Factors Affecting the Maximum Yield of Non Protein Nitrogen From Skeletal Muscles

Pages with reference to book, From 116 To 125
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Abstract

Attempt was made to study the factors that affect maximum yield of nonprotein nitrogen from various skeletal muscles. The results showed that sonification was the most, effective method out of the various extraction procedures used for the extraction of NPN, while the use of tungstic acid as protein precipitant was most suitable. The pH studies showed that pH 2 gave the optimal yield of NPN. Results of the coloured sample dilutions showed exponential relationship between optical densities and the concentralions of NPN used. The results are discussed in terms of the factors affecting maximum yield of NPN along with the significance of the term half optical density dilution. (JPMA 35 : 116, 1985).

Introduction

Proteins constitute the major building material of all the animal tissues. These are inturn composed of amino acids in which amino group is an essential part. Amino acids are also present in the free form in all the tissues of the animal, particularly in the muscles. The free amino acid pool of muscles especially 3-methylhistidine is an index of the rate of degradation of muscle proteins[1,2] Generally, the changes in the muscle proteins are measured either by directly estimating the quantity of proteins or by indirect estimation of free amino nitrogen. For skeletal muscles, usually the indirect method of assessing the rate of protein turnover is used because of the obvious reason of comparatively difficult solubiity of skeletal muscle proteins. Thus, amino nitrogen estimation for the study of net changes in muscle protein composition represents a much simpler approach to the problem and also helps in making a large number of observations. In addition, it is an important factor especially during investigations of the physiological changes occuring in the proteins of the diseased muscle

A variety of methods have been used to determine amino nitrogen. Earlier methods included the formol titration method[3] and a gasometric technique[4]. In the formol titration method, an adduct is formed at the amino group when formaldehyde is added to a solution of amino acids and free carboxyl groups are then titrated using phenolphthaline as an indicator. In the gasometric technique, the nitrogen formed by reaction of amino acids with nitrous acid, is measured. Both these methods however, suffered a lack of specificity. However, the techniques most commonly used presently include Nesslerizalion method[5] and Hardings ninhydrin method[6] for the estimations of non protein nitrogen and amino nitrogen respectively.

The maximum yield of a substance does not merely depend on the method of estimation used. It is also affected by various other factors, e.g. method of extraction, use of a suitable protein precipitant and pH of the protein free extract. In addition, dilution of the coloured samples is also important. Usually, the quantity of skeletal muscle tissue available for biochemical estimations is fairly large even in diseases of human muscles. In such conditions, if the colour intensity goes beyond the range of the standard curve, the only alternative is to dilute the samples to the readable intensity. To obtain the maximum yield of nonprotein nitrogen, a project was undertaken to study various factors which could affect the maximum yield of nonprotein nitrogen from skeletal muscles.

Material and Methods

1. Animals and Dissection:

The experiments were performed on skeletal muscles obtained from both the sexes of rats (Wister strain) weighing I 70-370g, rabbits (Oryctalogus cuniculus) weiging 0.7-1 .7kg and from pigeons (Columba levia) which weighed 200-400g. Whenever desried, the rats and rabbits, under ether anaesthesia, were killed by opening the thorax and the while diaphragm was dissected out. The two hemi-diaphragms were seperated from each other, the blood was wiped off and their attached bones and tendons were removed. The hemidiaphragngms were then weighed immediately. Similarly, a portion of the brachialis major muscle was dissected out from pigeon breast under ether anaesthesia and was weighed after wiping off blood. In addition, the sartorius muscles of both the rabbit and pigeon were dissected out from the thigh, their bones and tendons were removed and weighed.

2. Methods for tissue Extraction

In order to select a suitable method which would give optimal results, various, techniques were used for the prepan'tion of samples.

a) Homogenization: Pre-weighed rat hemidiaphragrn and pigeon sartorius muscles were chopped in 0.5ml distilled water and the contents, along with an additional . 1.5m1 distilled water, were transferred to a rough surface Pyrex 16 x 150mm tissue hand grinder and homogenized gently for about 30 minutes. The homogenized samples were then shifted to lOmi plastic centrifuge tubes and after precipitating proteins with 3ml of absolute ethanol, the tubes were centrifuged at 7000 rpm for 15 minutes in a Polish Unipan centrifuge, Type 3 10. The clear, transparent supernatents were then transferred into air tight sample bottles and refrigerated at 10°C.

b) Acetone Chopping and Acetic Acid

Heating: Preweighed rat hemidiaphragms and pigeon sartorius muscles were chopped in 0.5ml absolute acetone and the contents were transferred to glass test tubes containing 3m1 of 1% acetic acid. The tubes were heated for 10 - 15 minutes in a boiling water bath with the precaution to keep the test tube temperature below 40°C. Since heating of samples in acetic acid also coagulated proteins, the samples were directly centriguged for 10 minutes at 7000 rpm. The supernatents so obtained showed slight turbidity which could not be removed by absolute ethanol and recentrifugation. These samples were therefore, neutralized by a few drops of 1% $NaHCO_3$ and recentrifuged further for 10 minutes. The clear supernatants so obtained were stored at 10°C.

c) Boiling in Distilled Water Preweighed pigeon brachialis major muscles were either chopped in 0.5m1 distilled water or absolute acetone and transferred to test tubes containing 3.5m1 distilled water and heated for 10 - 15 minutes in a boiling water bath. The prcteins were precipitated by 1 ml absolute ethanol and the contents were centrifuged at 7000 rpm and the clear supernatents were stored at 10°C.

d) Sonification: Preweighed rat hemidiaphragm and rabbit and pigeon sartorius muscles were chopped in 0.5ml distilled water and the contents were transferred to a l0ml plastic centrifuge tube along with I ml additional distilled water. These samples were than insonated in a water bath for 15 minutes at 12000 Hz/sec using a sonic Dismembrator, Model 150 (Systems Corporation, Farmingdale, New York) according to the method described in Operating instructions leaflet. This was followed by the precipitation of proteins by 3ml absolute ethanol. The samples were centrifuged and the clear supernatents were stored. In those cases where effect of sonification time was studied on the extraction of free amino acids, the pigeon brachialis major muscles were insonated for 3, 6, 9, 12, 15 and 18 minutes. The proteins were precipitated as usual and after centrifugation, the clear supernatents were stored. In cases where the effect of periodical insonation was studied, 1 minute insonation was followed by a 5 minute re t, upto a total of 1 8 minutes of insonation.

3. Protein Precipitants

The protein precipitants used were absolute ethanol, sodium tungstate and 1 5% trichloroacetic acid (TCA). in cases of alcohol, 3m1 absolute ethanol was added to clear supernatents (pH 7). Biuret tests

were also performed to check the presence of proteins. In some cases, ethanol was evaporated from the clear supematents by heating samples in a water bath and the evaporated alcohol was replaced by equal amounts of distilled water. These aquous samples were then used for the estimation of nonprotein nitrogen.

Whenever sodium tungstate was used as protein precipitant, 1 ml of $NaWO_4$. $2H_2$ O and 1 ml of 2/3N H^2 SO_4 were added into each sample tube (pH 2). The samples were centrifuged and the clear supernatents were used for the estimation of nonprotein nitrogen. The presence of proteins was again checked by biuret test. Whenever TCA was used as protein precipitant, 5ml of 15% TCA was added into each sample tube (pH 2) followed by the usual procedure of nonprotein nitrogen estimation.

4. pH Fixation:

The effect of pH on nonprotein nitrogen estimation was studied in 3 series of experiments. in the 1st series, the proteins were precipitated by different precipitants and the pH of the extract was adjusted as follows:

Absolute ethanol (3 ml) pH reduced to 5.0 Sodium tungstate (2m1)_pH increased to 7.0 TCA 15% (5 ml)_pH increased to 7.0

In the 2nd series, 3 mixtures were prepared, each containing representatives of acidic, basic and neutral amino acid. The composition of these mixtures was as follows:

Mixture 1: The total concentration of amino acids in this mixture was 0.54 mg/ml and it contained the following amino acids: Aspartic and glutamic acid (acidic), lysine and arginine (basic) and leucine and glycine (neutral).

Mixture 2: The total concentration of amino acids in this mixture was 1 mg/mI and contained aspartic and glutamic acids (acidic), lysine and arginine (basic) and leucine, cystein, glycine, cysteine and tyrosine (neutral).

Mixture 3: The total concentration of amino acids in this mixture was 0.9 rng/ml and contained aspartic and glutamic acids (acidic), arginine and lysine (basic) and methionine, cystein, glycine, tyrosine, phenylalenine and cysteine (neutral).

The above mixtures were prepared by dissolving 0.1 gm of each amino acid in 1 litre of distilled water along with a few drops of concentrated HC1. For adjusting the pH, 90ml of the mixtures were taken in different bottlesand the pH was set at 8.0, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.0 and 2.0 with o.1N HC1 or NaOH using a Pye Unicam pH meter, Model-292. The changes in the volume of mixtures were also noted. in addition, a leucine standard of known concentration (107 ug/ml) was also prepared and its pH was set either at 7.0 or 2.0.

5. Sample Dilutions and Optical Densities:

For the study of the effect of sample dilution on the optical density of the coloured solution, nesslerized sampes of high intensity colour were prepared. These were then diluted to various degrees with distilled water and their O.D. was read at 500 nm,

6. Estimations of Tissue Nitrogen:

The nonprotein nitrogen was estimated by the standard nesslerization method[5] where as the amino nitrogen was estimated ninhydrin methods[6]. by Hardings ninhysrinmethod[6].

Results

1. Effect of Extraction Methods:

The results of various extraction methods on the yield of amino nitrogen from various skeletal muscles are shown in Table 1.

Table I

Effect of various extraction methods on the yield of free amino acids from skeletal muscles.

S. No.	Extraction Methods		Muscles	Amino Nitrogen	(mg%)	Biuret Test
1.	Homogenization	a)	Rat Diaphragm	0.030	(4)	Nil
		b)	Pigeon Sartorius	0.032	(3)	
2.	Chopped in acetone and heated in 1% acetic acid.	a)	Rat Diaphragm	0.018	(3)	+ ve
		b)	Pigeon Sartorius	0.025	(3)	+ ve
		c)	Pigeon Brachialis Major	0.045	(2)	+ve
3.	Boiled in distilled water					
	a) 10 min.	a)	Pigeon Sartorius	0.064	(3)	-ve
		b)	Pigeon Brachialis major	0.061	(3)	-ve
	b) 15 min.	c)	Pigeon Brachialis Major	0.046	(3)	-ve
4.	Dried in acetone and boiled in distilled water					
	a) 10 min.	a)	Pigeon Brachialis Major	0.049	(2)	-ve
	b) 15 min.	b)	Pigeon Brachialis Major	0.045	(3)	-ve

When muscles were chopped in acetone and heated in 1% acetic acid, the amino ,nitrogen yield from rat diaphragn and pigeon sartorius muscles was 40% and 22% smaller then those estimated from the homogenised samples where absolute ethanol was used as protein precipitant. The Biuret tests were positive in these cases indicating the presence of proteins. Amino acid extraction was comparatively much greater when pigeon sartorius and brachialis major muscles were boiled in distilled water for 10 minutes. A prolonged boiling reduced the yield of amino nitrogen by 25%. In pigeon brachialis major, acetone drying of the muscles prior to boiling in distilled water, reduced the amino nitrogen yield in the same muscles by about 20%. In addition, prolonged boiling for 15 minutes further reduced the amino nitrogen yield by about 8%.

Extraction of free amino acids by continued insonation of the chopped pigeon brachialis major muscle samples for 12 to 18 minutes, along with the use of absolute ethanol as protein precipitant, gave the maximum yield of amino nitrogen (Table II).

Table II

Effect of sonification time on the extraction of free amino acids from pigeon brachialis major muscles.

S. No.	Sonification Time (min)	Amino Nitrogen (mg%)
1.	3	0.060
2.	6	0.056
3.	9	0.059
4.	12	0.071
5.	15	0.065
6.	18	0.073
7.	1 - 15*	0.075

*In this case, 1 minute sonification was followed by a 5 minutes rest upto a total of 15 munutes sonification.

This yield of amino nitrogen was greater than those obtained by any of the methods described earlier. Periodical insonation of the muscles had no significant effect of the amino nitrogen yield.

2. Effect of Protein Precipitants

Effect of protein precipitants was studied on samples where free amino acids were extracted by insonation of the muscles for 15 minutes. The use of tungstic acid as protein precipitant gave the optimal yield of amino nitrogen from pigeon sartorius and brachialis major muscle as compared to absolute ethanol (Table III).

Table –III

Effect of various protein precipitants on the estimation of amino nitrogen. Free amine acids were extracted from skeletal museles by insonation at 12000 Hz/sec. for 15 minutes.

S.No.	Protein Precipitants		Muscles	Amino Nitrogen (mg%)		Biuret test
1.	Absolute Ethanol	a)	Rat Diaphragm	0.054	(10)	-ve
		b)	Pigeon Sartorius	0.073	(10)	-ve
		c)	Rabbit Sartorius	0.073	(4)	-ve
		d)	Pigeon Brachialis Major	0.070	(1)	Nil
2.	Absolute Ethanol (evaporation of alcohol and addition of water)		Rabbit Sartorius	0.052	(4).	Nil
3.	Tungstic Acid	a)	Rabbit Diaphragm	0.038	(4)	-ve
		b)	Pigeon Sartorius	0.101	(8)	-ve
		c)	Pigeon Brachialis Major	0.090	(3)	-ve
		d)	Rabbit Sartorius	0.045	(3)	-ve
4.	TCA (15%)		Rabbit Sartorius	0.047	(2)	Nil

However, the use of tungstic acid instead of: absolute ethanol reduced the amino nitrogen yield by about 34% in the rabbit sartorius muscles. Similarly, evaporation of ethanol and its replacement with distilled water also reduced amino nitrogen yield by about 23%. The use of 15% trichloro-acetic acid gave almost the same yield of amino nitrogen as was obtained with tungstic acid. It was further observed that irrespective of various protein precipitant used, the pigeon muscles had maximum amounts of amino nitrogen. The Biuret tests were always negative for different protein precipitants.

3. EffectofpH:

The initial pH of the insonated samples, after the use of absolute ethanol, tungstic acid and 15% trichloroace tic acid as the protein precipitants, was 7.0 and 2.0, respectively. When the pH of ethanol precipitated sample was reduced to 5.0, the yield of amino nitrogen from rabbit sartorius muscle decreased by about 66% (Table IV),

Table – IV

Effect of various protein precipitants and pH on the estimations of amino nitrogen. The extraction was carried out by insonation at 12000 Hz/see for 15 minutes.

. No	Muscle	Protein Precipitants	pH	Amino Nitrogen (mg%)	
1.	Rabbit Sartorius	Absolute Ethanol	pH 5	0.021 (3)	
				pH 7	0.068 (4)
2.	Rabbit Sartorius	Tungstic Acid	pH 7	o.022 (3)	
	Pigeon Brachialis Major	Tungstic Acid	pH 7	0.060 (3)	
				pH 2	0.090 (3)
3.	Rabbit Sartorius	TCA (15%)	pH 7	0.022 (3)	
				pH 2	0.047 (2)

Similarly, an increase in the pH of tungstic acid precipitated samples from 2.0 to 7.0, decreased the amino nitrogen yield by about 50% and 33.% in the rabbit sartorius and pigeons brachialis major

muscles. A similar increase in the pH of TCA precipitated samples from 2.0 to 7.0 was again found to decrease the amino nitrogen yield from rabbit sartorius muscle by about 50% (compare Table III and IV). These results clearly indicated the dependence of aminonitrogen yield on the pH of the samples after protein precipitation.

Effect of pH on the percent yield of amino nitrogen from various amino acids mixtures of known concentration demonstrated that the maximum yield was possible only at the lower pH of 2.0 (Fig. I).

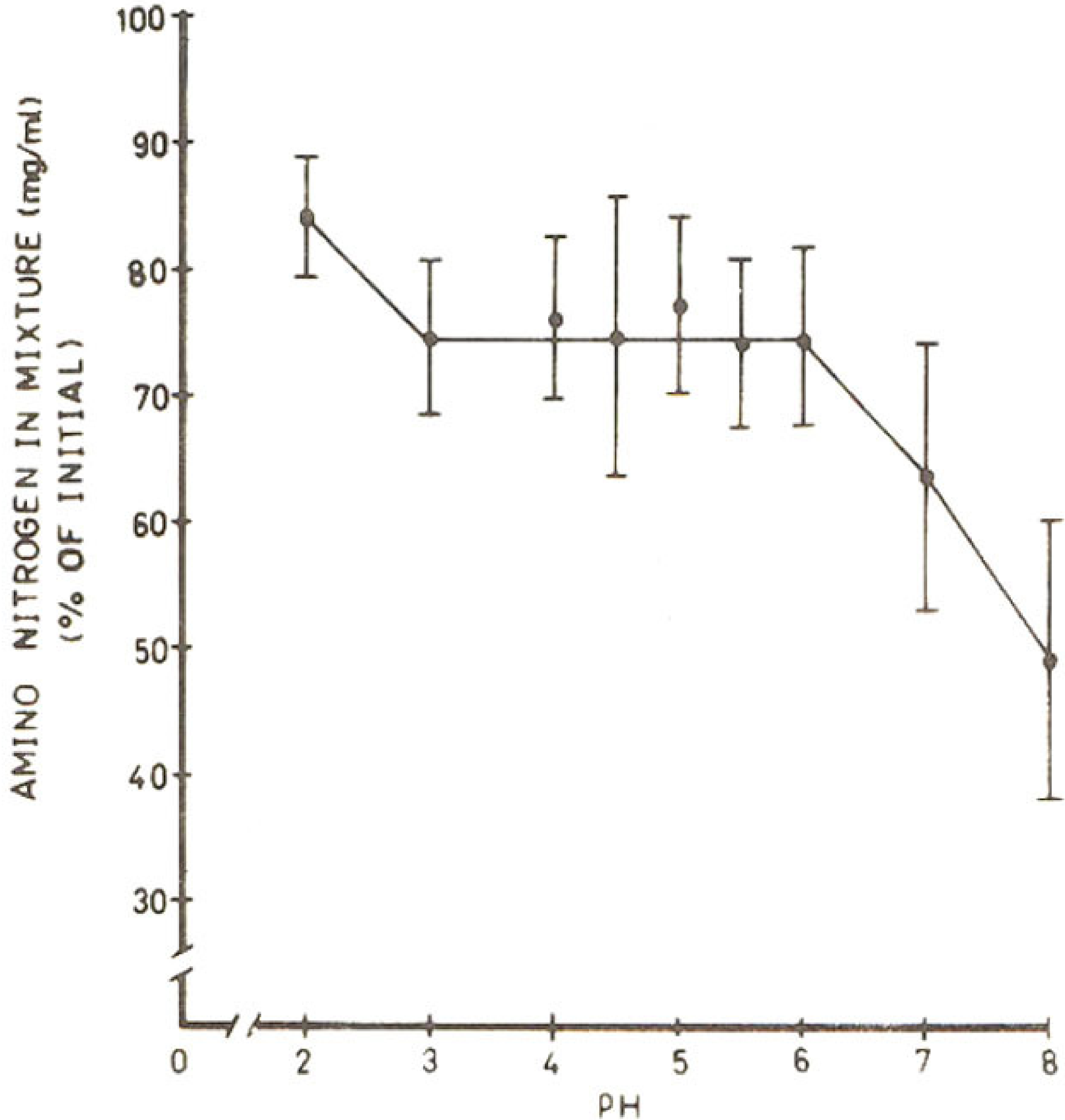

Fig. 1 Effect of pH on the % yield of amino nitrogen

An increase in pH from 3.0 to 6.0 reduced the amino nitrogen hield comparatively but the values remained more or less the same in this pH range. However, a further increase in pH to 7.0 or 8.0 had very prominent and significant drecreasing effect on the amino nitrogen estimation.

Since different methods were used for the estimations of amino nitrogen (Hardings ninhydrin method) and nonprotein nitrogen (Nesselerization method) and since our darlier experiments showed a high pH sensitivity for amino nitrogen yield, both the amino nitrogen and NPN were also estimated from the leucine standards of known concentration (107 pg/nil) at pH 2.0 and 7.0. The amino nitrogen was again found to be pH sensitive as the maximum yield was obtained at pH 2.0. (Table V).

Table V

Effect of pH on the estimation of amino nitrogen and NPN from the known leucine standard (107 μg/ml).

S.No.	Estimations	pH of Leucine Standard		P
		7	2	
1.	Amino Nitrogen (ug/ml)	98 ± 2 (26)	109 ± 3 (21	($P < 0.005$)
2.	NPN (ug/ml)	119 ± 6 (24)	116 ± 10 (21)	($P > 0.05$)

However, NPN estimations by Nesseleri. zation method gave slightly higher values and was not pH dependent. In addition, amino nitrogen and NPN values estimated at pH 7.0 showed a + significant difference between the two methods of estimations, the difference being highly significant ($P < 0.0005$). Similar estiniations at pH 2.0 gave more or less the same results, with there being no significant difference between the two methods of estimations used ($P < 0.05$).

4. Effect of Coloured Sample Dilutions on the Optical Density:

Effect of various degrees of coloured sample dilutions on the optical density was found to have an exponential relationship with the degree of colour dilutions (Fig. 2).

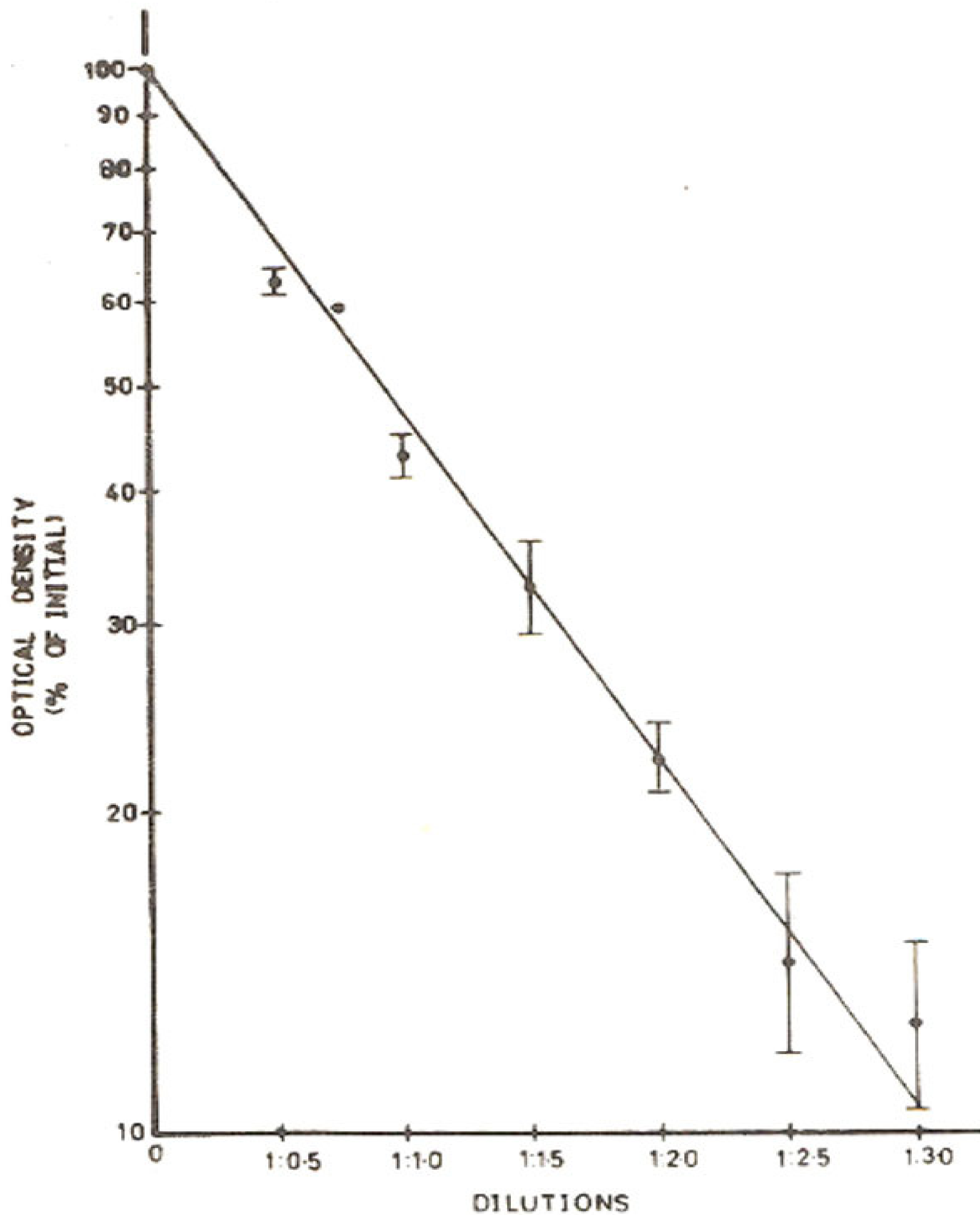

Fig. 2. Exponential relationship between sample dilution and the optical density.

In many of the experiments, the exponential straight line had two components, an initial slightly faster component and a later comparatively slower component. In order to differentiate between these two components, half optical density dilution (half 0. D. dilution) and minimum and maximum optical densities were also calculated (Table VI).

Table VI

Maximum and minimum optical densities and half O.D. dilutions read from the exponential sample dilution curves. The samples were diluted to various degrees with water after Nesslerization.

Expt.	Maximum Optical Density	Minimum Optical Density	Half O.D. Dilutions
1.	0.190	0.025	0.88
2.	0.180	0.030	0.80
3.	0.130	0.035	1.20
4.	0.610	0.150	1.05
5.	0.560	0.240	0.80
6.	0.210	0.055	0.80
7.	0.508	0.150	1.10

These results demonstrated that a 1 1 dilution of the coloured samples reduced O.D. by about 47%of its initial vilue instead of 50%. Thus, in those cases where half O.D. dilutions had a value less than l, it represented a greater decrease in optical density in relation to the degree of dilution. Similarly, a half O.D. value greater than 1 represented a lower rate of decrease in O.D.

Discussion

The present results showed the involvement of a number of factors which affected the maximum yield of amino nitrogen from various skeletal muscles. Thus, studies on extraction methods of nonprotein nitrogenous substances, i.e. hand homogenization, heating of muscle samples in 1% acetic acid or boiling the wet or acetone dried tissues in distilled water, did not prove satisfactory since neither of them were good enough for complete extraction. The maximum yield of non protein nitrogen was obtained only when extraction was carried out by disrupting the cell membrane with the help of an

ultrasonic vibrator or the sonic dismembrator. The rupturing of outer cell wall by ultrasound takes place by a machine-gun like phenomenon whereby extremely small cavitation bubbles, driven at very high speed from the probe tip of the sonic device, actually penetrate through the cell wall and disrupt its integrity[7]. This method of extraction has also been shown to be most satisfactory for extraction of enzymes and other substances from microorganisms[7]. In addition, ultrasound energy has also been used extensively now-a-days for diagnostic purposes in various fields particularly in muscle diseases[8]. Although, there are some disadvantages in the use of ultrasound, e.g. during high velocity virbations, heat is produced which can destroy some enzymes and proteins, but this can be prevented by carrying on sonification in a cold water bath[7]. The efficiency of this method is actually dependent on the power delivery of the instrument and also on the ability to keep a given cell within the effective area of the probe for a sufficient length of time so Lhat complete disruption of cells takes place.
For skeletal muscle, which usually have a large amount of connective tissues present, the most effective ultrasound frequency for complete disruption of the cell was 12000 Hz/sec provided that insonation was carried out uninterrupted for 15 minutes. Further, the use of l0ml plastic centrifuge tubes for insonation, using the probe of sonic dismembrator from Artex Systems Corporation, Formingdale, New York, helped to keep all the muscle cells within 2mm distance around the probe and thus in the maximum extraction from the cells. The advantage of this method is that the chances of errors are very small and the extraction is maximum. It was also found that 15 minutes sonification was sufficient for complete extraction and that continued or periodic sonification gave the same results..
The presence of protein interferes with many chemical determinations, primarily the analysis for compounds containing amino nitrogen and those involving reduction or oxidation of a metal ion. Proteins must be removed before such analysis can be made. Such separations are carried out by taking advantage of the unique nature of the protein molecule, e.g. the size and colloidal nature in solution. Proteins and high molecular weight polypeptides can be separated from low molecular weight compounds such as urea, glucose, creatinine etc. by dialysis, ultrafiltration and contrifugation as these giant molecules do not pass through the semipermeable membranes. Further, many substances are capable of causing proteins to precipitate. The choice of protein precipitant in an analytical procedure is dependent on a number of factors, including the type of proteinaceous material to be precipitated, other materials affected, possible interference of excess precipitant in the filtrate and later steps in an analytical procedure as well as ease of removal of the precipitant from the protein free filtrate[9]
Proteins are generally precipitated from solutions by salts of heavy metals (e.g. $HgC1_2$, $CuSO_4$ etc.) ; by certain acids (tungstic acid, trichloro acetic acid); by concentrated solution of salts (e.g. ammonium sulfate, sodium sulfate) and by dehydrating agents like ethyl alcohol and acetone. Although these reactions have been used for many years for the separation and characterization of proteins, there is still no definite evidence conceming the nature of the mechanisms involved[10] Some of this uncertainty is also due to the experimental difficulties involved in the isolation of the pure products formed in these reactions.
In general, the use of a protein precipitant has a two fold effect on the tissue sample preparation, particulary when one is not interested in the precipitated protein itself. Firstly, it precipitates proteins and secondly it changes the pH of the solution, the pH being dependent on the type of protein precipitant used. Since our major interest was to obtain a protein free muscle extract which could be used for amino nitrogen estimations, the choice of a suitable protein precipitant was of utmost importance. We ‘thus, used three different protein precipitants, i.e. ethyl alcohol (absolute), tungstic acid and TCA to study their effects on the maximum yield of amino nitrogen from muscle extracts. In our experiments, the maximum amount of amino nitrogen was estimated from those muscle extracts where proteins were precipitated by tungstic acid and the use of this acid had changed the pH of the extracts froni neutral to acidic (pH 2). The protein free extracts were crystal clear and the biuret tests

for this reaction were negative, indicating the complete removal of proteins. Similar pH change (from neutral to pH 2) was also observed with the use of 15% TCA but the amount of amino nitrogen estimated was comparatively less. In case of acids, the evidences suggest[10] that the proteins combine with the negative or acidic radical only to form insoluble salts of proteins on the acidic side of their isoelectric points. Since Biuret test was also positive for TCA and the protein free extracts were not crystal clear, as was also observed with tungstic acid, the presence of short chain peptides is suggested to be the interfeanng factor in the colorimetric determination of amino nitrogen. These extracts could however, be made crystal clear by heating the mixture for a few minutes at 90 - 95°C. Similarly, the addition of alcohol to electrolyte free solutions of proteins, converts them to suspensoids, which flocculate upon the addition of a few drops of salt solution. Precipitation by alcohol is most effective at the isoelectric point of the proteins. In our experiments, absolute alcohol was added to an electrolyte containing extract. Therefore, all the protein precipitation occured in the presence of muscle's own electrolyes. The pH of this mixture was 7. However, protein precipitation was complete as the biuret test was negative. On the bases of these results, it is suggested that the use of a given protein precipitant had its own effect on the maximum yield of amino nitrogen in our experiments.

Since pH was also suspected to be inter-fearing the estimations of amino nitrogen from muscle protein free extracts as well. For this purpose, the pH of tungstic acid and TCA precipitated muscle extracts was brought to 7 while the pH of ethanol precipitated samples was changed to 5. From all these samples,- significantly less amount of amino nitrogen was estimated, indicating that the pH of protein precipitants had a direct effect on the estimation of amino nitrogen In order to confirm further the dependibility of maximum amino acids yield on the pH of the solution, 3 mixtures of different amino acids were prepared by setting each at different pH. The results of these experiments again showed the maximum yield of amino nitrogen to be occuring at pH 2. This again indicated the influence of pH on amino nitrogen estimations, i.e. the maximum yield could be obtained only at acidic pH of 2.

The Hardings ninhydrin method used for the estimation of amino nitrogen, suggests to keep the pH of the solution in between 5 and 7. Further, it is suggested that a few drops of pyridine or a few crystals of sodium acetate are to be used to adjust the pH. In our experiments however, we have found that a. lowering or increase in pH reduced the amount of amino nitrogen estimated. This is probably due to the reason that we have used HCI and NaOH for lowering and increasing the pH of the solution. It is the presence of HCI or NaOH in the solution which probably interfered either in the conversion of ninhydrin into hydrindantin or in the conversion of the later into Rheuman's purple.

The nonprotein nitrogen (NPN) of the muscle is a collective concept and -includes the nitrogen present in all of the -constituents of the muscle which are not precipitated as proteins. Included in this fraction of muscles are urea, uric acid, creatinine, creatine, amino acids, glutathione and other compounds in small amounts, some of which are of unknown structure[10] The NPN content of muscles was estiniated by nesslerization method using the same muscle extract that was used for the estimation of amino nitrogen. Since a number of different proteins precipitants were used during muscle extraction, the pH factor was again involved in these estimations. Thus, in an attempt of amino nitrogen and NPN estimations from leucine standard using Harding ninhydrin and nesslarization methods at pH 2 and 7, the NPN yield was about 8.4% to 11.2% greater than that present in the standard and there was no effect of pH. The amino nitrogen was however, estimated about 9% less at pH 7 and this difference was significant statistically ($p < 0.001$). In addition, estimated amino nitrogen and NPN vaIues at pH 2 were almost same with there being (no significant difference between the two ($P > 0.05$). These results again indicated the dependibility of amino nitrogen estimations on the pH of the solution.

In the nesselarization method, the protein free filtrate is heated with alkaline copper solution, using a Folin-Wu tube to prevent reoxidation. The cuprous oxide formed is treated with a phosphomolybdic acid solution, blue colour being obtained which is compared with that of a standard. In the Hardings ninhydrin method, the

NH_2 group of amino acids is oxidized by reaction with ninhydthi to form an aldehyde along with the liberation of a molecule of each of ammonia and carbon dioxide.
The amino nitrogen estimations in our experiments were based uponthe use of a leucine standard curve. In general, 95% of the leucine standard curves constructed at various time periods had an angle below 450 and only in 5% trials, the curves were found to be perfect and passing at 450 angle. These latter standard curves were rarely obtained, usually after a large number of trials and were not feasable experimentally. However, if the former standard curves were chosen for calculation purposes, 10% less amino nitrogen was calculated. Thus, an standard curve of the mean values of 19 individual trials was constructed. This was found to be more close to the range of everyday estimations. We therefore, suggest to use a standard curve of mean values rather than a 450 angle perfect standard curve.
A general difficulty which the investigators usually encounter duritig nitrogen estimations is the higher intensity colours of the samples which some times even go beyond the range of the standard curve. Under such conditions, the samples are required to be diluted. In our experiments, when such high intensity coloured samples were diluted by equal volumes of distilled water, the optical density did not decrease to half but remained below 50% (Table VI). These results were in contradiction with Beer's Law which states that "optical density is directly related with concentration". When the results were however, plotted on a semilog graph paper, a straight line was obtained indicating an exponential relationship which could be expressed by the general exponential equaltion:
It = Iie-kt
Wher It = Intensity of transmitted light
Ii = Intensity of incident hight
e = Exponential
k = A constant which is known as absorption coefficient for 500nm wavelength and the absorbing medium used.
t = Time constant
From this exponential curve, half optical density dilution was calculated (Table VI). We define half optical density dilution as that dilution at which the optical density is reduced to half its initial value. The significance of calculating this parameter is that if half optical density dilution is one, multiplication factor will be two to obtaine the initial O.D. and if it is less than one or greater than one, the multiplication factor would be increased or decreased accordingly following the exponential equation given above.

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