

A
COMPARATIVE STUDY ON THE FREE AMINO ACID CONTENT
OF
SEMINAL PLASMA AND MOTILITY OF SPERMS
IN
THARPARKAR AND MURRAH BULLS

M. Sc. (A. H.) THESIS

**A. PRASAD
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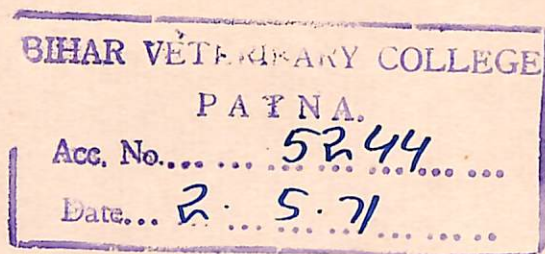
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THESIS.

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By

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1970

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P A T N A ,

Dated, the 23rd December, 1970.

Certified that the work described
in this thesis entitled "A COMPARATIVE STUDY
ON THE FREE AMINO ACID CONTENT OF SEMINAL
PLASMA AND MOTILITY OF SPERMS IN THARPARKAR
AND MURRAH BULLS" is bonafide work of Dr.
Akhileshwar Prasad carried out under my
guidance and supervision for the award of
M. Sc. (A.H.) degree.

 23.12.70.
(A. K. Ray)

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Akhil 23/12/70

(Akhileshwar Prasad)

Most Affectionately

Dedicated

To

My Revered Teacher

Dr. A.K.Ray,

Professor of Physiology,

Bihar Veterinary College,

PATNA.

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INTRODUCTION

"O Lord, may I have milk cows in thousands and tens of thousands."

Rajar Veda, Anuvak 212, Upanishad 4.

is: INTRODUCTION

Cattle is worshipped in India as one of the greatest of work animals and milk is of unique importance to the agricultural economy of the country and for the health and prosperity of the masses. Cattle is the main source of much needed animal protein in the diet of the predominantly vegetarian people of the Indian people.

CHAPTER - I.

INTRODUCTION.

Cattle being the main source of power for agricultural operations and responsible for the maintenance of fertility in the soil constitutes the backbone of Indian agriculture. About three-fourth of the population depends directly or indirectly on agriculture for their livelihood.

The Indian economy is very much dependent upon the livestock and its products. Besides cattle, buffaloes also play an important role in the development of agriculture of the country. Although cattle and buffalo are also important in the world being population (175.0 million cattle and 51.4 million buffaloes, as per 1961 census). The average milk production of a cow is 360 lbs. and that of buffalo 1075 lbs. per year as

INTRODUCTION

"O Agni, may I have milking cows in thousands and tens of thousands".

Yajur Veda, Anuvaka 219, Hymns 6.

(a) GENERAL:

Why cow is worshiped in India ? As cow being the producer of work bullocks and milk is of unique importance to the agricultural economy of the country and for the health and prosperity of the masses. It provides the only source of much needed animal protein in the diet of the predominantly vegetarian section of the Indian people. So in the "Vedas" it is declared a sacred animal.

Cattle being the main source of power for agricultural operations and responsible for the maintenance of fertility in the soil constitute the back bone of Indian agriculture. About three-fourth of the population depends directly or indirectly on agricultural pursuits for their livelihood.

The Indian economy is very much dependant upon the livestock and its product. Besides cattle, buffaloes also play an important role in the development of agrarian economy of the country. Although cattle and buffalo of India constitute 23% of the world bovine population (175.0 million cattle and 51.0 million buffaloes, as per 1961 cattle census). The average milk production of a cow is 369 lbs. and that of buffalo 1077 lbs. per year as

compared to 4126 lbs. in U.S.A., 5576 lbs. in U.K. and 7005 lbs. in Denmark per year.

According to Mahadevan (1966) "although there are some 22 cows to every 100 persons in India, it is estimated that, if all the milk produced is consumed as liquid milk, cattle provide only about 5½ ounces of milk per head of human population per day. This compare with a per capita consumption of 30 to 40 ounces per day in Australia and New Zealand".

In spite of the large population of cattle in the country, the milk yield and work efficiency of our cattle are among the lowest in the world. As a result the diet of our people is exceedingly poor in animal proteins which are essential for maintenance of good health.

This is due to improper breeding and the lack of a balanced diet for the animals. The result is weak animals of low milk yield and work efficiency. Weak animals have also a low resistance to the attack of diseases. A smaller number of well cared for and healthy cattle will be more economical to keep, and will contribute in a much greater measures to the diet of the people and to agricultural efficiency. The measures to be taken towards this end are: controlled breeding, balanced feeding and prevention of diseases in cattle. The great hindrance in the rapid development of cattle and buffalo wealth was the paucity of quality sires. The proportion of superior bulls in the country has been estimated at one in 250. In animal breeding there is a popular saying "The sire is half the herd". It is true, the real improvement of the animal

products depends upon good sires who can transmit their inherited characters of dairy, draught and meat to their progeny who should perform better than their parents. Hence, in a herd, a sire is always valued more than the cow as she can produce hardly about half a dozen progeny in her life time, while a sire has the potentiality to produce progeny even in thousands especially with the use of the advanced technique of Artificial Insemination (A.I.). Thus, fortunately, A.I. has made that difficulty a thing of the past, so that the initial battle of paucity of bull can be said to have been won.

But success of A.I. is very much dependant on the ability in preserving the "in vitro" viability, and fertility of spermatozoa. A.I. is now practised throughout the world and is accepted as the most practicable zootechnique for improvement of livestock. All these cattle development and breeding programmes are mostly dependant on the availability of semen from good and proven sires. To have a maximum benefit of an outstanding sire the semen is extended by diluting with semen diluters for bringing conception in a large number of females. The diluted semen can be transported to a long distance, where it can be used. But the success of programme depends on the quality and fertilizing ability of semen at the time of insemination, even though it has been obtained from superior bull. To bring perfection of the diluting fluids a day to day improvement of the semen diluter is being carried out from the basic 'egg yolk-phosphate diluent' found by Phillips and Lardy (1939, 1940). From the purely practical point of view, which

matters greatly, the ability of spermatozoa to "survive" i. e. retain their remarkable properties under condition of long term storage "in vitro" is of great importance, specially in a country like India, where preserving facilities for semen under conditions are difficult and expensive. The advantages to be derived from the practice of Artificial Insemination, increase as the keeping qualities of semen are prolonged. The greater the length of time ^{that} semen can be stored and still retain its viability and power of fertilization, the greater the number of cows which may be inseminated. Of prime importance in this connection is the fact that semen could be transported greater distance for insemination purposes. For this, the keeping quality of semen is of great importance.

Role of Buffalo:

Buffaloes are more economical producers of milk in India as compared to the cow. Male buffaloes after castration can be used for heavy transport as well as ploughing avoiding hot part of the day. Buffaloes can thrive on such coarse fodder as may not be relished by cattle. They produce 59.9% of the total milk produced, although they constitute only 30% of the total milch animals in the country. The average fat percentage in buffalo's milk is 7.66 which is much higher as compared to cow's milk. As such, it is more frequently used for the manufacture of dairy products e.g. butter, ghee, cheese, khoa etc. as it yields a much greater return.

So attempts for the improvement of our native buffalo is the immense need of the hour. A great deal of genetic variability

exists in buffaloes and there is wide scope for the improvement in productive potentiality of these animals by modern breeding technique.

(b) OBJECTIVES OF INVESTIGATION:

Much is now known about the occurrence of various substances in semen. From the time semen is collected, till it is used for, many factors like temperature, humidity, rainfall, season of the year have an adverse effect on the same either directly or indirectly. The magnitude of these adverse effects have been investigated previously by many workers.

Biochemistry of semen is a relatively modern, but rapidly expanding, field of physiology; consequently, our attention gone towards particularly as regards the biological significances of various chemical constituents of semen - a major role in the process of fertilisation.

Besides complete chemical and biological examinations of semen have not been made, and the ultimate source of many of the individual components is not known.

According to Mann (1954) there is little information apart from some immunological studies, on chemical differences between sperm proteins of various species. An early attempt in this direction was made by Faure-Premiet (1913), who purified 'ascardine', a protein peculiar to the testicular tissue, and probably also to spermatozoa, of *Ascaris megalocephala*.

Mann further stated that there are indications that the

amino acids and proteoses present in the seminal plasma may be of some importance to the spermatozoa. It may be recalled that excessive dilution of semen exerts a deleterious effect on spermatozoa and that this can be counteracted, partly at least, by the inclusion in the diluting media of amino acids such as glycine, alanine, valine, leucine, lysine and glutamic acid.

Bhattacharya and Prabhu (1950, 1951, 1952 and 1953) recorded great variation in quality and quantity of semen production between cow bulls and buffalo bulls.

Larson and Salisbury (1954) also stated that although proteins are contained in the commonly used diluent, there is still a need for fuller information on the proteins normally present in the seminal plasma.

Keeping in view the above mentioned ideas the present study have been categorised as follows :-

- (i) To find the role of main free amino acids in seminal plasma of cow and buff bulls preserved semen upto 72 hours.
- (ii) To ascertain motility change in preserved neat semen from 0 to 72 hours at 24 hour interval.
- (iii) To observe crystallisation pattern of preserved neat semen from 0 to 72 hours at 24 hours interval.

The paper chromatographic technique was adopted for estimation of different amino acids.

*

LITERATURE REVIEW

THE LITERATURE REVIEW ON ANIMAL ACTION IN BIRDS

(1970)

The function of the nervous system in birds is to coordinate the activities of the various organs and tissues of the body. It is the central control system of the bird, and it is responsible for the bird's behavior. The nervous system is made up of the brain, the spinal cord, and the nerves. The brain is the center of the nervous system, and it is responsible for the bird's thoughts and feelings. The spinal cord is the main pathway for the transmission of information between the brain and the rest of the body. The nerves are the branches of the spinal cord that carry the information to the various organs and tissues of the body.

CHAPTER - II.

LITERATURE REVIEW.

THE LITERATURE REVIEW ON ANIMAL ACTION

The literature review on animal action is a vast field, and it is impossible to cover it all in a single chapter. However, it is possible to give a general overview of the field. The literature review on animal action is divided into two main parts: the study of the behavior of individual animals and the study of the behavior of groups of animals. The study of the behavior of individual animals is the older of the two, and it has a long history. The study of the behavior of groups of animals is a more recent development, and it has a shorter history. The literature review on animal action is a very important part of the study of animal behavior, and it is essential for the understanding of the behavior of individual animals and groups of animals.

(1970)

LITERATURE REVIEW

(A) LITERATURE REVIEW ON AMINO ACIDS IN SEMEN.

(a) INTRODUCTION:

The functions of all living systems are carried out by means of one of the important class of molecules known as, protein. Thousands of proteins occur in nature with multivariant functions assigned to them. A large number of them known as enzymes act as catalysts for biological reactions. Other may be structural proteins like muscle proteins; some others like haemoglobin, act as carriers of oxygen and so on. Chemically, protein molecules are also long chains composed of 20 or odd different amino acids. Hence the importance of amino acids is crystal clear and needs no explanation.

(b) ROLE OF SEMINAL PLASMA:

"Seminal plasma is of great biochemical interest". It is certain that the natural mating process could scarcely be expected to function smoothly and efficiently without the provision of seminal plasma as a normal diluent and vehicle for the thick mass of closely packed epididymal spermatozoa; no more could the blood corpuscles act as oxygen carriers in vivo, without the blood plasma. It is possible to induce pregnancy by the artificial insemination of epididymal spermatozoa in guineapig or rabbit. The seminal plasma also exerts a distinct stimulating effect on sperm motility (Mann, 1954; pp. 24).

Albright et al (1957) reported that concentrated

spermatozoal samples showing little or no motility after 2 - 4 hours incubation at 37°C could be stimulated by the addition of seminal plasma.

Werthessen et al (1957) observed that horse semen samples of apparently normal spermatozoa content are often infertile. It was found from study of some 1,300 individual matings, that the capacity of the seminal fluid to maintain motility of spermatozoa varied widely, and was inversely proportional to the non-protein sulphhydryl concentration; this capacity correlated well with actual foaling percentages.

Olbrychtowa and Walkowski (1959) noticed not unfavourable, but a beneficial effect on motility when bull spermatozoa were suspended in stallion seminal plasma.

The chemical and physical properties of semen (Mann, 1964; White, 1958; White and Macleod 1963) are largely determined by the seminal plasma which constitutes its bulk, particularly in the boar and stallion.

Sharma and Mahajan (1961) found the removal of seminal plasma to be harmful to the motility and livability of buffalo spermatozoa during storage and on the other hand addition of seminal plasma at low level (obtained from the same semen samples) on the preservation showed the motility and the livability of the spermatozoa improved significantly with the addition of increasing volumes of seminal plasma to the diluted semen.

In the year 1965, Davis observed in cimex Lectularius L. that seminal fluid is essential in insemination; without it

spermatozoa do not migrate.

(c) AMINO ACIDS IN SEMINAL PLASMA:

The separate existence of sperm and seminal plasma was fully appreciated as far as 1791 by Louis Nicolas Vauquelin, the author of the first treatise on the chemical composition of semen. Friedrich Miescher (1870, 1878, 1897) was the first to point out in Salmon, for instance, the high dry weight and protein content of semen was almost entirely due to spermatozoa and seminal plasma. He was the man whose fundamental studies provided the earlier information on the chemical nature of some of the sperm proteins. The seminal plasma contained not more than 0.78% of dry matter, for this 0.65% mineral and only 0.13% organic material. However a more recent analysis of salmo frontinalis has shown a content of 1.76% nitrogen and 0.43 percent phosphorus in the seminal plasma (Felix, Fischer, Krekels and Mohr, 1951). Sea-urchin (Arbacia punctulata) seminal plasma has about 0.25 percent protein (Hayashi, 1945).

Sarkar, Luecke and Duchan (1947) assayed the amino acids contents of seminal plasma by microbiological methods in protein hydrolysate. The following is the reported table by Sarkar et al :-

TABLE - 2.1

Table showing protein composition of bull-semen.

<u>In dried material, ash and lipid-free (mg/100 g)</u>	
<u>Spermatozoa</u>	<u>Seminal plasma</u>
1	2

Con'd Table 2.1

	<u>Spermatozoa.</u>	<u>Seminal plasma.</u>
	<u>1</u>	<u>2</u>
Total nitrogen	17.61	12.05
Arginine.	25.47	7.91
Histidine.	2.54	2.31
Lysine.	5.08	4.86
Tryptophan.	1.59	2.63
Phenylalanine.	3.81	3.42
Methionine.	1.81	1.61
Threonine.	3.78	3.20
Leucine.	5.20	3.81
Iso-leucine.	3.42	2.79
Valine.	3.73	3.11
Glutamic acid.	8.33	7.75

(Cf. T. Mann, "The Biochemistry of Semen", Methuen & Co. Ltd. N.Y. 1954; page 84.)

The data, shown in the above table, indicate that spermatozoa have a much higher concentration of proteins than the seminal plasma. Further data on the composition of bull sperm protein have been presented by Porter, Shankman and Melampy (1951), who found in extensively washed, lipid-free and dried spermatozoa 16.7 percent nitrogen; in addition to the amino acids recorded previously, they identified aspartic acid (5%), glycine (1.7%), Proline (3.1%), serine (4.5%) and tyrosine (4.3%).

Gray (1928, 1931) showed that it is possible to delay

the decline of activity in sea-urchin sperm by replacing the sea-water with egg-water, a similar effect has been later demonstrated with solutions of the egg-surrounding jelly. Both sperm motility and respiration can be extended, for instance, by addition of seminal plasma, and there are several indications that this is due to proteins and their breakdown products in seminal plasma.

Hayashi (1945, 1946) experimenting with sea-urchin demonstrated the occurrence with seminal plasma of a non-dialysable constituent beneficial to the viability and fertilizing capacity of spermatozoa.

Wicklund (1949, 1952) demonstrated a favourable influence of serum albumen on the fertilizing power of washed or aged sperm of *Psammechinus miliaris* was retained much longer following dilution with albumin solutions than with sea-water.

Tyler (1950) and Tyler and Atkinson (1950) found that the life-span of sea-urchin sperm can be considerably extended by the addition of certain peptide and amino acids.

Tyler and Rothschild (1951) examined the sperm metabolism of (sea-urchin) and *Lytechinus* in sea-water enriched with amino acids and noted that under such experimental conditions the initial increase the respiration characteristic of the 'dilution effect', was less pronounced but the subsequent decline in activity was considerably delayed, and the total amount of oxygen consumed greatly increased. These facts, coupled with evidence of non-utilization of the added amino acids, indicated that the amino acids enabled the spermatozoa to make fuller use of their endogenous

substrate, probably by inducing the formation of complexes with copper and other toxic heavy metals commonly present in sea-water. This hypothesis has gained additional support from the results of further work on the detoxicating effect of metal-chelating agents such ethylenediamine tetra acetate (versene), diethyldithiocarbamate, L-benzoinoxime (cupron) and 8-hydroxquinoline (Tyler, 1953). Perhaps the beneficial action of proteins (Metz, 1945; Wicklund, 1949) and of seminal plasma (Hayashi, 1945, 1946; Chang, 1949) on spermatozoa is also, partly any rate, due to the similar processes. The beneficial action of these amino acids is believed to depend primarily on their metal-binding capacity (Tyler and Rothschild, 1951).

Metz and Donovan (1950) demonstrated that in the starfish certain amino acids promote the agglutination of spermatozoa by egg-water of this species; in the absence of these amino acids agglutination does not take place. Besides several other effects of amino acids have been observed with the sperm of lower animals.

Jacobsson (1950) and Lundquist (1952) reported fourteen free amino acids in the seminal plasma of man; glycine, threonine, alanine, valine, leucine, isoleucine, cystine, proline, tyrosine, phenylalanine, lysine, arginine, aspartic acid and glutamic acid.

In the year 1952, Gassner and Hopwood found serine, glycine, alanine, aspartic acid and glutamic acid in the bovine seminal vesicle secretion and in the ampullar fluid; the latter containing in addition a trace of tyrosine.

(d) AMINO ACID CONTENT OF SPERM AND ITS ROLE IN SEMEN PRESERVATION:

In the year 1856, Koelliker pointed out that spermatozoa rendered motionless by dilution with water, can be revived by prompt addition of salts or concentrated solution of certain organic substances such as sucrose, glucose, lactose, glycerol, urea and various proteins. He investigated in some detail the activating influence of blood serum, male accessory gland secretions and of a variety of inorganic and organic substances on sperm motility.

The work of Miescher and his contemporaries, however, dealt largely with fish spermatozoa. However more rapid advances were made soon after Elic Ivanor (1907) and several other pioneers in the field of artificial insemination, perfected the technique of semen collection from domestic animals. On the mammalian side, bull semen has received much attention from protein analysis.

The occurrence of free amino acids also known in fish semen, as long as 1923. Steudal and Suzuki (1923) isolated leucine, lysine and alanine in pure form from protein free extracts of herring testicles.

Knoop and Krauss (1944) showed that diluents containing 1.09 percent glycine or "1" proline in addition to sodium and potassium phosphate and egg yolk maintained 36 and 49 percent more living bovine spermatozoa after 12 days storage than did egg yolk-gelatin and egg-yolk diluents respectively.

Tosic and Walton (1945; 1946a, b, 1950) studied the oxidative deamination of amino acids by bull spermatozoa. In

addition to sugars and fatty acids, spermatozoa are capable of oxidizing a number of amines and amino acids.

In the course of their study, Tosic and Walton examined several pure amino acids and found that spermatozoa oxidize three naturally occurring amino acids, namely L-tyrosine, L-phenylalanine and L-tryptophane. According to Tosic (1947, 1951) the hydrogen peroxide forming aerobic process in bull semen is an oxidative deamination catalysed by the L-amino acid oxidase of spermatozoa.

Martini (1947) incubated bovine semen or washed sperm at 37°C for 2 hours with sodium phosphate buffer, sodium arsenite, and the amino acid under test. To measure the extent of deamination trichloroacetic acid was added, followed by 2, 4 - dichloronitrophenyl hydrazine, and the absorption coefficient measured in a Pulfrich Photo-meter. This gives the amount of keto-acid produced. Whole semen showed a considerable production of keto-acid under these conditions without the addition of amino acids; this presumably arises from amino acid was added. Tyrosine was deaminised to the greatest extent followed in descending order by methionine, leucine, valine, tryptophane, histidine, cysteine, and isoleucine. The deamination of alanine, glutamic acid, arginine and lipine was negligible.

Sarkar, Luecke and Duchan (1947) determined concentration of 11 amino acids in a composite of 149 semen samples of 40 different bulls of the Holstein-Friesian, Gurnsey and Jersey breeds. Sperm and seminal plasma contained 17.61 and 12.05 percent nitrogen respectively, when corrected for moisture, fat and ash. Spermin

was not detected in semen. The amino acid composition of sperm and that of seminal plasma was very similar, except as regards arginine (25.47% in sperm V. 7.91% in seminal plasma), leucine (5.20% in sperm V. 3.81% in seminal plasma and tryptophan (1.59% in sperm V. 2.63%).

Lorenz and Tyler (1951) showed extension of motile life span of spermatozoa of the domestic fowl by amino acids and proteins. Glycine, when added to saline diluents at concentrations between 0.003 and 0.133 molar, extends the life span of spermatozoa of the domestic fowl as measured by persistence of motility at 22°C. Glycine was relatively more effective on dilute than on concentrated semen suspensions. Certain egg white proteins had a similar effect on the life span of sperm. Purification of the saline diluent alone served to prolong the life span of spermatozoa and correspondingly reduce the effects of glycine and proteins.

Giese and Well (1952) found that glycine (0.05 M) protected the spermatozoa of sea-urchin *Strongylocentrotus purpuratus* from the detrimental effect of light. Prolonged spermatozoal survival with the use of glycine containing salt diluent was obtained with storage at 38°C, but not at 4°C., when compared with the yolk-citrate diluent (Tyler and Tanabe, 1952).

Roy and Bishop (1954) reported that in equal volumes of egg yolk and 4% glycine solution in distilled water there was longer spermatozoal survival at 5°C. than in the yolk phosphate or yolk-citrate diluents. Beneficial effects on spermatozoal survival were also reported by Rakes and Stallcup (1956), Baier et al (1957) and

Saha and Singh (1958).

Roy et al (1956) used egg yolk medium with glycine and noticed that there was a recovery of about 80 percent of the initially motile spermatozoa when frozen samples were thawed at room temperature.

Cegielka, in the year 1958, placed a preliminary report on qualitative study on the amino acids in the semen from ox, dog, and pig.

Seperation of amino acids in the semen of bull, dogs, and pigs was done by Paper Chromatography method, which showed that the number varied according to species. He found that amino acids were liberated when the semen was stored 4-6 hours at room temperature, but later they disappeared. Dog semen had more amino acids than the bull and pig semen.

In 1958, Lake et al reported, the unpublished observations of Lake and McIndoe, that there is a high concentration of free glutamate in the seminal plasma of cocks. Reported glutamate concentration as follows :-

TABLE - 2.2

Glutamate concentration in seminal plasma of cocks.

Average concentration (mg/100 ml.)	S. D. of (1)	Range of (1)	Average concentration (in equiv/l)
(1)	(2)	(3)	(4)
Glutamate 1033	91	890-1140	70

Lake and McIndoe (1959) identified chromatographically free glutamic acid in a concentration of about 1 g./100 ml. in the seminal plasma of cock.

Besides small amounts of alanine, aspartic acid, glycine, serine, and an unidentified ninhydrin-reacting substance were detected, and about 100 mg. of creatine/100 ml. were present. About 80% of the non-protein nitrogen in the seminal plasma is accounted for by glutamic acid plus creatine. It is probable that these substances are mainly of testicular origin and that the glutamic acid plays a major part in maintaining the osmotic pressure and pH.

Roy (1959) estimated the full and half life of buffalo and bull spermatozoa in semen samples diluted with 4% egg yolk glycine diluent (4% GEY), 4% GEY + 1% fructose, and yolk citrate. He found that 4% GEY and 4% + 1% fructose maintained full and half life of both buffalo and bull spermatozoa for a much longer period than yolk citrate.

Tomar (1959) reported that spermatozoa lived for 23 days in egg yolk glycine ds compared with 12 days in sodium bicarbonate-glucose-fructose, although with buffalo semen, sodium bicarbonate-glucose-fructose buffer maintained the motility score of 3 for a longer period than yolk citrate or yolk glycine.

Using microbiological and paper chromatography methods, Novak, Blum, Taber and Liuzzo (1960) quantitatively determined the seminal plasma and sperm amino acids of the honey-bee, *Apis mellifera*. Arginine content of both seminal plasma and spermatozoa of the honey bee to be greater than that of any of the other 14

amino acids found.

Heijkenskjeld and Nordlander (1960) determined the content of alpha-amino nitrogen in human semen plasma and found no correlation between sperm morphology and amino nitrogen content.

Hopwood, Gassner (1962) found the pattern of free amino acids in semen of normal mature bulls is predictably constant. Seminal amino acids, by qualitative paper chromatographic procedures, were about 5 times as concentrated as those of blood plasma, and glutamic acid was the predominant component in the seminal plasma. Castration, vasectomy and vasoligation reduced the levels of seminal amino acids while androgen treatment increased them with the exception of glutamic acid. The seminal free amino acid pattern was altered by scrotal insulation which produced aspermia. Analysis of testes and accessory sex organ tissues and fluids for free amino acids indicated that glutamic acid is mainly a product of the testes and epididymides since it was most concentrated in these tissues rather than in the seminal vesicles and ampullae or their secretions. Seminal amino acid level were correlated with the age of the bull as well as the quality and breeding potential of the semen.

El-Ridi et al (1962) reported that the content of amino acids and proteins of human seminal plasma is also related to fertility, but to a small extent than acid phosphate activity. Rate of proteolysis is high in fresh semen, even at room temperature.

Chubb and Copper (1962) detected consistently twenty-three free amino acids by filter-paper chromatography of alcoholic

extracts of fowl seminal plasma; these included several not previously reported in seminal plasma of the domestic-fowl. In addition, several unknown ninhydrin-reacting substances were found. Glutathione and ergothioneine were not detected.

Ahluwalia (1963) investigated amino acids content of fowl seminal plasma and spermatozoa. The amino acid components of turkey seminal plasma were also determined. He reported that almost all the so-called essential amino acids were present in large quantities in the seminal plasma of high fertility semen than in that of low fertility semen, but almost all the so-called non-essential amino acids were present in large quantities in low fertility seminal plasma. He suggested that the importance of amino acids in relation to fertility is to be investigated. The general picture of the amino acids in turkey semen was similar to that in fowl semen.

Barris, Nelson, and Rafael Mancilla (1963) studied free amino acids in the semen of stallions by paper chromatography and found: glutamic acid, aspartic acid, alanine, glycine, threonine and histidine. A comparative analysis of free amino acids in domestic animals was made.

Buruiana et al (1964) reported the chemical analysis and polarographic behavior of the proteins from bovine, porcine, ovine and human seminal fluid and proved that they (proteins) are of glycoproteic character.

Ahluwalia and Graham (1966) recorded a total of 18 amino acids quantitatively in the seminal plasma and spermatozoa of fowl semen. Glutamic acid was the major component in both fractions. In

the spermatozoan fraction, only 6 amino acids were detected, namely glutamic acid, lysine, arginine, serine, aspartic acid and threonine. Except for serine and lysine, the amino acid concentrations in the spermatozoan fractions were much smaller than in the seminal plasma. In general, the amino acid content of turkey seminal plasma is similar to chicken seminal plasma with a few quantitative differences.

Rottenberg (1966) studied on boar seminal plasma proteins, carbohydrates and amino acids of the water-insoluble glycoprotein (Fraction B) from boar seminal plasma and found about 83% of protein and amino acid analysis indicated the presence of significant amounts of all the normally encountered amino acids. No amino acid predominated.

Hood et al (1967) identified twenty common amino acids in the porcine spermatozoa and their relative proportions were determined in 2 ejaculates from each of 6 yorkshire boars. No significant differences were found among boars or between ejaculates within boars for any of the amino acids.

Roussel and Stallcup (1967) studied some amino acid aspects of bovine semen. The content of 17 amino acids in spermatozoa and seminal plasma of 13 young Holstein-Friesian bull was determined. Simple correlations between the amounts of the individual acids in spermatozoa are tabulated, and also correlations of amino acid content of spermatozoa and the plasma with semen characters. The highest correlations in respect of percentage motility, sperm concentration and percentage of live spermatozoa were with

Threonine content of spermatozoa (-0.85, -0.89 and -0.95 respectively) and proline content of spermatozoa (-0.58, -0.63, and -0.68), the amino acid activity been adjusted to a sperm concentration of 1000×10^6 per ml. In general, amino-acid levels were of little significance in the determination of semen quality.

Setchell et al (1967) studied the amino acids in ram testicular fluid and semen and their metabolism by spermatozoa. The testis of the ram secretes considerable amounts of amino acids (200 micro moles/day) into the fluid collected from the efferent ducts. The principal amino acid in this testicular fluid is glutamate, which is present in concentrations about 8 times those in testicular lymph or in blood from the internal spermatic vein. The concentration of glutamate in seminal plasma from the tail of the epididymis is about 10 times that in testicular fluid, and, though glutamate is the major amino acid in ejaculated seminal plasma, its concentration is less than in epididymal plasma.

Zlatarev (1967) studied the effect of organic acids in a diluent for the storage of bull's semen and reported that survival of the semen of the different bulls depended on their age. He recorded a tendency towards a better storage of the semen in younger bulls in that particular diluents used.

Joshi, Rawat and Roy (1968) studied the preservation effects of spermatozoal viability in diluents made up of amino acids with milk and/or egg yolk as base. Semen samples from 9 Murrah buffalo and 4 Haryana bulls were used. A 4% solution of alanine or serine with 25% egg yolk resulted in sperm motility comparable to

that obtained in 4% glycine with 25% egg yolk. Motile spermatozoa in these diluents averaged 60-64% after 6 hours storage and 14-20.8% after 126 hours storage, with 4% alanine giving the best results at 126 hours. The addition of glycine, serine, alanine, succinate and egg yolk to whole milk diluents was not advantageous to buffalo semen. By paper chromatography, nine amino acids were found in the seminal plasma obtained from Murrah and Hariana bulls. Besides, Threonine was present in the semen of Hariana bull but absent in that of Murrah bull. The free amino acids present in seminal plasma from Murrah and Hariana bulls are given in Table 2.3.

TABLE - 2.3

Free amino acids present in the seminal plasma of
Murrah and Hariana Bulls, as determined
by Paper Chromatography.

Murrah bull	Hariana bull
Alanine.	
Arginine.	
Aspartic acid	
Glutamic acid	
Glycine	And Threonine.
Lysine.	
Histidine	
Serine	
Tyrosine	

Popov (1968) preserved the bull semen in glucose-citrate diluent with EDTA and arginine.

(e) HISTORY OF ISOLATION OF AMINO ACIDS:

Tracing the history of isolation of amino acids, Braconnet (1820) obtained the first amino acid Glycine (Gr.glykys=sweet), although eighteen years elapsed before Braconnet's "Sugar of gelatin" was shown to contain nitrogen; the substance is amino-acetic acid, $\text{CH}_2(\text{NH}_2)\text{COOH}$. It was a matter of chance observation rather than of systematic research. In the same way Aspartic acid was first obtained by precipitation of its barium or calcium salt by alcohol. Emil Fischer (1901) conducted the first outstanding advance in the systematic separation of protein hydrolyzates and it was he, who showed that esters of amino acids can be distilled without decomposition; as fractional distillation is not possible since amino acids melt at relatively high temperatures, usually with decomposition. The quantitative isolation of amino acid constituents is difficult. Separation by fractional crystallization is subject to the limitation that many of the acids fall into groups of structurally similar substances differing but little in solubility.

T A B L E - 2.4

Amino acids isolated from proteins.

A. Neutral Amino Acids

Name	Formula	Investigator	Year
1	2	3	4
1. Glycine.	$\text{CH}_2(\text{NH}_2)\text{COOH}$	Braconnet	1820

Cont'd Table 2.4

1	2	3	4
2. Alanine	$\text{CH}_3\text{CH}(\text{NH}_2)\text{COOH}$	Weyl	1888
3. Serine.	$\text{HOCH}_2\text{CH}(\text{NH}_2)\text{COOH}$	Cramer	1865
<u>B. Acidic Amino Acids.</u>			
4. Aspartic acid.	$\text{HOOCCH}_2\text{CH}(\text{NH}_2)\text{COOH}$	Ritthausen	1868
5. Glutamic acid.	$\text{HOOC}(\text{CH}_2)_2\text{CH}(\text{NH}_2)\text{COOH}$	-do-	1866

(Cf. Louis F. Fieser and Mary Fieser, "Organic Chemistry", 2nd Ed., D.C. Heath & Co. N.Y. 1950; Page 431-432).

(f) DIFFERENT METHODS FOR THE DETERMINATION OF AMINO ACIDS:

Kossel and Kutscher (1900-1901) presented a method for the determination of basic amino acids in which precipitation of group followed by subsequent separations of the amino acids were involved. Emil Fisher (1901) described the ester procedure for the isolation of monoamino acids. The more recent methods often do not require preliminary separation of the basic amino acids.

There are various methods applicable to the determination of several amino acids. The general methods employed are as follows :-

(1) Specific Precipitants:

Block and Bolling (1945) observed that most of the accumulated data on amino acid composition of proteins were obtained by isolation methods. These depended upon the precipitation by

reagents supposedly specific for single amino acid or groups of related amino acids. Further purification by recrystallization yielded fractions which, after having been characterized as pure by elemental analysis and melting point could be weighed. Thus, information concerning the amounts of the amino acids in the original protein is obtained. The method involves laborious processing.

(2) Microbiological Methods:

Hawk, Oser, Summerson (1954) described that the fundamental principle involved in microbiological assays is to measure the response of bacteria, yeasts, or molds to graded increments of the sample and of a standard solution added to media furnishing all the nutrients required by the microorganism, except the amino acid under assay. The graded response may be measured by the increase in population of the micro-organisms (i.e. turbidometrically) or by their products of metabolism (acid or CO_2 production). The methods are advantageous, less expensive, simple but not devoid of limitation.

It furnishes very reliable quantitative results (Snell).

(3) Enzymatic Decarboxylation:

Olcott (1951) expressed that in this method of amino acid determination, enzyme systems that have the property of specifically decarboxylating one amino acid are employed. An aliquot of a neutralized protein hydrolysate is mixed with such an enzyme system in a warburg or van slyke apparatus and the carbon dioxide

involved is measured by standard techniques. The total amount liberated is equivalent to the amino acid present in the hydrolysate. The method seems to be rapid and capable of accuracy provided the enzyme preparation is available.

(4) Chromatography:

Chromatography has been defined as "a procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material" (Gordon, Martin, Synge 1944). They developed a system of partition chromatography which was used by Tristram (1946) for determining the monoamino, mono-carboxylic acid contents of several proteins. In the method, the amino acids in protein hydrolysate are acetylated with acetic anhydride. The resulting acetamino acids are then subjected to separations that involve partitions between an aqueous phase and a non-aqueous solvent mixture. The aqueous phase is held stationary in the form of a column of wet silica gel. A suitable indicator is adsorbed in the silica. The acetamino acids are then dissolved in the non-aqueous solvent and the solution is permitted to percolate down through the column. As it does so, the different amino acids move at different rates and so separate into bands that are detectable by means of colour changes in the indicator. As bands move out of the column, they are caught in separate containers and then subjected to further separation procedures, or to direct determination by titration.

(1) Paper Chromatography:

Consden, Gordon and Martin (1944) used for the first

time, partition chromatography on a sub-micro scale. Cellulose in the form of a strip of filter paper is used as the stationary phase. Majority of the methods which have been devised for paper chromatography are based on the principle, "separation of substances from a mixture by the passage of solvent in a definite direction and selective fixation" (Weil, 1953). "A drop of solution containing a mixture of substances is placed on a piece of filter paper one end. Next, this end is placed in a suitable solvent within a closed container. The solvent passes the spot where the solution had been applied. Each substance in this mixture will ideally move along with the solvent at a unique rate, so that after a while all the components of the mixture will occupy a distinct position somewhere along the path of the solvent" (Block and Zweig, 1958).

For qualitative and quantitative estimations of amino acids paper chromatography is widely used.

(ii) Adsorption technique:

For basic amino acids, the technique necessitates an adsorbent mixture and a filter aid.

(iii) Ion-exchange chromatography:

The synthetic ion-exchange resins are essentially solid acids or bases. They possess the capacity to hold bases or acids with which they come in contact. They are attractive analytical reagents and because of the displacement phenomenon by which a strong acid displaces a weak one or a strong base a weak base

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(Olcott, 1951).

(iv) Gas Chromatography:

Zlatkis Or'o and Kimball (1960) demonstrated that amino acids can be converted by ninhydrin into volatile aldehydes. These were separated by gas chromatography and converted to methane which was subsequently estimated in a thermal conductivity cell.

(5) Ionophoresis:

Martin and Synge (1945) defined ionophoresis a "process concerned with the movement in a electrical field of relatively small ion", electrophoresis as "movement of large molecules and particles", but the distinction between them is ignored. Blackburn (1965) expressed that electrophoretic method was capable of determining amounts of amino acids in micro quantities.

The advantages of the high-voltage electrophoresis method are for their speed and high resolving power. Both, the paper chromatographic technique and electrophoresis are equally efficient except that the latter requires less time.

(6) Isotope dilution method:

Shemin (1945) stated, "this method is based upon the principle that the usual laboratory procedures for isolating amino acids donot separate isotope containing molecules from their normal analogues. If a known amount of an amine acid, that has been labelled by the incorporation of a stable or radioactive isotope

element, is added to a protein hydrolysate, and then the same amino acid is isolated from the mixture and purified, a determination of the concentration of isotope in the sample will give a direct measure of the amount of the amino acid originally present in the hydrolysate. The decrease in the concentration of the labelled element from that present in the added amino acid to that present in the isolated amino acid indicates the extent to which the labelled amino acid has been diluted".

The technique has so far been applied only with amino acids labelled with N^{15} . The measurement of N^{15} requires mass spectrograph, an instrument which is not generally available, although the method has the possibility of being the most accurate and reliable.

(B). LITERATURE REVIEW ON MOTILITY AND CRYSTALLIZATION
PATTERN OF SEMEN

EVALUATION OF PROGRESSIVE MOTILITY OF SEMEN:

Legerlof (1936) said "The motility of spermatozoa is a necessary physiological phenomenon for fertilisation".

Asdell and Salisbury (1941) reported "The motility of spermatozoa serves as a means for properly distributing spermatozoa throughout the female reproductive tract, in which ovum is found as a fertilising unit thus insuring the statistical criteria of spermatozoa ova meeting".

Motility gives a fair indication of the quality of semen and signifies a high degree of physiological integrity of the spermatozoa. It is influenced by a number of factors viz. pH of semen, temperature, live percentage, age, health condition, time of collection, frequency of ejaculation, feeding, exercise, season, sex vigour of the bull etc.

A number of methods were described for estimating motility of spermatozoa. The commonest one for field condition was reported by Herman and Swanson (1941) - five grades for representing sperm motility from 0 - 5, where 0.5 represent 10% motility. Swanson and Herman (1944) stated that conception increased as motility increased upto 3.0 rating, and there after

increased motility did not result in important increase in conception rate. The index of correlation has also revealed a significant curvilinear correlation between motility and rate of conception. Initial motility was also stated to be dependant upon the activity of spermatozoa as well as on sperm concentration, (Maule, 1952).

Prabhu and Bhattacharya (1951) studying comparison of first and second ejaculates in Indian water buffalo found an initial motility of 3.07 and 3.32 respectively. The same authors who conducted experiments with local Uttar Pradesh buffalo bull with teasers on heat and not on heat cows, recorded a motility rating of 2.47 and 2.32 for first ejaculates and 3.46 and 2.85 for the second ejaculate of the same collection.

Prabhu and Sharma (1954) studying the comparative effect of 4 successive ejaculates recorded an initial motility of 2.69; 2.31; 1.89 and 1.01 respectively for successive ejaculates of buffalo bull.

Studying the seasonal variation in semen characters Kushwaha et al (1955) recorded an initial motility of 3.57 and 3.74 during 1949-50 and in the subsequent year being best in spring 4.15; 3.75 in summer, 3.4 in winter and 3.34 in autumn.

Sayed and Oloufa (1957) studying the effect of frequency of collection on Egyptian buffalo bull, stated the motility

as 84.71% and 55.95% with collections of once a week and thrice a week.

Hafez and Darwish (1956) reported an initial motility of 7.25 for fresh semen in buffaloes and observed drop down of motility after third ejaculate.

Oloufa et al (1958) recorded a motility of 75.37% in summer with an average of 64.02 for the year under study i.e. 3.5 and 3.0 respectively.

Tomar and Desai (1958-59) while studying preservation of buffalo semen rated motility in ten categories from 0 to 5 every 0.5, 10 percent motile spermatozoa.

Kale (1962) stated an initial motility of 4.0 to 4.5 in buffalo bull semen.

Mishra and Sengupta (1964) recorded a motility of 1.58 ± 0.09 in summer with buffalo bull having wind protection and 0.87 ± 0.30 without protection from hot winds while in the same year Sengar and Sharma (1964) recorded 1.83 as initial motility for the first ejaculate.

CRYSTALLISATION PATTERN OF SEMEN:

In the year 1966, Tomar, Johari, Misra and Singh observed that there did not seem any information indicating the

differences in keeping quality of buffalo (*Bos bubalus*) and Zebu (*Bos indicus*) semen under undiluted condition in the not known whether there was any gross distinctiveness with dried seminal film of the two species. Being fascinated with their observations they under take study to find out any differences which might exist in keeping quality and crystallisation behaviour of the undiluted semen of Murrah buffalo and Haryana (Zebu) bulls and found differences in the crystallisation patterns of zebu and buffalo semen. Zebu semen showed distinct channels in the film while buffalo semen film was devoid of such channels and presented uniform distribution of spermatozoa.

*

CHAPTER - III

MATERIALS AND METHODS.

MATERIALS AND METHODS

COLLECTION OF SEMEN SAMPLE AND PRESERVATION:

Semen sample of four Tharparkar (Zebu) and four Murrah (buffalo) bulls were made available from this study. The management of all zebu and buffalo bulls were same. Semen was collected in artificial vagina (A.V.) and its processing was started within one hour after collection. To meet the required volume of semen sample generally two successive collection were taken, specially in the case of buff-bulls.

The semen of buffaloes and zebu bulls were obtained from Semen Bank, Patna and Government Cattle Farm, Patna respectively and brought to the laboratory in thermosflask. The usual transporting procedure was strictly followed.

In the laboratory the semen sample was divided in four parts and preserved in the sterilized test tubes of the size 1 cm. x 10 cm. plugged with sterilized cotton, taking all the precautions of cold-shock and contamination. Then the samples of 24 hours, 48 hours and 72 hours were kept in refrigerator in a beaker containing water in order to cool the sample gradually. After 30 minutes the water from the beaker was thrown out and the samples were kept in the beaker for onward preservation.

PREPARATION OF STANDARD AMINO ACID SOLUTIONS:

The techniques of Cowgill and Pardee (1962) were

adopted with some modifications for preparing the standard solution and their subsequent applications on the chromatograms for development. Thus 5 standard curves were obtained for reference amino acids to ascertain the quantities of amino acids.

0.2 millimole of each of the amino acids was weighed accurately, in a previously weighed clean and dry test tube. The quantities for individual amino acids were as follows :-

(i) DL-a- Alanine (B.D.H.)	17.82 milligram.
(ii) DL- Aspartic acid (B.D.H.)	26.62 "
(iii) Glycine (B.D.H.)	15.02 "
(iv) L-Glutamic acid (E. Merck).	29.43 "
(v) DL- Serine (B.D.H.)	21.02 "

Then to each test tube, added 10 ml. of 10% isopropanol in distilled water containing dil-HCl. Shaken the tubes carefully till the amino acids got dissolved. Presence of HCl helped this process. Then further added 10 ml. of this solvent to each test tube to make the five amino standard solution i.e. alanine, aspartic acid, glycine, glutamic acid and serine. 1 microlitre of each of the standard solution contained 0.01 micromole of the respective amino acid. Stoppered the test tubes and kept for the preparation of standard curves.

The weight of the amino acids contained in standard solutions used for preparation of standard curves as follows :-

TABLE - 3.

The weight of the Amino Acids (in Microgram)
contained in standard solutions

Quantity in Micro moles	Alanine	Aspartic acid	Glutamic acid	Glycine	Serine
0.01	0.8910	1.3311	1.4714	0.7509	1.051
0.02	1.7820	2.6622	2.9428	1.5018	2.102
0.03	2.6730	3.9933	4.4142	2.2527	3.153
0.04	3.5640	5.3244	5.8856	3.0036	4.204
0.05	4.4550	6.6550	7.3570	3.7545	5.255
0.06	5.3460	7.9866	8.8284	4.5054	6.306
0.07	6.2370	9.3177	10.2998	5.2563	7.357
0.08	7.1280	10.6488	11.7712	6.0072	8.408
0.09	8.0190	11.9799	13.2426	6.7581	9.459
0.10	8.9100	13.3110	14.7140	7.5090	10.510
0.11	9.8010	14.6421	16.1854	8.2599	11.561
0.12	10.6920	15.9732	17.6568	9.0108	12.612
0.13	11.5830	17.3043	19.1282	9.7617	13.663
0.14	12.4740	18.6354	20.5996	10.5126	14.714
0.15	13.3650	19.9665	22.0710	11.2635	15.765
0.16	14.2560	21.2976	23.5424	12.0144	16.816
0.17	15.1470	22.6287	25.0138	12.7653	17.867
0.18	16.0380	23.9598	26.4852	13.5162	18.918
0.19	16.9290	25.2999	27.9566	14.2671	19.969
0.20	17.8200	26.6220	29.4280	15.0180	21.020

PREPARATION OF REFERENCE AMINO ACIDS MIXTURE:

Since, the amino acid solutions were already prepared for the purpose of utilising them for standard curves, 10 ml. from each of the five standard solutions were pooled in a clean and dry test tube. It was prepared for applying it by the side of the unknown mixtures in the chromatograms for the sole purpose of identification of the resolved amino acids.

LABORATORY FOR CHROMATOGRAPHIC WORK:

All the chromatographic works were done in the laboratory spared for the purpose, which was free from ammonia and fumes. The room had minimum of window space and was located in the centre of the building, which prevented sudden draft and radical changes of temperature. These conditions permitted to obtain the developed chromatograms with regular spots of amino acids and with straight solvent-fronts.

The following equipments were used for the estimation of amino acids in the present investigation :-

- (i) Two dimension paper chromatography cabinet (Sirco make) size - 24"x12"x24", which had the glass and wood construction (paraffined) along with 3 solvent troughs, 3 bended glass supports, and 6 anti-siphon rods.
- (ii) A packet of 100 sheets of whatman chromatographic filter paper no. 1 (size 46 cms. x 57 cms.).
- (iii) Hair dryer, glass atomizer, table lamp and centrifuge machine.

(iv) Pipettes, micropipettes, funnels and other routine glass wares.

(v) Working table with glass plate.

There was also an almirah containing different chemicals all belonging to 'Analar' grade.

CHROMATOGRAPHIC METHOD:

Utmost cleanliness was observed during the chromatographic work. The glass wares along with glass plate, solvent troughs and rods were thoroughly washed with hot soap water and distilled water. The sequence for cleaning of micropipettes was, hot soap water, distilled water, and acetone. It was a routine practice that the appliances after cleaning were perfectly dried before use with adequate care to prevent contamination. During work, both the hands were being kept clean as far as practicable. Clean rubber hand-gloves were also being used when necessary particularly while touching the chromatograms.

The method of Block and Zweig (1958) was adopted with some modifications according to the convenience. One dimension descending paper chromatography with a single solvent system was employed throughout the chromatographic work. It involved the following main procedures :-

1. Preparation of Sample.
2. Application of Sample (Spotting).
3. Development of Chromatogram.
4. Drying of Chromatogram after development and

detection of spots.

5. Quantitative elution of Ninhydrin treated amino acids.

1. Preparation of Sample:

Semen collected from Tharparker and Murrah bulls was centrifuged at 3,000 r.p.m. for 15 minutes. One volume of seminal plasma was added to five volumes of 95 percent alcohol in a centrifuge tube to precipitate the proteins. It was then centrifuged for ten minutes at 2,500 r.p.m. (Joshi et al , 1968).

The supernatant extract was reduced to dryness at 50°C in air oven and the dry residue diluted with 10 percent isopropyl alcohol to one-half of the original volume (Gassner et al , 1952). This extract concentrate was then ready to be applied on the chromatograph paper.

2. Application of Sample (spotting):

A sheet of whatman no. 1 filter paper (46 cm. x 57 cm.) was layed traught on clean glass plate. A line 8 cm. distant and parallel from the edge of the lesser length of the paper was faintly drawn with a light pencil. Five tiny pencil points spaced at 4 cm. interval were faintly marked on the line drawn from either ends and a small circle (4 mm. dia.) was made on each point. Two points on either extremes had the locations of 4 cm. away from the respective edges. Then the paper was divided throughout the length on lesser length i.e. 46 cm. with faintly drawn pencil. This was

done in order to facilitate in cutting the developed chromatogram for drying in air oven. While doing so, perfectly clean hands were used and care was taken not to touch the filter paper with bare naked hands. Hand gloves were used. From the right corner four circles had been meant for the first semen sample of 10-10 μ l, and the fifth one for reference amino acids mixture and in the same pattern from left corner four circles for second semen sample and the fifth one for amino acid mixture.

Now, the solutions were applied by means of different micropipette to the allotted circle as follows :-

Prior to taking up the work of spotting, manual manipulations of the other micropipettes were practised, to get used to their applications in the actual procedure of spotting the sample.

One of the clean, dry micropipette having 100 micro litres capacity and subdivisions indicating 1 micro litre was dipped in the solution which was to be applied on the paper. The micropipette was allowed to get filled by capillary action. When the desired quantity of the solution travelled in the pipette, it was then withdrawn and the outer sides were wiped off with a clean and dry ordinary filter paper. To prevent the loss of the solution to the glass plate during spotting, a watch glass was put below the chromatographic paper. The watch glass was being moved on that line after each spotting. The solutions were applied at the designated points already marked and encircled with the soft pencil along with the identification marks below each one of them. The "modus operandi" adopted while spotting the solution of the specified volume was, by

gently touching the paper, a number of times. While doing so, usually an angle of 45° was formed between the micropipette and the paper. After each such operation, the material was allowed to dry with the exposure to ordinary table lamp. For best results, only 5 μ l aliquots were applied at one time. Since, spot size, the uniformity of spot and shape of it can effect the subsequent spot of the developed chromatogramme such meticulous care was taken with each application so that ultimately, each spot was small, round and uniform. The spots were then allowed to dry completely. After it, the paper was folded sharply along a line 5 cm. from the edge. Such folding kept the distance of the spots 2.5 cm. from the fold. Finally, the paper was transferred to the chromatographic cabinet where it was put in the manner was described below :-

Both edges near the fold were caught hold, by hand protected by clean rubber globes and the paper was pushed gently in glass trough which was resting on the upper grooves of the cabinet. The paper was fixed by the help of glass support which had bended ends. During operation care was taken that paper rested over the antisiphon rod which too, was placed parallel to the trough on the upper grooves. The paper was thus in a hanging position and the antisiphon rod was in a position to control the excess flow of the solvent. Then the paper was allowed to equilibrate in the vapour of ammonia escaping from the beaker (containing 80 ml. of 0.3% NH_3) kept on the floor of the cabinet.

3. Development of Chromatogram:

Phenol : Water mixture (Joshi et al , 1968) was the

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3. Development of Chromatogram:

Phenol : Water mixture (Joshi et al , 1968) was the

only solvent used throughout the chromatographic work. The solvent has the following advantages :-

- (i) It has appreciable resolving power for amino acids found in the seminal plasma of Zebu and Murrah bulls.
- (ii) The solvent system does not destroy the amino acids while drying the developed chromatograms.
- (iii) In this solvent system the chromatographic paper do not require lengthy pre-equilibration time.
- (iv) It is simpler to prepare and easily available.
- (v) Moreover it is economical.

(a) Preparation of Solvent:

A bottle of 500 gm. of Phenol of 'Analar' grade was taken and kept in air-oven, adjusted to 50°C to make the crystals to liquify. This was done without opening the bottle. When the crystals liquified, the bottle was cooled at room temperature. Then to it 166.6 ml. of glass distilled water was added to make Phenol : water in ratio of 75W: 25 V and stoppered and gently shaken for a while.

(b) Development of Chromatogram:

Enough of this solvent was poured into the troughs by means of a pipette through the holes in the upper lid of the closed chamber, so that the troughs were 3/4th full. During solvent - development of the chromatogram it was an usual practice to check

whether the trough contained ample quantity of the mixture. When necessary, some more was poured. After 20 -22 hours, when the solvent - front had travelled a distance of 44 - 46 cms. and approximately 5 - 3 cms. away, from the other edge, development was stopped. The chromatogram (developed) was taken out with hand covered with gloves and kept for drying in the room at room temperature.

4. Locating the spots in Chromatogram:

Methods of Giri et al (1952) was adopted for drying and colour development of the chromatogram and also during subsequent elution of the ninhydrin spot for quantitative estimation.

The developed chromatogram was hanged with plastic clips. Perfect drying of the paper was ensured by exposure to ordinary table lamp for 10-15 minutes. Then the paper was sprayed with 0.5 percent ninhydrin in 95 percent acetone. The acetone was preferred due to the noted reasons -

- (a) Amino acid are insoluble in acetone and donot spread.
- (b) There is no offensive smell (e.g., n-butanol).
- (c) It is economical.
- (d) It conserves space.

Spraying was done with the glass atomizer, 50 ml. capacity, having a rubber bulb which was connected to the glass tube of the glass container, by small connecting rubber tube. The following procedures were adopted during the spraying operations:-

Glass container with the colouring reagent was caught

hold with the left hand and kept in slightly filled position, at the distance of 15-18 inches from the developed chromatogram. Gently, the rubber bulb was manipulated, with the result, thin spray of the colour reagent appeared which were allowed to fall on the right corner of the paper above. Gradually, the container was moved to the left on the same line but continuing the operation, till it reached on the other corner. Same procedure was repeated below this application, and continued till the entire paper was sprayed with the reagent which left a wetting mark on the paper. Care was taken, not to over flood the chromatogram to prevent formation of diffused and ill-defined spots. The spraying technique was followed as described, since it prevented over-flooding of the reagent. The whole process took 10-15 minutes and consumed about 30 ml. of the reagent. Then the sprayed chromatogram was allowed to remain for another 15-20 minutes till it got finally dried. After it, the sprayed paper was cut with the help of a dry scissors along the already drawn line. It was then folded and transferred to the hot air oven and kept at 65°C for 30 minutes, with particular care for the temperature control. Precautions were taken, to put clean and dry, but ordinary papers, as linings to the inner walls of the oven. The chromatogram was then taken out and put in the hanging position. Examined the resolutions of the sample and located the ninhydrin spots against the corresponding known amino acid (reference amino acid).

5. Elution of Ninhydrin spots:

Distinct zones of ninhydrin spots that had different

colorations were formed depending upon the amino acid. The spots, on identification against the reference amino acids, were cut, one after another and put into separate clean and dry test tubes, already marked with glass pencil for their identity. Care was taken to hold paper cuts with clean forceps not by bare fingers, and while cutting the paper clean and dry scissors were used. The paper cuts from the place of original application of the sample and from some randomly selected portion of the paper were taken for blank determination.

As the colour obtained by amino acid of the sample spot was found to be too weak to be measured in the Klett-Summerson photoelectric colorimeter, the four spots of the amino acid were taken together in the test-tube already allotted to amino acid. Then, to each test tube 4 ml. of 75 percent Ethyl alcohol containing 0.2 mg. of copper sulphate was added, and thoroughly shaken till the ninhydrin colour was eluted from the paper. The coloured solution so obtained was read in a Klett-Summerson photoelectric colorimeter using green filter (540 m. μ). Colorimetric readings were noted and averaged. For the standard amino acid solutions these were plotted on the graph paper against the respective quantities of amino acids originally taken for application on the paper, the points were joined with straight line. The colorimetric readings obtained from the unknown mixture or extract were utilised, for evaluation of quantities, with the help of the standard curves.

To know the quantity of amino acids in unknown mixtures and extracts the standard curves were always used. The amino acid

on identification in the chromatogram followed by elution, indicated its quantitative transfer to the solvent (i.e. 4 ml. 75 per-cent Ethylalcohol containing 0.2 mg. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). The optical density of the solution, when known by colorimetric reading, shows the concentration of the amino acid or unknown substance in the solution. It was known by reference to the standard curve prepared for that particular amino acid.

In the same way estimation of amino acids in the semen sample, of zebu and buffalo bulls after preservation (in refrigerator) of 0 hour, 24 hours, 48 hours and 72 hours were determined by paper chromatography method.

METHOD FOR CALCULATION

Amino acid content in deproteinized (Alcoholic extract) Seminal plasma:

First, the amount of amino acid was estimated in 40 micro litres of the sample and then, from this estimated amount the amino acid content present in 1 ml. of deproteinized plasma was known by calculations. Standard curves and Table 3 were used during such calculations.

For instance, in case of Glutamic acid, when the colorimetric reading indicated 60 on the scale, the reading was referred to the standard curve which showed 0.04 micromoles of that amino acid. Evidently, Table 3 as quoted above, pointed to 5.8856 micrograms of glutamic acid. However, from practical stand point the last two decimal places were omitted. This value indicated the quantity of amino acid present in 40 micro-litres of deproteinized seminal plasma. To know the total quantity of amino acid present in 1 ml. of deproteinized seminal plasma, it required usual multiplications and divisions to arrive at the value, which was then represented in milligrams of the amino acid. To simplify the matter, it can be represented as follows :-

Since 0.5 ml. of sample was prepared from 1 ml. of deproteinized, alcoholic extract, seminal plasma.

Therefore 1 ml. of it represents 2 ml. of the deproteinized seminal plasma. When A represents the total amount of amino acid present in 40 micro litres of the sample, and T the amount of

amino acid in 2 ml. of the deproteinized seminal plasma.

$$\text{Then } T = \frac{A \times 1000}{40}$$

= A x 25 micro grams (i.e. amount present
in 2 ml. of deproteinized seminal plasma).

$$= \frac{A \times 25}{1000} \text{ mg.}$$

or $\frac{A \times 25}{1000 \times 2}$ mg. of amino acid present in 1 ml.
of the deproteinized seminal
plasma.

or $\frac{A \times 25 \times 100}{1000 \times 2}$ mg. = Total amount of amino acid
present in 100 ml. of the
deproteinized seminal plasma.

or A x 1.25 mg. = -do- -do-

REAGENTS USED

Reagents used for the estimation of amino acids:

- (1) 10% isopropanol in distilled water (containing dilute HCl.).

10 ml. isopropanol, 1 ml. 6N HCl, 89 ml. distilled water (when it was used for the sample HCl was being omitted, and distilled water was taken in 90 ml. quantity).

- (2) 6 N HCl.

- (3) Phenol : Water mixture (75 : 25, W/V).

- (4) 95% Ethanol.

- (5) Ninhydrin solution (for colour development).

0.5 gm. ninhydrin ($C_6H_4CO.CO.CO.H_2O$) was dissolved in 100 ml. of acetone (Analar).

- (6) 75% Ethyl alcohol (containing 0.2 mg. $CuSO_4 \cdot 5H_2O$ in 4 ml.).

50 mg. $CuSO_4 \cdot 5H_2O$, was dissolved in 210 ml. of distilled water. To this solution 790 ml. of Ethyl alcohol was added to make it one litre.

EVALUATION OF PROGRESSIVE MOTILITY OF SEMEN:

Working procedure:

The simple conventional method of grading motility of semen for routine examination, devised by the Russian workers, was adopted.

The following scores are given for various types of motility:-

- | | | |
|----------------|---|---|
| 0 | = | No motility. |
| 1 or / | = | Poor motility, approximately about 1-20% of sperms are in motion. Their movement is mostly weak and oscillatory. |
| 2 or / / | = | Fair motility, approximately 20 to 40% sperms have progressive motility but movement is undulating. |
| 3 or / / / | = | Good motility; about 40 to 60% of sperms are in motion. The movement is vigorous but limited to individuals sperms. |
| 4 or / / / / | = | Very good motility, approximately 60-80% sperms are in rapid vigorously progressive movement. |
| 5 or / / / / / | = | Excellent motility approximately 80-100% sperms are very rapid and vigorous progressive movement in straight line. |

CRYSTALLISATION PATTERN OF SEMEN:

Working procedure:

A thin film of semen was made on the clean and dry glass slide with a pin head. The film was dried in air and examined under microscope (10x) for crystallisation pattern and spermatozoal behaviour.

*

CHAPTER - IV.

RESULTS & DISCUSSION

RESULTS AND DISCUSSION

(a) SEMINAL AMINO ACIDS.

Choice of solvent:

It is well established fact in the field of chromatography that choice of the developing solvent plays a central role in the ultimate efficiency of the process of resolutions and the solvent ultimately chosen in this work was based on the findings of previous works.

In 1944, Consden et al used the phenol solvent for chromatographic procedure. Patton (1950), preferred the use of phenol : water mixture (80% phenol) in separation of amino acids by paper chromatography. Gassner and Hopwood (1952) adopted the 80% phenol in the separation of amino acids of seminal plasma and successfully separated aspartic acid, glutamic acid, serine, glycine and alanine by one dimension paper chromatography.

Kowkabany (1952) explained that slower moving solvents produced rounder and less diffused spots.

Joshi et al (1968) used phenol : water mixture (75:25; W/V) in separating the free amino acids present in the seminal plasma of Murrah and Hariana bulls using one dimensional descending chromatography. Block and Zweig (1958) also recommended it, as one of the solvents of choice, for the analysis of amino acids on paper.

Taking cue from others, it was considered proper, to use phenol solvent used by Joshi et al (1968) throughout the

chromatographic work. The advantages enumerated earlier, made it more alluring for the purpose.

Fixation of location of Amino Acid in a chromatogram:

The specific distance travelled by the particular amino acid during the solvent development, indicates its resolution from the mixture of amino acids or deproteinized sample applied on the chromatographic filter paper. Different amino acids have different positions in the chromatogram. Their locations on the paper along with the distance travelled by the solvent front, assists in calculating its Rf. values. Calculations of the Rf. values have been appreciated as the guidelines for identification of the amino acids. However Zimmerman (1953) enumerated a number of points that may cause variations in Rf. values. Block and Zweig (1958) cautioned that such calculated values must not be depended upon entirely. They further recommended that while applying the aliquot of a sample on paper, a good number of spots of reference standard amino acids solution (mixture) may be applied. The application should be such, as to facilitate identity of unknowns. They also advocated that such applications in the same chromatogram cancelled the probable experimental variations. Therefore, in the beginning, it seems to be a necessity, to have an idea of the approximate locations of each amino acid of the standard solution. The Rf. values were calculated by the following recognized formula :-

$$Rf. = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent front.}}$$

Two filter papers were taken and numbered as 'A' and 'B'. In filter paper 'A' five standard amino acid solutions were applied in quantities of 5 and 10 micro litres. Duplicates were also made in filter paper 'B'. Filter paper 'A' was developed only once, but 'B' was run for the second time after 24 hours drying in between two solvent stages in a room for better separation. For rest of the procedures, there was no difference.

Both the solvent fronts were measured. Various ninhydrin spots were also measured (distance from the point of application to the ninhydrin spot). Rf. values were calculated. The calculated values are represented in Table 4.1. Apparently, the locations of aspartic acid, glutamic acid, serine, glycine and alanine were in the descending order. The Rf. value for each one was different (for each amino acid).

It was noticed that the quantity of amino acid (in 10 micro litres) made bigger ninhydrin spots, whereas, the spot-size in 5 micro litre aliquot showed small area.

TABLE - 4.1

Showing the distance of solvent fronts, amino acids in a single run and redeveloped chromatograms with the calculated Rf. values.

Solvent develop-ment.	Description (Application (in micro litres)	Aspartic acid		Glutamic acid		Serine		Glycine		Alanine	
		5	10	5	10	5	10	5	10	5	10

Single Run 'A' Distance travelled by solvent front (in cms.)...
..... a.

45.4 cms.

Distance travelled by the amino acid (in cms.)
..... b.

7.3 7.4 12.5 12.5 16.5 16.6 19.2 19.2 28.4 28.5

Room temp.
33°C.

Rf. = $\frac{b}{a}$

0.16 0.16 0.27 0.27 0.36 0.36 0.42 0.42 0.62 0.62

Re-developed 'B' Distance travelled by solvent front (in cms.) ...
..... a.

45.5 cms. - 1st run.
46.0 cms. - 2nd run.

Distance travelled by the amino acid (in cms.)....b.

14.7 14.8 22.8 22.6 29.0 29.0 31.2 31.3 38.0 38.4

Room temp.
34°C.

*Rf. = $\frac{b}{a}$

0.32 0.32 0.49 0.49 0.63 0.63 0.69 0.69 0.82 0.83

* The distance of solvent front in chromatogram in second run (46 cms.) was taken for calculation of *Rf. values.

Assessment of the resolution of the constituent amino acids from a mixture:

To test the efficiency of phenol : water mixture, steps were taken to examine the resolutions of different amino acids. Two filter papers, designated as No. I and No. II were taken. The applications of an aliquot of 10 micro litres were made to 10 spots only in this order i.e. Mixture - Aspartic acid - Mixture - Glutamic acid - Mixture - Serine - Mixture - Glycine - Mixture - Alanine. In this work, reference amino acid mixture and five standard solutions were used. Applications were made on the same pattern, as it was done in chromatogram No. I in the case of second chromatogram. Chromatogram No. II was re-developed, after 24 hours drying in between the two solvent stages. Separated amino acids were identified by the help of known amino acids (reference). Visual assessment of separations were done for both the chromatograms after colour development.

The chromatogram No. II showed the very distinct separation of serine and glycine, alanine.

The solvent re-development technique advocated by Majumdar and Ramanujsen (1965), Pasieka and Logan (1966) was thus varified and found to be more effective. The results indicated for its further application in the subsequent analysis.

Preparation of standard curve:

During the preparation of standard curves all the procedures, as detailed in the chapter - "Materials and Methods", were scrupulously followed. Solvent redevelopment technique was, however, introduced to provide better separation of amino acids.

Majumdar et al (1965) reported that better separations are achieved by carrying out two consecutive runs in the same direction with the same solvent.

A standard amino acid solution for Glutamic acid was taken and an aliquot of 2, 4, 6, 8, 10 and 12, 14, 16, 18, 20 micro litres were applied on whatman chromatographic filter paper, one after another. Duplicate applications of these quantities were also spotted on separate sheet of paper. Utmost care was taken while application of the different volumes of the solution. The chromatograms were developed with phenol : water solvent (Joshi et al , 1968), for 21 hours and then re-developed after 24 hours of drying in between the two solvent stages. Colour development with 0.5% ninhydrin in 95% acetone provided regular, uniform, ninhydrin spots. The ninhydrin spots were then cut and eluted. Optical density measurements of the elutriates were made and colorimetric readings were recorded. The values of different elutriates obtained from the second chromatogram were also noted. Ultimately, the average values were calculated. These were plotted on the graph paper against the corresponding quantities of amino acid applied initially, on the chromatographic paper. A straight line was drawn



STANDARD CURVE FOR GLUTAMIC ACID

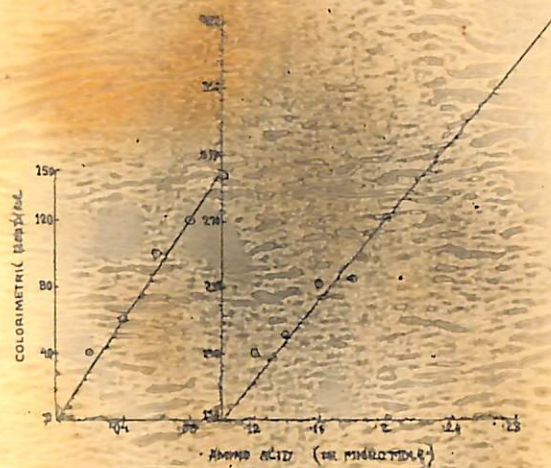


FIG - 2

STANDARD CURVE FOR SERINE.



AMINO ACID (IN MICRO MOLE)

FIG-3

STANDARD CURVE FOR GLYCINE

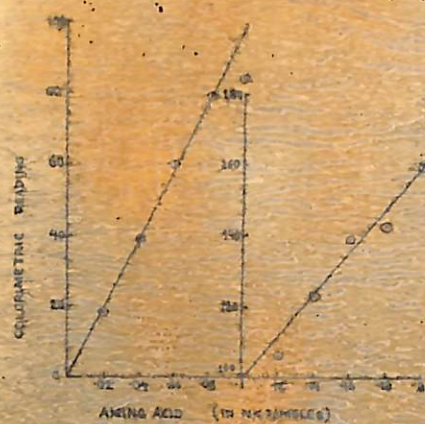


FIG. 4

STANDARD CURVE FOR ALANINE

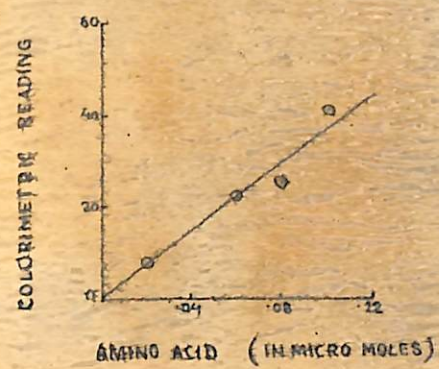


FIG-5

which consequently, provided the standard curve. It was observed that aliquots which had more quantity of amino acid during application on the paper, provided bigger spots.

Same procedures were repeated in case of aspartic acid, serine, glycine and alanine. The five standard curves thus obtained were used for reference during estimations of amino acid in the deproteinized seminal samples.

The standard curve for aspartic acid, glutamic acid, serine, glycine and alanine have been represented graphically in figure 1 to 5 respectively.

Determination of optimum concentration of the sample for use in the chromatographic work:

Earlier experience pointed to the necessity of keeping the concentration of applications on chromatographic paper to a minimum while containing the optimum amount of amino acids. During chromatographic analysis optimum concentration of a mixture is essential for better resolutions of different amino acids. And it was also observed during the preparation of standard curves that there existed a correlation between the amounts of amino acids and the corresponding sizes of ninhydrin spots. As a consequence, particular attention was devoted to ascertain the suitable quantity of the prepared sample to be spotted on the chromatogram for analysis. A aliquot of 50, 20, and 10 micro litres were applied on the chromatographic paper. Duplicate application for the same was also made. A application of 10 micro litre of reference amino acid

mixture was spotted by the side of the duplicate. The chromatograms were re-run and colour developed. After colour development, the chromatogram was put in the hanging position and was examined through naked eyes for the resolutions of the amino acids. As stated earlier, the spots formed from the aliquot of a reference amino acid mixture were used, while identification of resolved amino acids from the sample.

Taking into consideration the result of visual examination it was finally concluded that 10 micro litres was the best volume to give clear distinct and non diffuse spots of amino acids to be analysed than 20 micro litres, where as 50 micro litres gives non distinct and diffused spots of amino acids.

Estimation of amine acids in seminal plasma of
Tharparkar (Zebu) and Murrah (Buffalo) bulls:

The procedures adopted for preparation of the deproteinized seminal plasma have been already described in the chapter - "Materials and Methods".

The semen samples of zebu and buffalo were processed for the Preparation of Sample to be chromatogrammed as described in Materials and Methods, chapter and were finally transferred to the respective clean and dry test tubes. The test-tubes were then stoppered and shifted to the laboratory meant for chromatography.

A sheet of chromatographic filter paper was taken out from the packet and put on the table with care so that it laid traught over the glass plate. Four spots of each 10 micro litres

from each sample and one spot of 10 micro litres of reference amino acid solution after the fourth spots were applied as described in Materials and Methods chapter under sub-heading 'Application of Sample (Spotting)'. After re-developing and colour developing of chromatogram, it was put for removing the ninhydrin spots. With the aid of the ninhydrin spots of the respective amino acids obtained as a result of the resolution of the reference amino acid mixture, and the same amino acids in the sample were identified. After elution, each eluate was measured for its optical density in Klett-Summerson photoelectric colorimeter using green filter (540 m. μ).

Thus reading for aspartic acid, glutamic acid, serine, glycine and alanine were obtained for each sample. The readings were then referred to the respective standard curves and the quantities of the amino acids were thus known. The value for the particular amino acid was thus obtained and expressed in milligram per 100 ml. of deproteinized seminal plasma by calculations.

For illustration, the actual reading and calculating made for ascertaining the content in 2 ml. of the sample are indicated in Table 4.2 below in respect of glycine.

TABLE - 4.2

Showing the details of calculation for ascertaining
glycine content in 2 ml. of deproteinized
seminal plasma.

Volume used for spotting (micro litres)	Eluate	Colorimetric reading.	Amount obtained on reference to standard curve (in micro moles)	Weight in micro gram	Total glycine in 2 ml. of deproteinized sample (micro-gram).
10	1	160	0.2	15.02	$\frac{15.02 \times 1000}{40}$
10					
10					
10					
					= 375.5

Same procedures were adopted to estimate aspartic acid, glutamic acid, serine, glycine and alanine in the prepared samples from the semen of zebu and buffalo bulls.

The photos of the redeveloped, coloured chromatograms of the deproteinized seminal plasma-samples of Tharparkar (zebu) and Murrah (buffalo) bull are being presented in figure 6 and 7 respectively. The resolution of the reference amino acids mixture and the same amino acids in the sample were distinctly complete in the chromatograms.

Estimated values of aspartic acid, glutamic acid, serine, glycine and alanine of zebu and buffalo bull seminal plasma of 0 hour, 24 hours, 48 hours and 72 hours at $4 \pm 1^{\circ}\text{C}$ preservation in Refrigerator are presented in Table 4.3 to 4.7. The results are of proximate value.

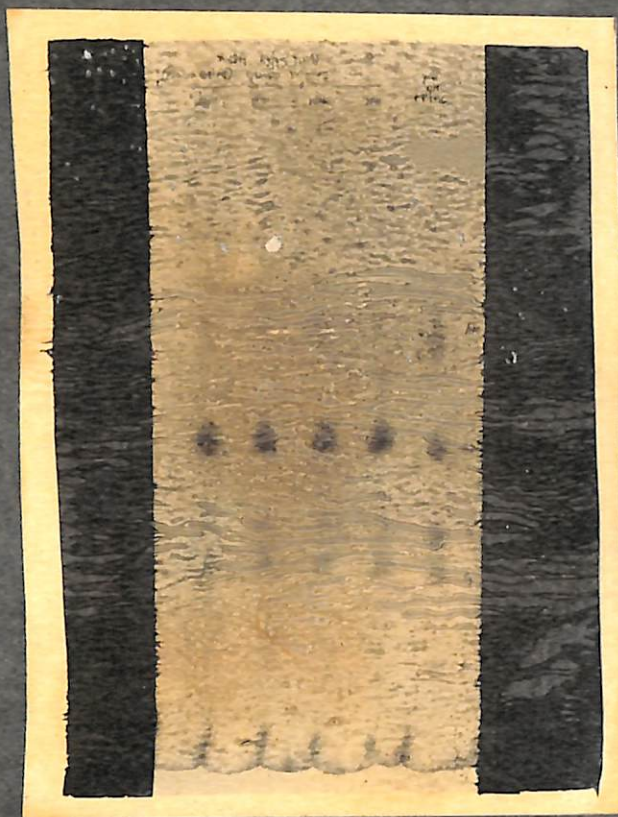


Fig-6

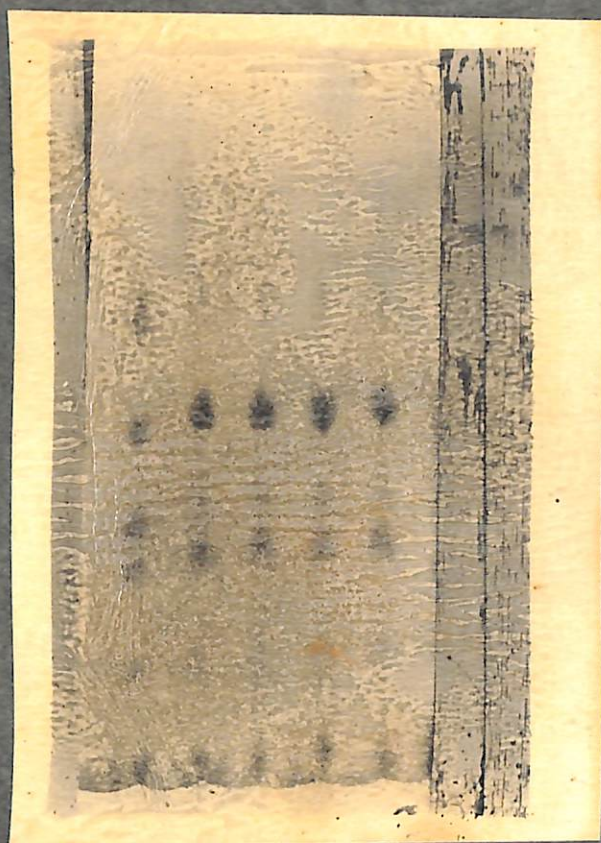


Fig-7

TABLE - 4.3

Table showing quantity of Aspartic acid (milligram/100 ml. of seminal plasma) present in deproteinized seminal plasma of Tharparkar (Zebu) and Murrah (buffalo) bull under different hours of preservation.

	No. of obser- vation	0 hour Average quantity with S.E.	24 hours Average quantity with S.E.	48 hours Average quantity with S.E.	72 hours Average quantity with S.E.
Zebu bulls.	4	3.28 \pm 1.05	4.62 \pm 0.43	7.02 \pm 1.57	8.07 \pm 1.56
Buffalo bulls.	4	3.03 \pm 1.08	4.37 \pm 1.02	3.24 \pm 0.82	3.83 \pm 0.69*

* denotes average of three observations.

TABLE - 4.4

Table showing quantity of Glutamic acid (milligram/100 ml. of seminal plasma) present in deproteinized seminal plasma of Tharparkar (Zebu) and Murrah (buffalo) bull under different hours of preservation.

	No. of obser- vation	0 hour Average quantity with S.E.	24 hours Average quantity with S.E.	48 hours Average quantity with S.E.	72 hours Average quantity with S.E.
Zebu bulls.	4	42.90 \pm 10.20	38.07 \pm 4.74	35.66 \pm 6.60	28.00 \pm 6.97
Buffalo bulls.	4	37.33 \pm 7.74	34.76 \pm 6.83	44.55 \pm 5.21	31.75 \pm 5.23

* denotes average of three observations.

TABLE - 4.5

Table showing quantity of Serine (milligram/100 ml. of seminal plasma) present in deproteinized seminal plasma of Tharparkar (Zebu) and Murrah (Buffalo) bull under different hours of preservation.

	No. of observation	0 hour Average quantity with S.E.	24 hours Average quantity with S.E.	48 hours Average quantity with S.E.	72 hours Average quantity with S.E.
Zebu bulls.	4	3.38±0.63	* 4.33±0.09	5.03±0.43	4.80±0.93
Buffalo bulls.	4	3.02±0.39	3.71±0.17	3.71±0.52	2.93±0.76*

* denotes average of three observations.

TABLE - 4.6

Table showing quantity of Glycine (milligram/100 ml. of seminal plasma) present in deproteinized seminal plasma of Tharparkar (Zebu) and Murrah (Buffalo) bull under different hours of preservation.

	No. of observation	0 hour Average quantity with S.E.	24 hours Average quantity with S.E.	48 hours Average quantity with S.E.	72 hours Average quantity with S.E.
Zebu bulls.	4	5.88±0.90	* 7.94±0.48	8.11±1.36	7.37±1.49
Buffalo bulls.	4	12.60±1.21	13.51±0.93	23.87±1.76	14.64±1.89*

* denotes average of three observations.

TABLE - 4.7

Table showing quantity of Alanine (milligram/100 ml. of seminal plasma) present in deproteinized seminal plasma of Tharparkar (Zebu) and Murrah (Buffalo) bull under different hours of preservation.

	No. of obs- ervation	0 hour Average quantity with S.E.	24 hours Average quantity with S.E.	48 hours Average quantity with S.E.	72 hours Average quantity with S.E.
Zebu bulls.	4	0.61±0.77	* 2.75±0.87	3.50±0.80	3.42±1.14
Buffalo bulls.	4	2.28±1.47	2.42±0.90	1.61±0.76	1.11±0.79*

* denotes average of three observations.

TABLE - 4.8

Showing the amino acid content of seminal plasma (by chromatographic method; Larson & Salisbury, 1953)

Seminal Plasma (Bull)	mg/ml.
Aspartic acid.	0.09
Glutamic acid.	0.35
Serine .	0.13
Glycine .	0.09
Alanine .	0.25

TABLE - 4.9

Table showing effects of different hours of preservation on amino acid content of seminal plasma of Tharparker (Zebu) and Murrah (Buffalo) bulls.

	Aspartic acid		Glutamic acid		Serine		Glycine		Alanine	
	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.	d.f.	M.S.
Zebu bulls	0	0	0	0	0	0	0	0	0	0
Between hours.	3	26.716*	3	124.39 N.S.	3	158.92 N.S.	3	3.99 N.S.	3	7.26 N.S.
Within hours.	11	7.326	11	243.65	11	234.78	11	5.51	11	2.79
Buffalo bulls.	0	0	0	0	0	0	0	0	0	0
Between hours.	3	1.11	3	2286.47**	3	169.76 N.S.	3	257.43 N.S.	3	15.64 N.S.
Within hours.	11	4.43	11	193.97	11	231.45	11	163.27	7	14.82

N. B. :- * indicates significant at 5% level.

** indicates significant at 1% level.

N.S. denotes not-significant.

In case of Aspartic Acid content of zebu bulls seminal plasma the critical difference (3.586 for 4 replications and 4.532 for 3 and 4 replications) between different treatments revealed that there is difference in this acid content of seminal plasma between 0 and 48 hours of preservation and also between 0 and 72 hours of preservation but not among other hours of preservations.

In case of Glutamic acid content of seminal plasma of buffalo bulls it was found that the 72 and 48 hours of preservation differed significantly from each other (C. D. 9.85) whereas the 24 and 48 hours of preservation do not differ significantly but their difference is very close to the C. D. (9.768 as against the C.D. value 9.85).

TABLE - 4.10

Mean Gradation of progressive motile spermatozoa before and after preservation at different intervals.

	0 hour		24 hours		48 hours		72 hours	
	No. of observer- variation (n)	Mean motility + S.E.	No. of observer- variation (n)	Mean motility + S.E.	No. of observer- variation (n)	Mean motility + S.E.	No. of observer- variation (n)	Mean motility + S.E.
Zebu bulls.	4	4.75 ± 0.254	4	2.875 ± 0.076	4	2.250 ± 0.289	4	1.750 ± 0.333
				(60.52%)		(47.37%)		(36.84%)
Murrah bulls.	4	3.5 ± 0.28	4	1.89 ± 0.10	4	1.38 ± 0.37	4	0.75 ± 0.25
				(53.71%)		(39.42%)		(21.42%)

TABLE - 4.11

Analysis of variance of gradation of motile spermatozoa.

Source of variance	d.f.	S.S.	M. S.	F.
--------------------	------	------	-------	----

FOR ZEBU

Between intervals.	3	20.67	6.89	
Within intervals.	12	15.44	1.290	5.34*
Total.	15	36.11		

FOR BUFFALO

Between intervals.	3	20.54	6.85	
Within intervals.	12	4.94	0.41	16.71**
Total.	15	25.48		

N.B.: - * indicates significant at 5% level.

** indicates significant at 1% level.

GRAPH SHOWING THE DIFFERENT MAIN FREE AMINO ACIDS
CONTENT OF SEMINAL PLASMA OF THARPARKAR (ZEBU) AND
MURRAH (BUFFALO) BULL.

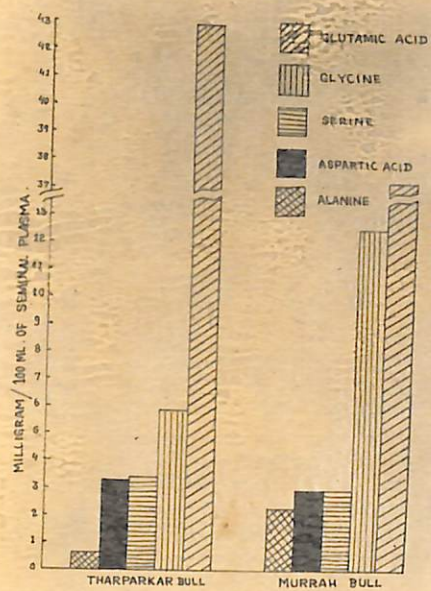


FIG - 8

AMINO ACIDS IN SEMINAL PLASMA OF THARPARKAR (ZEBU)
AND MURRAH (BUFFALO) BULLS.

Stein (1946) emphasized the necessity of having available two kinds of amino acid analytical techniques, "primary standard" and "routine". Primary method need not necessarily be a convenient, or rapid method, nor adaptable to handling of small quantities of protein. Olcott (1951) pointed to "isotope dilution method" which closely approached to the criteria of primary standards. The rest of the methods classified as routine may have some inherent experimental errors. Most of the estimated values for amino acids were obtained from microbiological assay, which too have certain limitations viz. changes in micro-organisms, synthetic potentialities of the organisms, presence of inhibitors in the hydrolysates or samples.

(1) Aspartic Acid:

It was observed that the estimated average values of aspartic acid calculated as mg/100 ml. of seminal plasma were initially (0 hour) 3.28 ± 1.05 and 3.03 ± 1.08 for zebu and buffalo respectively (Table - 4.3) and graphically represented in Figure 8. Seemingly close similarity between the two species was marked. Little information on the aspartic acid content of zebu and buffalo seminal plasma was available. The estimated values of aspartic acid in zebu seminal plasma obtained in the present study were not in close agreement with value reported by

Larson and Salisbury (1953); Table - 4.8. The higher value of aspartic acid found in exotic breed as reported by Larson and Salisbury (loc. cit.), might be due to difference in breeds and also due to difference in experimental procedures.

On preservation it was seen that the aspartic acid content increased gradually from 0 hour to 72 hours with maximum at 72 hours, 9.07 mg. in case of zebu; but the same is not true in case of buffaloes (Figure 9). At 24 hours of preservation the aspartic acid content increased from the initial concentration, then followed by decrease and again there was a sharp rise at 72 hours. The maximum concentration in respect of buffalo semen was at 24 hours preservation indicating thereby fluctuation in the concentration of aspartic acid on preservation.

In respect of zebu it can be easily inferred from the above observation and motility Table - 4.10 that as the concentration of aspartic acid increased the motility decreased from 39.48 to 63.16% ($P \leq 0.05$). So it can be said safely that increase in concentration of aspartic acid must be one of the contributing factors for the decline of motility of zebu semen.

The significant differences in aspartic acid concentration due to preservation supports the above finding (Table - 4.9).

Unlike the above findings the differences in the aspartic acid content at different hours of preservation in case

of buffalo was not significant (Table - 4.9).

The increase in concentration of aspartic acid on preservation might be due to proteolytic action of enzymes, signifying degradation of proteins with high molecular weight to smaller fragments and constituent amino acids (Lundquist, 1953). Moreover from the success of the protein-rich systems like peptone, gelatine, egg and milk in semen diluents and amino acid rich media, in their ability to maintain motility and fertility of sperms during storage, it may be inferred that besides exerting a protective action, the proteins may be broken down through successive enzymatic degradations to amino acids and simpler substances for energy purposes (Sharma, 1962).

The oxidative degradation of different amino acids leading to the formation of H_2O_2 in bull semen does not involve the five amino acids under present study (Tosie and Walton, 1950).

So in case of zebu, proteolytic action of enzymes is probably one of the main reasons in increasing the concentration of aspartic acid under preservation. Besides lack of deamination power might be the secondary probable reason.

As regards buffalo the reason or reasons for decrease in concentration after 24 hours become a point of consideration. The deamination process might be the only reason to be imagined at present level, owing to lack of information on buffalo semen.

(11) Glutamic Acid:

The estimated values of Glutamic acid calculated in mg/100 ml. of seminal plasma were 42.90 ± 10.20 for zebu and 37.33 ± 7.74 for buffalo at 0 hour (Table - 4.4) and graphically represented in Figure 8. There was a marked difference in the concentration between the two species. Literature was scarcely available on the glutamic acid content of zebu and buffalo seminal plasma except present estimated value of amino acid, was not in complete agreement with the value obtained by Larson and Salisbury (1953). However, the higher value for the glutamic acid among all the five amine acids confirmed the findings of Larson and Salisbury (loc. cit.). Hopwood and Gassner (1962) also reported that glutamic acid was predominant component in the seminal plasma of bull.

From Table - 4.4 and graphical representation in Figure 9, it was observed that the concentration of glutamic acid decreased gradually on preservation in case of zebu from 42.90 ± 10.20 to 28.00 ± 6.97 , whereas in case of buffaloes there was a decrease in concentration at 24 hours but it again rose at 48 hours and finally showed a decreasing trend in subsequent hours of preservation. This fluctuation in the concentration during different hours of preservation was significant (Table - 4.9).

In respect of zebu it can be inferred from the above observation that the deamination of glutamic acid might probably

be one of the factors in diminishing the concentration of glutamic acid leading to gradual decrease in motility. But, Martini (1947) reported that deamination of glutamic acid in bovine semen was negligible, when incubated at 37°C for 2 hours.

Setchell et al (1967) reported that testicular and ejaculated ram spermatozoa oxidized (U-¹⁴C) glutamate to a small extent and small amount of radioactivity was detected in protein from ejaculated ram spermatozoa after incubation with (U-¹⁴C) glutamate. This may be the probable second factor in decrease of the glutamic acid on preservation.

On the contrary in the case of buffalo the peak concentration of glutamic acid was recorded in 48 hours preservation. This may be accounted for by the fact that the proteolytic action of enzymes in the case of buffaloes might be starting late resulting in great accumulation of glutamic acid (due to enzymatic degradation of proteins), which could not counteracted by deamination process at equal rate immediately. The deamination process might be thought to be the only reason at present level, owing to lack of information on buffalo semen.

(III) Serine :

At 0 hour the estimated average value of serine in seminal plasma comes to 3.38 ± 0.63 and 3.02 ± 0.39 mg/100 ml. for zebu and buffalo bulls respectively (Table - 4.5 and figure 8). There was initially no marked difference between the

two species. The estimated values of serine were not in agreement with the reported value of Larson and Salisbury (1953), Table - 4.8. The values of zebu and buffalo were about one fourth of the concentration found in exotic breed (Larson and Salisbury; loc. cit.). Little literature was available for serine content in seminal plasma of zebu and buffalo.

From Table - 4.5 it was observed that the concentration of serine in both species gradually increased upto 48 hours of preservation, then there was a decline. There was a rise in concentration of 0.95 mg. at 24 hours and 1.64 mg. at 48 hours over the initial value in zebu seminal plasma, whereas 0.69 mg. rise in buffalo seminal plasma at 24 hours remained static upto 48 hours preservation.

From the findings of Joshi et al (1968) it might easily be inferred that serine plays an important role in maintaining the motility of spermatozoa (at a definite concentration) in both cases. The solution of 4% serine was found suitable for maintaining motility (14 - 20.8%) beyond 126 hours storage with the diluents of milk combined with 25% egg yolk.

Appreciable increase in concentration of serine upto 48 hours storage in case of zebu as compared to buffalo semen was noticed. This increase in serine might indirectly be correlated with the maintained motility picture upto 48 hours which was actually observed in zebu breed.

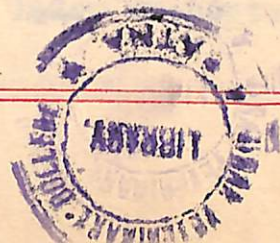
The fall in concentration after 48 hours of preservation in both cases might probably be due to deamination of serine, which was the only probable cause that could be thought of. In case of buffalo the fall in concentration of serine at 72 hours storage over the initial concentration might be the reason for rapid fall in motility.

Hours of preservation were found to little influence the serine content of seminal plasma for both zebu and buffalo bulls. This might be due to less number of observations and sampling fluctuation.

(iv) Glycine :

The estimated average value of glycine of seminal plasma were 5.88 ± 0.90 mg/100 ml. for zebu and 12.60 ± 1.21 mg/100 ml. for buffaloes at 0 hour (Table - 4.6 and Figure 8). This indicated species difference. But the findings of the present study did not conform with the reported value of exotic breed by Larson and Salisbury (loc. cit.); Table - 4.8). Little available literature on estimates of glycine content of seminal plasma of zebu and buffalo bulls were found.

From Table - 4.6 it was observed that in case of zebu there was increase in concentration of glycine upto 48 hours then there was a decline of 0.23 mg/100 ml. of seminal plasma, whereas in case of buffalo bull semen there was a gradual increase in



concentration upto 72 hours at a slower pace.

Statistical analysis revealed non significant effect of hours of preservation in case of zebu and buffalo bulls.

It could be inferred from the present findings that proteolytic action of enzymes might be one of contributing factors for increasing the concentration of glycine. The fall in concentration at 72 hours preservation in case of zebu might be due to sampling fluctuation.

The other probable reason would be that glycine was metabolized by the sperms. It had been well established by radio-isotope studies that glycine was metabolized by the sperms regardless of the presence or absence of glucose (Flipse, 1955a, 1956).

The beneficial effect of glycine solutions in combination with egg yolk or milk to increase survival time of bovine spermatozoa at 5°C is a well reported fact (Roy and Bishop, 1954; Flipse and Almquist, 1955, and Rakes and Stallcup, 1956).

Knoop and Krauss (1944) recommended that inclusion of glycine in egg yolk buffer diluent to improve its quality. This was also supported by Tyler and Tanabe (1952).

The observation of Roy (1959), also confirms its beneficial effect in preserving zebu and buffalo semen. Besides the works of Gabriel (1955), Bair et al (1957), Hahn (1957), Albright et al (1958) and Saha and Singh (1958)also supported

the beneficial effect of glycine in the bovine semen diluent on livability of sperms with improved survival.

Recent work had shown that glycine at 0.01 M concentration was detrimental to bovine spermatozoan survival (Roy and Bishop, 1954; Rakes and Stallcup, 1956) and where as 0.1 M glycine had been shown to increase spermatozoan survival (Roy and Bishop, 1954; Rakes and Stallcup, 1956 and Flipse and Almquist, 1955). From all these it could be concluded that at lower concentration glycine did not show beneficial effect. The estimated concentration of glycine in both zebu and buffalo fresh semen was far below the undesirable concentration of 0.01 M.

So the finding of other workers that extra addition of glycine to semen enhanced the livability on spermatozoa might have been due to the beneficial effect of its desirable concentration.

(v) Alanine:

From Table 4.7 and figure 8 the estimated value of Alanine for both zebu and buffalo bulls were found to be very low and even zero for some samples. The colorimetric readings of different samples of both zebu and buffalo bulls varied between 0 to 22, which was less reliable reading due to low optical density. However the estimated average values of alanine in seminal plasma were 0.61 ± 0.77 and 2.28 ± 1.47 mg/100 ml. at

0 hour for zebu and buffalo respectively. Little literature on estimates of Alanine content of seminal plasma of zebu and buffalo bulls were available. The reported data of exotic breed by Larson and Salisbury (1953), was very high which was not in agreement with the estimated value found during in the present study for both species. Breed difference and the procedure of estimation might be the reasons for this.

On preservation it was seen that the Alanine content of zebu seminal plasma increased gradually from 0 hour to 48 hours. A fall of 0.084 mg/100 ml. of Alanine from 48 hours to 72 hours was negligably low since it might be due to experimental errors, which was admissible upto 5% (Block and Zweig, 1958). On the contrary in case of buffaloes there was gradual decline in Alanine content from 2.28 mg. to 1.11 mg. on preservation.

The increase in concentration in zebu might be attributed to the proteolytic action of enzymes (Lundquist, 1953).

Similarly the decrease in concentration of Alanine in buffalo semen might be due to deamination of Alanine (Martini, 1947).

The work of Joshi et al (loc. cit.) showed the beneficial effect of Alanine using 4% solution with 25% egg yolk in maintaining the survival even after 126 hours of storage with motility of 14- 20.8%. The addition of 4% Alanine with mixed with milk diluent gave the best results in comparasion to other amino

acids in case of both Hariana and Murrah buffalo semen. On the basis of above findings it might be inferred that decline in Alanine concentration in buffalo semen might be one of the possible causes for the fall in motility.

(b) MOTILITY:

Mean gradation of progressive motile spermatozoa before and after preservation at $4 \pm 1^{\circ}\text{C}$ at every 24 hours interval upto 72 hours are given in Table - 4.10.

The figures within brackets indicate motility expressed in percentages over the corresponding value before preservation. The average motility was found to be + 4.75 and + 3.5 for zebu bull and buffalo respectively. It is evident from the ~~above~~ ^{4.10} table that the loss of motility due to preservation at $4 \pm 1^{\circ}\text{C}$ upto 72 hours ranged 39.48 to 63.16% for zebu bull and 46.29 to 78.58% for buffalo. The loss of motility from 0 hour to 72 hours was found to be gradual in case of zebu but in buffalo there was a sharp decline after 48 hours preservation. The results thus indicate distinct specificity in the keeping quality of zebu and buffalo semen. The differences in the keeping quality of semen may be discussed as one of the factors of metabolism or of aging potentiality of spermatozoa (Tomar and Desai, 1961 a, b, c). The metabolic pattern of semen may influence the keeping quality by increased metabolic rate or by higher phosphatase activity.

Both the phenomena will lead to increased acidity on account of lactic acid accumulation and thus may cut short the livability of spermatozoa.

The data on gradations of progressive motile spermatozoa were analysed statistically by the application of the analysis of variance to see whether hours of preservation influenced the motility or not (Table - 4.11).

From Table - 4.11 it revealed that the variations in gradation of progressive motile spermatozoa were highly significantly affected by the hours of preservation.

(c) CRYSTALLISATION PATTERN:

The crystallisation pattern of the fresh ejaculates and stored for 24 hours, 48 hours and 72 hours at $4 \pm 1^{\circ}\text{C}$ were studied in both zebu (Tharparkar) and buffalo (Murrah) bulls.

The dried films of zebu and buffalo semen gave no distinctly pictures. The spermatozoa of both semen were uniformly distributed in the film.

The present result was in agreement with the observation of Tomar et al (1966) in respect of buffalo (Murrah) but differed in respect of zebu bulls. The observations of Tomar et al (1966) were on Haryana bulls, whereas the present work on Tharparkar bulls. The breed difference might be one of the causes for

the differences. This might be attributed to specificities of chemical composition of seminal plasma (Tomar et al , 1966).

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RESULTS

Different from amino acids (e.g. Aspartic acid, Glutamic acid, Serine, Glycine and Alanine) were determined quantitatively in serumal plasma of Tharparkar and Murrah bulls at different hours (0, 24, 48 and 72 hours) of preservation at $4 \pm 1^{\circ}\text{C}$ by paper chromatography method.

Resolution of the constituent amino acids from the mixture was studied and was found satisfactory for aspartic acid, glutamic acid and alanine. Glycine spots were very close to each other. Aspartic acid was found to give better resolution of the amino acids.

CHAPTER - V

SUMMARY

Standard curves for the individual amino acids were prepared with amounts of the acid ranging from 0.05 to 0.5 micro mole of each.

In case of Tharparkar bulls, the average amount of aspartic acid, glutamic acid, serine, glycine and alanine were recorded to be 3.88 ± 1.08 , 37.90 ± 10.20 , 3.35 ± 0.63 , 0.55 ± 0.20 and 0.61 ± 0.27 $\mu\text{mole}/100\text{ ml}$ serumal plasma respectively; whereas in Murrah bull, they were 3.03 ± 1.08 , 37.35 ± 7.74 , 3.12 ± 0.32 , 12.26 ± 1.21 and 2.22 ± 1.47 respectively.

Significant increase in the aspartic acid content at 48 and 72 hours of preservation of serum over those of initial

S U M M A R Y

Different free amino acids (e.g. Aspartic acid, Glutamic acid, Serine, Glycine and Alanine) were determined quantitatively in seminal plasma of Tharparkar and Murrah bulls at different hours (0, 24, 48 and 72 hours) of preservation at $4 \pm 1^{\circ}\text{C}$ by paper chromatography method.

Resolution of the constituent amino acids from the mixture was studied and was found satisfactory for aspartic acid, glutamic acid and alanine; but serine and glycine spots were very close to each other. Re-development technique was found to provide better resolution of the amino acids.

Standard curves for the individual amino acid were prepared with amounts of the acid ranging from 0.02 to 0.2 micro mole of each.

In case of Tharparkar bulls, the average amount of aspartic acid, glutamic acid, serine, glycine and alanine were recorded to be 3.28 ± 1.05 , 42.90 ± 10.20 , 3.38 ± 0.63 , 5.98 ± 0.90 and 0.61 ± 0.77 mgs/100 ml. seminal plasma respectively; whereas in Murrah bull, they were 3.03 ± 1.08 , 37.33 ± 7.74 , 3.02 ± 0.39 , 12.60 ± 1.21 and 2.28 ± 1.47 respectively.

Significant increase in the aspartic acid content at 48 and 72 hours of preservation of semen over those of initial

concentration was observed in Tharparkar bulls. On the contrary, in case of buffalo bulls aspartic acid content of seminal plasma was not affected due to different hours of preservation.

Non-significant differences in glutamic acid content in seminal plasma at different hours of preservation in Tharparkar bulls, was recorded. But in buffalo (Murrah) bulls its content in seminal plasma at 48 and 72 hours of preservation deffered significantly from each other whereas the content at 24 and 48 hours of preservation did not differ significantly.

The different Amino acid content of seminal plasma of Tharparkar and Murrah bulls viz. Serine, Glycine and Alanine were not influenced by the hours of preservation.

Significant decline in motility due to preservation in case of both Tharparkar and Murrah bull semen was observed.

Unlike the Hariana bull semen no crystallisation pattern was found in Tharparkar bull semen.

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