatments was observed on reducing equivalents (NADPH) generating enzymes. When combination of hormones was added to the culture medium it led to the increase in the specific activities of glucose-6-phosphate dehydrogenase (from 51.81, 53.28 and 54.25 to 83.82, 86.75 and 91.25), 6-phosphogluconate dehydrogenase (from 54.57, 57.70 and 60.57 to 82.52, 92.98 and 105.80) and of NADP-isocitrate dehydrogenase (from 196.90, 214.50 and 209.50 to 327.37, 368.57 and 315.61).

The addition of glucose to the culture medium increased the specific activities of glucose-6-phosphate dehydrogenase (61.45, 68.25 and 74.31), 6-phosphogluconate dehydrogenase (68.31, 79.64 and 87.46) and NADP-isocitrate dehydrogenase (287.57, 337.27 and 295.14). The maximal increases in the specific activities of these reducing equivalents generating enzymes were observed when combination of hormones and glucose were added together to the culture medium. The specific activity of NADP-isocitrate dehydrogenase was observed increasing upto 24 hours of incubation only. Whereas, the specific activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increased linearly upto 36 hours of incubation under the influence of all the three treatments.

Acetyl-CoA carboxylase was purified from the goat mammary gland. The initial units of the acetyl-CoA carboxylase in the cytosol were 255.4 and the specific activity of acetyl-CoA carboxylase in the cytosol was 0.018. The ammonium sulphate precipitation of the cytosol, led to the increase in the specific activity of the enzyme to 0.045 with 2.5-fold purification. Further, overnight dialysis of the fraction increased the specific activity to 0.052. The enzyme was purified to 2.9-fold. The negative adsorption on DEAE-Cellulose, increased the specific activity to 0.090 (5-fold purification). The subsequent ammonium sulphate fractionation led to 23-fold purification of the enzyme with the specific activity of 0.409. The last step involving the Sepharose-2B column

chromatography resulted in further purification of the enzyme to 45.5 fold, with the specific activity of 0.818. The yield of the enzyme obtained at the end of whole procedure was 19.2%. This purified enzyme gave single band on polyacrylamide gel electrophoresis.

Fatty acid synthetase was also purified from cytosol obtained from goat mammary gland, which contained 25225.50 units of fatty acid synthetase with specific activity of 13.22. The first ammonium sulphate fractionation increased the specific activity to 47.48, leading to a 3.6 fold purification. Further treatment of this fraction with calcium phosphate gel, resulted in the purification of the enzyme to 4.06-fold, with the specific activity of 53.73. DEAE-Cellulose column chromatography led to the further increase in the specific activity of the enzyme to 197.76, which showed a 14.6-fold purification. The last step consisting of second ammonium sulphate fractionation yielded a purified enzyme with specific activity of 294.37, representing a 22.3 fold purification of the enzyme over cytosol. The total yield of the enzyme at the end of purification procedure was 11.95%. This purified enzyme on polyacrylamide gel electrophoresis gave one protein band, which confirmed the homogeneity of the enzyme.

Antibodies were raised against the purified enzymes (acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase) in rabbits and their immunospecificity was checked by double immunodiffusion technique. The formation of the single precipitin lines

on immunodiffusion of the antibodies with the respective antigens, reflected the immuno-specificity of the antibodies. Furthermore, the formation of single precipitin line in each case confirmed the homogeneity of the purified enzymes.

While estimating the quantities of the lipogenic enzymes, employing immunological techniques, it was observed that when combination of hormones was added to the explant culture medium the syntheses of acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase were increased to 216%, 167% and 166% of the control, respectively. When only glucose was added to the culture medium, the increase in the amounts of these enzymes was observed to be in the order of 169%, 127% and 129% of the control, respectively. Whereas, the combined effect of combination of hormones and glucose in culture medium resulted in maximal increase (260%, 241% and 185%) of the control.

On the basis of the above mentioned findings, it is concluded that the increase in the specific activities of acetyl-CoA carboxylase, fatty acid synthetase and glucose-6-phosphate dehydrogenase, under the influence of hormones and glucose, were mainly due to the increase in the synthesis or amounts of these enzymes, rather than activation of the pre-existing less active enzymes molecules. It appeared that the enzymes responsible for lipid metabolism are stimulated by the lactogenic hormones complex (insulin, prolactin and cortisol) from the mid-

pregnant stage onward. This invariably reflects that by altering the hormonal status of an animal, the lipid synthesizing capacity of mammary tissue can be regulated by hormonal induction. Furthermore, the pronounced effect on the lipid metabolism can be induced by the combined action of hormones and glucose, which potentiate the action of each other. This study can further be extended to investigate the role of these hormones on the lipogenic capacity of the mammary tissue at different physiological stages of an animal i.e. pregnancy and lactation as well.

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