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Base-combining capacity of muscular tissue and muscle proteins from some fish and invertebrates.

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Base-combining Capacity of Muscular Tissue and Muscle Proteins from some Fish and Invertebrates.

Just as the properties of blood as a carrier of carbon dioxide depend upon the acid-base equilibrium in corpuscles and plasma, so the properties of muscular tissue as a mechanism where lactic acid is formed and removed during functional activity are dependent upon the acid-base equilibrium in the tissue. The muscle is conceived as consisting, among other things, of protein partly ionised as acid, of other weak acids such as phosphoric acid and phosphoric acid esters and of strong base, mainly potassium. The potassium salts of these weak acids constitute the buffer substances of the muscle, and of these the proteins are probably the most important.

The means for carrying out the investigation of the part played by these substances are only partially developed. The experiments to be described represent a preliminary treatment of part of the problem, directed mainly to characterising the proteins of the muscle in their capacity as acidic substances. For this purpose the tissue proteins were treated all together and no attempt has been made to fractionate them.

METHODS.

The principle of the method used is that in the presence of alcohol and formal in the acid dissociation constants of organic substances are greatly increased so that weakly acid and amphoteric substances can be titrated with caustic soda using Phenol Phthalein as an indicator. To do this successfully with tissues or proteins from tissues the material must have time to react but there must not be excess of alkali present. Suitable conditions for the purpose are obtained by treating the material with Sodium Phosphate (Na_2HPO_4) in the presence of alcohol and formalin, filtering and titrating the filtrate. The usefulness of the method depends upon the fact that the reaction of pure Na_2HPO_4solution is very close to the turning point of Phenol Phthalein and that the relationship is not altered by alcohol and formalin. With suitable proportions of these reagents the proteins are precipitated while the acid phosphate is not. At the same time large excess of alkaline phosphate can be used without having the filtrate too highly buffered by it. The reaction with the tissue can be written $Na_2HPO_4 + HA = NaH_2PO_4 + NaA$ and in the titration $NaH_2PO_4 + NaOH = Na_2HPO_4 + H_2O$ so that the caustic soda used is equivalent to the free acid present, if there was initially sufficient excess of Sodium Phosphate.

Four seperate determinations have been made: (1) Total Acidity or Base-combining Capacity of the Tissue, representing all

acidic groups in the tissue not bound by base whether proteins or otherwise: (2) Protein Acidity or Base-combining Capacity determined on the whole protein of the tissue denatured and washed free of other material. The figure represents the free acid groups of the protein material near the isoelectric point. At the reastion of the living tissue some of these will be neutralised by base. (3) Acid combining Capacity of the Protein Ash. In washed protein treated as for (2) there is often a measurable amount of alkaline ash. This is taken as representing the firmly bound base and therefore the highly dissociated acidic groups of the protein. In the course of this determination it is convenient to find (4) the weight of washed protein in the tissue. It will be seen in the table that the weight of protein found is much lower than that frequently given for fish muscle; apparently the higher figures are obtained by multiplying the total nitrogen of the tissue by 6.25 and calling it protein. The figures obtained here agree satisfactorily with Clark and Almy's (1918) results for fish muscle.

The reagents used are as follows:-

Alcohol. 95% Methylated spirit redistilled over Caustic Soda, to every 100 c.c. of which is added 5 cc. of 0.5% Phenol Phthalein in alcohol.

Formalin-Phosphate 3 parts of 6% Sodium Phosphate ($Na_2HPO_4,12H_2O$) and 1 part of 40% Formalin purified by Charcoal.

Standard Soda. Carbonate free Caustic Soda about 0.15N is used; Standardized with Potassium Hydrogen Phthalate and delivered from a microburette graduated in 0.01 c.c. divisions, with a fine jet delivering about 0.01 c.c. per drop.

The procedure is as follows:- 4 to 6 gms of tissue finely pounded or minced are weighed to 0.1 gm in a tared stoppered conical flask, of 100 to 200 c.c. capacity. For each gm. of tissue 4 c.c. of alcohol are added. If the muscle is to be obtained at a stage prior to full development of lactic acid the mincing is done in the cold and the tissue added to a tared flask containing alcohol and cooled in freezing mixture. The tissue can be left under alcohol for several days without measurable change. Subsequent procedure varies according to whether (1), (2) or (3) and (4) are determined.

(1) Total Acidity. 4 c.c. of Formalin-Phosphate per gm. of tissue are added, the flask shaken and left overnight (12 to 15 hours). The flask is weighed to 0.1 gms and the contents filtered into two or more 25 c.c. tared stoppered conical flasks. The first portions of filtrate are used to rinse out the flasks. About 10 gms of filtrate are collected in each and weighed to 0.01 gm. The filtrates are titrated to the first definite change of colour in the direction of pink. 3 to 4 drops more soda should produce

a pale but distinct pink. The colour is permanent in a stoppered flask. It is preferable to titrate a number of samples together and match them as closely as possible. Different filtrates differ in colour and turbidity so that exact matching is out of the question.

A blank titration is done on the reagents with water in place of tissue, and deducted from the values found. This is more convenient than exactly neutralizing the reagents. The result is calculated in terms of milliequivalents per 100 gms of tissue (N/100).

(2) Protein Acidity The alcohol and tissue are heated just to boiling to complete precipitation of proteins. The alcohol is filtered off through a closely woven linen filter on a Büchner funnel. The flask and filter washed repeatedly with hot water till at least 500 c.c. of filtrates are collected. If the filter becomes clogged it can be cleared by rubbing with a glass rod. The cloth is lifted out and returned to the flask with its contents, after wiping round the funnel. 4 c.c. of alcohol are added for each gm. of original sample and dealt with as under (1).

(3) and (4) Protein Dry Weight and Ash. The protein is coagulated and washed as in (2) but preferably in an Alundum crucible. With more labour, but not much more error it may be treated on a cloth filter, scraped off when almost dry and transferred to a crucible. The protein is dried at 110°C and weighed to 0.01 gm. It is then ashed at moderate temperature; 5 c.c. of standard HCl about 0.03 N run in and sucked through into a filter tube, the crucible well washed with hot water and the whole filtrate heated in boiling water for 5 minutes to expel CO_2. Phenol Phthalein is added and the excess of HCl titrated. The result is expressed as in (1).

The variation in the results can be seen from the figure for Total and Protein Acidity in Haddock muscle.

	Mean	Max.	Min.	No. of Animals	No. of Titrations
Total	17.0	18.5	15.8	10	36
Protein	9.5	11.1	8.5	8	28

The variations obtained for the total acidity in some invertebrates were greater, due in part at least to the difficulty of getting such gelatinous material in a finely divided state.

Where, as in most cases, the determinations of total acidity were done on muscle containing maximum lactic acid the titre of the resting muscle can be calculated by deducting the

titre of the lactic acid content. The figures published by Furusawa and Kerridge (1927) show that most tissues have a resting pH very close to 7.0 so that the calculated value corresponds to the titre at this pH. The lactic acid production in invertebrate muscle (other than lobster) is very small and the correction on this account is negligible.

In the determinations on protein all the protein material in the tissues, soluble and insoluble has been treated together, and it is in all cases denatured. In fact it seems impossible to handle the protein in its natural state. There is no evidence to show whether denaturing alters the acid-base relations of these proteins. The protein figures are not altered by lapse of time after death or by the process of denaturing, whether by heat, alcohol, toluene or chloroform, though chloroform seems to result in slightly higher values for total acidity. The method of treatment used gives a product containing only a very small ash in the case of mammalian and frog's muscle and the titration value is very little affected by the method of washing i.e. whether water only is used or very dilute acetic acid followed by water or whether the washing is done after previous treatment with phosphate. That is to say the product obtained by washing with water is substantially isoelectric, and contains very little if any strongly bound base, or in other words the protein contains few highly dissociated acid groups. With fish and invertebrates a significant difference is observable, there is an appreciable amount of strongly bound ash, which can be at least partly removed by washing with dilute acetic acid, the titre being proportionately increased.

It might be preferable to wash the protein with a buffer solution of pH equal to the isoelectric point of the protein if this were known; but in most cases it is not known and it would in any case be difficult to correct the titration value for the adherent buffer solution. The method of washing with water only is not perfect theoretically but is easy, gives reproducible results and has moreover revealed a significant difference between different types of muscle.

RESULTS.

A summary of the experimental values obtained are given in Table I.

TABLE I.

	PROTEIN.					TOTAL IN RIGOR.		
	A.	B.	C.	D	E. $\frac{AX10^5}{D}$	F.	G.	H.
Species and type of Tissue	Weight percent.	Acidity N/100	Ash N/100	B & C.	Equiv.	Acidity N/100	Lactic Acid N/100	F-G.
Haddock body muscle	13.8	9.5	1.1	10.6	1300	17.0	3.2	13.8
Cod " "	12.9	8.8	1.6	10.4	1240	16.8	2.3	14.5
Hake " "		8.6	(1.6)	10.2		15.4	0.7	14.7
Eelpout " "		8.9				18.6		
Sculpin " "		9.6				16.8		
Raja erinacea "wing" muscle		9.1				24.0		
" stabuliforis jaw muscle		(7.7)				(21.1)		
" stabuliforis ventricle of heart		(6.5)				19.9		
Lobster abdominal muscle		9.8				39		
Pecten large adductor muscle	11.7	7.0	1.4	8.4	1400	46		
Pecten small adductor muscle	12.4	6.3	1.5	7.8	1590	33		
Modiolus retractor on foot		7				23-34		
Cucumaria frondosa longitudinal muscles	11.4	5.3	2.0	7.3	1560	13.7		

EXPLANATION OF TABLE 1

Results in brackets are doubtful or obtained from a single sample only.

A. Dry weight of washed protein as percentage of moist tissue.

B. Base combining capacity of washed protein, mgm equivalent per 100 gms. of tissue.

C. Acid combining capacity of ash of protein, mgm equivalent per 100 gms. of tissue.

D. Total base combining capacity of protein, mgm equivalent per 100 gms. of tissue. Sum of B. and C.

E. Equivalent wieght of protein as acid from A. and D.

F. Total base combining capacity of tissue, mgm equivalent per 100 gms. of tissue.

G. Calculated base combining capacity of lactic acid present in rigor.

H. Calculated base combining capacity of resting tissue.

The conclusions drawn from them are as follows:-

(1). The differences among the teleostean fishes are small and doubtfully significant. It is suggested that the difference in lactic acid production in rigor between the Haddock and the Hake is not due to a corresponding difference in buffering capacity of the tissue, as any large difference would probably be accompanied by a difference in base combining capacity. This conclusion also accords with the fact that the lactic acid production in rigor was found to exhause the precursor in the muscle, since no more could be obtained by treatment with alkaline phosphate solution, an indication that hydrogen ion concentration might not be the limiting factor in fixing the lactic acid maximum. Hence the differences in the character of rigor and of the post mortem changes in the muscles of teleostean fishes are probably to be connected with the differences in the original content of glycogen and other substances capable of giving lactic acid.

(2) Elasmobranch fishes evidently differ considerably from teleosteans but the data are too scanty for any further conclusion.

(3) The invertebrates are markedly different. The very high total acidity of some is of doubtful significance. It may be the result of the large amount of organic extractive material.

(4) There is evidently some correlation between high protein base combining capacity (and protein content) and muscular activity as a whole, while the ash titre varies inversely. Possibly the high ash value in invertebrate muscle in connected with the presence of proteins of a mucoid type which are usually strongly acidic.

Two further conclusions obtained from other results need to be mentioned.

(5) The titration of the total tissue of the Haddock immediately on capture and during and after rigor gave the following results.

FISH	III	VI	VII
At once	14.9 N/100	15.0	15.5
Rigor	16.6	15.7	17.2
After Rigor	16.1	16.4	--

The change in titre between killing and full development of rigor is of the same order as the increase of lactic acid found in 1925, i.e. 0.11% of acid equal to 1.2 N/100. The figures also show that there is no appreciable change when rigor passes off. A number of determinations on the different species showed that within 48 hours of death once rigor had fully developed there was no measurable change either in total or in protein acidity.

This result is contrary to the finding reported previously but that was made by a very inadequate method giving no stable end point for titration. The apparent change in titre was probably simply due to a change in consistency of the tissue allowing more rapid neutralization of the added alkali.

The view now advocated is that the passing off of rigor is not due to any hydrolytic process in the protein, or if it is to one that increases the acid groups by less than 10 per cent, between the height of rigor and its complete disappearance. This is in agreement with the well known fact that within 24 hours of death there is no increase in the soluble nitrogen of vertebrate muscle. The cause of disappearance of rigor is more probably to be sought in a change of the relationship between water and proteins or other colloids of the muscle fibre. The fact that fish muscle has more tendency to "bleed" when rigor has passed off than during the height of rigor may be indicative of the type of change concerned.

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