The Free Amino Acids of Fish

1-METHYLHISTIDINE AND β-ALANINE LIBERATION BY SKELETAL MUSCLE ANSERINASE OF CODLING (*GADUS CALLARIAS*)

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Anserine (β-alanyl-1-methylhistidine), carnosine (β-alanylhistidine) and their component amino acids have been the subject of renewed interest following the reported implication of the dipeptides in respirative phosphorylation (Severin & Meshkova 1950, 1952, 1953; Meshkova & Malysheva, 1951; Meshkova & Zaitseva, 1953). Previously, little was known of their function (du Vigneaud & Behrens, 1939), although Bate-Smith (1939) had noted important buffering properties in muscle. The precise point and method of participation of carnosine and anserine in the glycolysis system have not been elucidated, and other aspects of their metabolism remain obscure. Most information is available on carnosine. *In vivo* synthesis from histidine and β-alanine was demonstrated by Yudaev (1950*a*). Meshkova & Zolatarevskaya (1937) and Hanson & Smith (1949) failed to detect carnosine-cleaving activity in preparations of swine intestinal mucosa—a good source of the then known metal-activated dipeptidases. The latter workers confirmed a demonstration by Garkovi (1937) of 'carnosinase' activity in kidney, spleen and liver. They further succeeded in partially purifying the enzyme, which was found to be activated by Mn^{2+} and Zn^{2+} ions. Parshin & Dobrinskaya (1938) demonstrated hydrolysis of both carnosine and anserine by yeast autolysates. Little other work has been carried out on the latter dipeptide, but *in vivo* synthesis from 1-methylhistidine and β-alanine has been demonstrated in the rabbit by Yudaev (1952).

The present paper, which describes an anserine-cleaving system in codling muscle, arose from an investigation into changes which occur in the free amino acids of skeletal muscle of fish, gutted and iced under practical fish-trade conditions. Such changes, deriving from the action of bacteria (Reay & Shewan, 1949), leaching (Jones, 1954*a*) and autolytic agents are not only of general biochemical interest but are also important in relation to the palatability of flesh and ease of Maillard (1912)-type browning reaction (Tarr, 1954; Jones, 1954*b*).

This study on codling 'anserinase' was much facilitated by the knowledge that anserine is present in the muscle to the total exclusion of carnosine (Yudaev, 1950*b*; Shewan, 1953). A short preliminary report of some of this work has appeared (Jones, 1954*c*).

EXPERIMENTAL

Materials

Codling. These were line-caught in Aberdeen Bay by the Station vessel *Keelby* and maintained in aerated sea-water tanks.

Anserine. This was isolated from frozen pike (*Esox lucius*) which have been shown chromatographically to be a good source of the dipeptide (personal communication from Dr J. M. Shewan). Thawed muscle (2 kg.) was minced and extracted into 80% (v/v) ethanol at 60°. Cooled extract (30 l.) was passed through an ethanol-equilibrated H^+ form 4 % cross-linked sulphonated polystyrene (P.S.X.) cation-exchange resin column (Partridge & Brimley, 1952); dimensions 5·5 × 50 cm., particle size 60–100 mesh. The column was washed fat-free with 80% (v/v) ethanol, freed from ethanol with distilled water, and exchanged cations were displaced with 0·1 N-NaOH through successive columns of P.S.X., dimensions: (i) 4 × 25 cm., 60–100 mesh; (ii) 2·5 × 15 cm., 80–100 mesh; (iii) 1·5 × 9·5 cm., 100–120 mesh. Fractions (25 ml.) were collected, a series of which contained uncontaminated anserine as ascertained by paper chromatography with phenol–ammonia (Block, Le Strange & Zweig, 1952), phenol–HCl (Yudaev, 1949) and butanol–acetic acid (Woiwod, 1949). Other mixed fractions containing anserine were subjected to secondary separation on Dowex 2 anion-exchange resin following the procedure of Partridge & Brimley (1952): further uncontaminated anserine fractions were obtained. Bulked anserine fractions were concentrated under reduced pressure and twice recrystallized from aqueous ethanol to give 3·1 g. white crystals, m.p. 240° decomp. (uncorr.). Linneweh & Linneweh (1930) record m.p. 238–9°. (Found: N, 23·2%. Calc. for $C_{10}H_{16}O_3N_4$: 23·3%.)

Amino acids. β-Alanine (Light and Co. Ltd., Poyle Trading Estate, Colnbrook, Bucks) and DL-1-methylhistidine (Shandon Scientific Co., 6, Cromwell Place, London, S.W. 7) were recrystallized from aqueous ethanol. The latter compound discolours if stored in the light. Cysteine (Light and Co. Ltd.). Amino acid chromatographic standards, 0·01 M in 10% (v/v) *iso*propanol (Shandon Scientific Co.).

Resins. Sulphonated polystyrene, nominal divinylbenzene content 4 % (Permutit Co. Ltd., Gunnersbury Avenue, London, W. 4). Dowex 2 (R. W. Greef and Co. Ltd., Finsbury Avenue, London, W.C. 2).

Salts. A.R. grades where available.

Buffers. Acetate–veronal (Michaelis, 1931); phosphate, KH_2PO_4–NaOH.

Ninhydrin reagent. This was prepared according to Moore & Stein (1948).

Methods

Tissue-free amino acids. Tissue (10 g.) was repeatedly extracted with 75% (v/v) ethanol at room temperature to final extract volume 250 ml. Samples (25 ml.) were shaken with 75 ml. $CHCl_3$ (Awapara, 1948), and, on separation of the emulsion, 4 ml. samples of the upper, aqueous layer were freeze-dried. Freeze-dried preparations were dissolved in 400 μl. water and 5–20 μl. samples were spotted in successive 0·2 μl. droplets by microsyringe on to 8 in. squares of Whatman no. 1 paper for two-dimensional chromatography in phenol–ammonia and collidine–lutidine (Dent, 1948). Air-dried chromatograms were sprayed on both sides with 1% (w/v) ninhydrin in wet butanol, and colour allowed to develop over 2 days at room temperature (Thompson, 1951). β-Alanine and 1-methylhistidine spots, with blanks of equivalent adjacent area were cut out and eluted into 2 ml. 50% (v/v) acetone. They were evaluated in a Hilger Spekker microcell arrangement at 570 mμ. Square chromatograms (8 in.) in batches on frames (Datta, Dent & Harris, 1950) enabled a number of standards to be run in the same tank. Amino acids (4–10 μg.) could be determined with an accuracy of $\pm 7\%$. Determinations were triplicated.

Anserine hydrolysis. Determined by two methods.

(*a*) Incubates (1 ml.) were deproteinized by addition of 1–2 ml. 1% (w/v) picric acid. Supernatant liquid after centrifuging was analysed for amino-N by a direct ninhydrin colorimetric procedure (Moore & Stein, 1948). Hydrolysis was determined from the difference between corrected colour values given by original and hydrolysed dipeptide using proportionality factors as described by Fleisher (1953) for blood peptidases in a modification of the method of Schwartz & Engel (1950).

(*b*) Incubates were deproteinized by addition of ethanol to 80% (v/v) at 60° and centrifuging. Supernatant extracts were passed through microcolumns of 80% (v/v) ethanol-equilibrated P.S.X. cation-exchange resin. After washing the columns with 80% (v/v) ethanol and distilled water, organic cations were displaced with 0·075N ammonia. They were concentrated and freed from ammonia by freeze-drying over P_2O_5. Freeze-dried preparations were chromatographed in butanol–acetic acid for evaluation of β-alanine by spot elution. The β-alanine content of the preparations was a measure of anserine hydrolysis.

Muscle and enzyme preparations

Sterile muscle suspensions. Samples (10 g.) of muscle were dissected from freshly killed codling and macerated into 240 ml. sterile water under aseptic conditions. Toluene (0·5 ml.) and $CHCl_3$ (0·5 ml.) were added to the macerate.

Cell-free muscle extracts. Muscle (20 g.) from freshly killed codling was homogenized by a 'Nelco' homogenizer into 100 ml. water. The suspension was centrifuged at 3500 r.p.m. for 10 min. at 2° and Seitz-filtered.

Freeze-dried dialysed muscle extract. Muscle (3 kg.) was minced with ice into 14 l. water and allowed to lyse with 100 ml. $CHCl_3$ for 4 hr. at 0°. The slurry was filtered through muslin and poplin and clarified through a Sharples Supercentrifuge at 24500 rev./min. Extract (13 l.) was concentrated to 1·5 l. under reduced pressure (15 mm. Hg/18°) in the presence of a little Silicone M.S. Antifoam A (Hopkin and Williams Ltd., Chadwell Heath, Essex) with little loss of activity and centrifuged at 2800 rev./min. for 20 min. at 0°. Supernatant concentrate was dialysed against a total of 40 l. water in successive 8 l. volumes in the presence of 0·5 ml. toluene and 0·5 ml. $CHCl_3$. A copious white precipitate which formed was centrifuged down and discarded. Dialysed concentrate supernatant was freeze-dried to give a readily soluble white powder (90 g., N content 154 mg./g.).

Acetone-fractionated enzyme. Fresh codling muscle (1 kg.) was minced with ice into 2 l. water, lysed with 20 ml. $CHCl_3$ at 0° and extract squeezed, clarified and concentrated under reduced pressure. Centrifuged concentrate (50 ml.) was adjusted to pH 7·5 at 0° and acetone added dropwise with constant stirring, temperature being progressively lowered to −4°. Precipitates were centrifuged down after additions of successively (i) 25 ml., (ii) 50 ml. and (iii) 75 ml. acetone. They were immediately suspended in 25 ml. distilled water at 0° and dialysed for 2 days. Acetone-concentrate preparation remaining after the last centrifuging (iv) was similarly dialysed. After dialysis preparations were recentrifuged and the supernatant extracts freeze-dried. Yields, (i) 520 mg.; (ii) 300 mg.; (iii) 490 mg.; (iv) 290 mg.

Acetone powders from muscle. Muscle (10 g.) dissected from chilled, freshly killed codling was macerated into 40 ml. distilled water at 0°. Acetone was then added dropwise to 60% (v/v) at 0° over 60 min. Suspensions were centrifuged down and washed with successively 80 ml. 60% (v/v) acetone at 0° and 80 ml. acetone at −10°. Acetone was removed from the powders *in vacuo* and they were ground at −30°.

RESULTS

Free β-alanine and 1-methylhistidine in fresh and stored iced muscle

The free 1-methylhistidine and β-alanine contents of fresh codling muscle have been determined, and changes which take place in the muscle of fish iced under conditions simulating those of the practical fish trade investigated. Considerable increases were found to occur during storage in ice (Fig. 1), although it is known that considerable leaching losses in free amino acid may take place under these conditions (Jones, 1954*a*). While the initial free β-alanine content is almost invariably low, some variation (which is the subject of continued long-term study and beyond the scope of the present paper) has been found in the initial free 1-methylhistidine content.

The increases in free β-alanine and 1-methylhistidine contents of iced codling muscle during the first 2 days arise before the onset of bacterial invasion of the tissue (Shewan, personal communication). Accordingly, the possibility of autolytic-type action was examined by following levels of the amino acid in sterile muscle suspension incubates at 0 and 15°. At both temperatures free β-alanine and 1-methylhistidine increased in unboiled incubates. No increase was found in boiled incubates (Fig. 2).

The identities of the substances under measurement in Figs. 1 and 2 were then confirmed by isolation from muscle mince incubates.

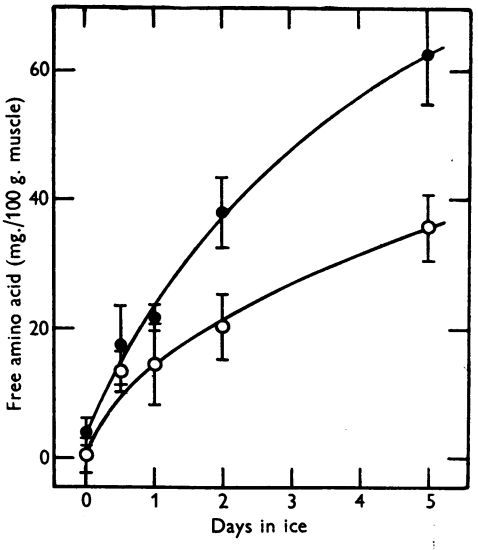

Fig. 1. Increase in free 1-methylhistidine and β-alanine in skeletal muscle of iced gutted codling. (Zero abscissa values are of fresh codling control.) Codling were killed by decapitation and samples immediately removed for analysis. Other codling were gutted and packed in ice in boxes at an ambient temperature of 2·5°. Periodically groups were removed for chromatographic analysis of free amino acids. Values are per 100 g. wet muscle as sampled. Points represent means of values from groups of five to six fish. Vertical lines are a measure of scatter within groups (S.D. $=\sqrt{[\Sigma d^2/(n-1)]}$). 1-Methylhistidine, ●—●; β-alanine, ○—○.

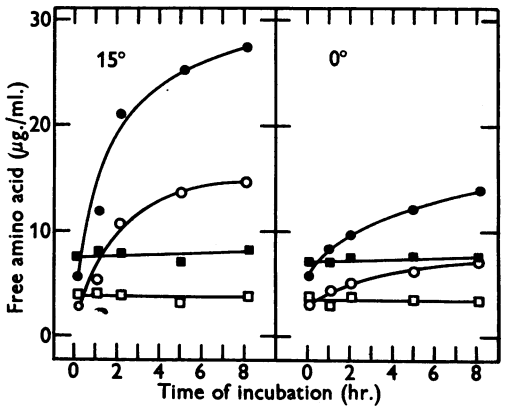

Fig. 2. Free 1-methylhistidine and β-alanine in muscle suspension incubates. Suspensions (10 g. muscle into 240 ml. water) were incubated at 0° and 15°, boiled and unboiled, in the presence of $CHCl_3$ and toluene. 1-Methylhistidine and β-alanine were determined by the chromatogram spot-elution method. ●—●, 1-Methylhistidine; ○—○, β-alanine in untreated suspensions; ■—■, 1-methylhistidine; □—□, β-alanine in boiled suspensions.

Fresh codling muscle (3 kg.) was passed through a sterilized mincer into 7 l. sterile distilled water. Chloroform (100 ml.) was added to suppress bacterial growth and the slurry incubated overnight at 12·5°. Ethanol was then added to 80% (v/v) and the temperature raised to 50° for 5 min. after which time the extract was filtered off and the residue re-extracted. Combined ethanol extracts were separated on ethanol-equilibrated P.S.X. cation-exchange columns as described in the anserine isolation. Two groups of mixed fractions contained (i) creatine, β-alanine in large quantity, trimethylamine oxide and a trace of tyrosine and (ii) trimethylamine oxide, creatinine, 1-methylhistidine in quantity, traces of histidine, lysine and anserine. These two groups were then subjected to further separation on Dowex 2 anion-exchange columns. From (i) uncontaminated β-alanine fractions were concentrated under reduced pressure and, twice recrystallized from aqueous ethanol, gave 1·1 g. β-alanine, m.p. 200° (uncorr.). Holm (1905) reported m.p. 200°. (Found: N, 15·4%. Calc. for $C_3H_7O_2N$: N, 15·7%.) Difficulty was experienced in securing a clean separation from (ii). Finally after repeated recrystallization from aqueous ethanol, 400 mg. uncontaminated 1-methylhistidine crystals were obtained, m.p. 250° decomp. (uncorr.). Linneweh & Linneweh (1930) reported m.p. 248–252°. (Found: N, 22·8%. Calc. for $C_7H_{11}O_2N_3, H_2O$: 22·5%.)

Table 1. *Free β-alanine and 1-methylhistidine in extract incubates*

Samples of extract (2 ml. diluted to 3 ml. incubate volume) were incubated for 8 hr. at 15° unbuffered, initial pH 6·8. Free amino acids were determined by chromatography and spot elution.

Preparation	1-Methylhistidine (μg./ml.)	β-Alanine (μg./ml.)
Extract before incubation	20	8
Extract after incubation	110	51
Boiled extract before incubation	28	9
Boiled extract after incubation	25	10

Table 2. *Free β-alanine and 1-methylhistidine in extract incubates*

Extract (2 ml.) was incubated with and without anserine (10^{-3} M); incubation time, 6 hr.; initial pH 6·9, unbuffered; temp. 15°; incubate volume, 3 ml. Estimated as in Table 1.

Preparation	1-Methylhistidine (μg./ml.)	β-Alanine (μg./ml.)
Extract before incubation	18	8
Extract after incubation	119	53
Boiled extract before incubation	24	11
Boiled extract after incubation	23	11
Extract + anserine after incubation	190	82
Boiled extract + anserine after incubation	24	10
Anserine control	0	0

Cell-free muscle extracts

Incubation of cell-free extracts at 15° led to accumulation of 1-methylhistidine and β-alanine (Table 1). The effect was not found in boiled extracts,

indicating the presence of a thermolabile factor or factors liberating the amino acids in muscle. As in this experiment, and in iced fish muscle, 1-methylhistidine and β-alanine were liberated in roughly stoicheiometric proportion, it seemed probable that they were deriving from a common compound. Accordingly, anserine was incubated with cell-free aqueous muscle extract and it was found that β-alanine and 1-methylhistidine liberation was increased (Table 2). This indicated probable cleavage of the dipeptide by the thermolabile factor.

Incubation of dialysed muscle extract (2 ml.) (an intermediate stage in the preparation of 'freeze-dried extract' reported in the Experimental section), which contained neither free amino acid nor anserine, with the dipeptide (1 μmole) resulted in over 90% destruction of the dipeptide, as ascertained by two-dimensional chromatography and spot-elution following ethanol-deproteinizing of 8 hr./15° incubates. Heating ninhydrin-sprayed chromatograms at 105° for 5 min. resulted in easy identification of anserine which gave a yellow spot; the 1-methylhistidine spot was blue-green and that of β-alanine red-purple.

Properties of freeze-dried muscle extract

Effect of pH *and buffers.* Freeze-dried extract dissolved in distilled water to give an opalescent solution, pH 6·8. Incubation with anserine at that pH, unbuffered, resulted in cleavage of the dipeptide. After deproteinizing by reducing the pH value to 1·2 and centrifuging, the supernatant extract, readjusted to pH 6·8, had no anserine cleaving activity (Table 3).

Fig 3 shows a typical reaction curve in the presence of acetate–veronal buffer. Fig. 4 shows the effect of pH on the anserine-cleaving activity of the preparation in the presence of 40 mM acetate–veronal and phosphate buffers and in the absence of buffers. Non-buffered systems had slightly greater activities. pH 7·3 was optimum for all three systems.

Table 3. *Anserine hydrolysis activity of freeze-dried extract and acid-deproteinized freeze-dried extract*

Freeze-dried extract (50 mg.) was dissolved in 10 ml. water. Reaction was adjusted to pH 1·2 with 6N-HCl at 0° and the precipitate which formed centrifuged at 0°. Supernatant liquid, readjusted to pH 6·8, was made up to 50 ml. Samples (2 ml.) were incubated for activity test with 1 μmole anserine, final volume 3 ml.: initial pH 6·9, unbuffered; incubation time 8 hr., temp. 15°. Control incubates with 2 mg. extract were carried out in parallel. Percentage hydrolysis of incubated anserine was determined by the direct ninhydrin colorimetric procedure.

Preparation	Hydrolysis (%)
Extract + anserine	58
Boiled extract + anserine	0
Deproteinized extract + anserine	0
Anserine control	0

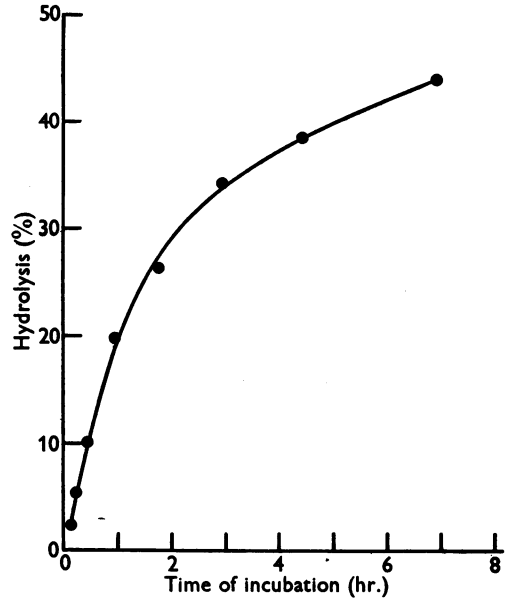

Fig. 3. Hydrolysis of anserine by freeze-dried extract at different times. Extract (5 mg.) was incubated with anserine (2 μmoles), NaOH to adjust reactants to pH 7·5 and acetate–veronal buffer, pH 7·54 (to 40 mM in a total incubate volume of 4 ml.). Incubation was at 15° under toluene. Periodically 0·2 ml. samples were removed for direct colorimetric analysis.

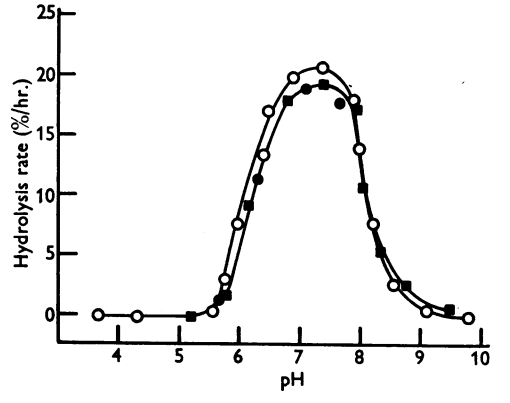

Fig. 4. Hydrolysis of anserine by freeze-dried extract at different pH values. Incubate conditions as for Fig. 3 except for differences in buffer. NaOH or HCl was added to bring reactants to correct pH in the absence of buffer. Buffer strengths, 40 mM. O—O, No buffer; ●—●, phosphate; ■—■, acetate–veronal. Values relate to the initial linear portions of reaction/time curves (Fig. 3).

Activators and inhibitors. (*a*) Metal ions. In an investigation into the effects of metal ions, some activation by 10^{-4}M-Co^{2+} was found (Table 4). Of the other ions listed, all other than K^+ and Mg^{2+} were inhibitory at the concentration tested. Zn^{2+} was found to be particularly activating at certain concentrations and was subjected to more detailed examination (Fig. 5). The optimum added Zn^{2+} concentration was $10^{-3.7}$M. At higher concentrations activation fell rapidly and, above 10^{-3}M, Zn^{2+} became inhibitory. The pH optimum of Zn^{2+}-activated freeze-dried extract was 7·3 (Fig.6), the same as that of the non-activated extract.

Table 4. *Activation and inhibition of anserine cleavage by freeze-dried extract*

Incubates of 1 mg. extract with and without additions were carried out in 40 mM acetate–veronal, pH 7·54; incubate volume 2 ml. Hydrolysis of 0·5 μmole anserine was determined by the direct colorimetric procedure after 2 hr. incubation at 15°. Inhibition and activation are expressed as percentages of the activity in absence of additions.

Ion	Concentration (M)	Inhibition (%)	Activation (%)
$Co^{2+}(CH_3.COO^-)$	10^{-3}	10	—
	10^{-4}	—	20
$Mn^{2+}(SO_4^{2-})$	10^{-3}	30	—
	10^{-4}	5	—
$K^+(Cl^-)$	10^{-3}	—	—
$Pb^{2+}(CH_3.COO^-)$	10^{-2}	95	—
	10^{-3}	80	—
	10^{-4}	20	—
$Ag^+(NO_3^-)$	10^{-3}	90	—
$Fe^{3+}(Cl^-)$	10^{-3}	94	—
$Mg^{2+}(Cl^-)$	10^{-4}	0	—

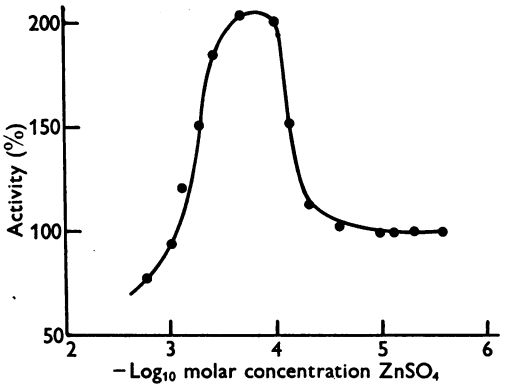

Fig. 5. Anserine cleavage by freeze-dried extract in the presence of Zn^{2+}. Incubation conditions and measurements as Table 4. 40 mM acetate–veronal buffer, pH 7·5. Activity is expressed as a percentage of that of non-activated systems.

(*b*) Other substances. Tested as in the previous experiment cysteine was found to be 90% inhibitory at 10^{-3}M and 40% inhibitory at 10^{-4}M. Fluoride was 10% inhibitory at 10^{-3}M. Inhibition by cyanide is illustrated by Fig. 7. In another

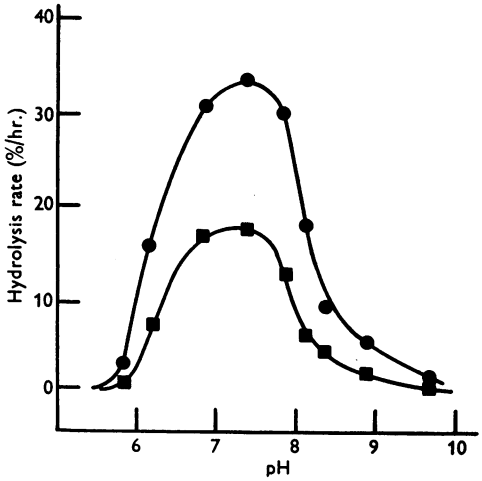

Fig. 6. Anserine cleavage by freeze-dried extract at different pH values in the presence (●—●) and absence (■—■) of 10^{-4}M-Zn^{2+}. Conditions as in Fig. 5.

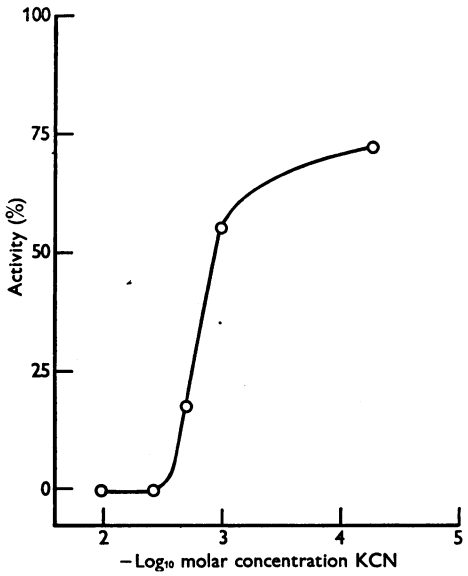

Fig. 7. Anserine cleavage by non-activated freeze-dried extract in the presence of CN^-. Incubates of extract (1 mg.) with and without additions were carried out in 40 mM acetate–veronal, pH 7·54, incubate volume 2 ml. Hydrolysis of 0·5 μmole anserine was determined by the direct colorimetric procedure. Percentages relate to non-inhibited values and are taken from the initial linear portions of reaction/time curves.

experiment the presence of $10^{-3.4}$M-Zn^{2+} appeared to afford some protection at lower CN^- levels, possibly owing to formation of the insoluble salt.

Properties of acetone-fractionated enzyme

When incubated with 10^{-3}M anserine in the presence of 10^{-4}M-Zn^{2+}, over 90% of the cleaving activity of freeze-dried preparations was found in that (fraction ii) from the precipitate formed over the range 33–50% (v/v) acetone. Incubation of this fraction, activated by Zn^{2+}, with different levels of anserine showed that cleaving activity appeared to have reached maximum value at $10^{-2.9}$M (Fig. 8). A value at half-maximum activity gave a numerical equivalent of $3{\cdot}8 \times 10^{-4}$ for the Michaelis constant.

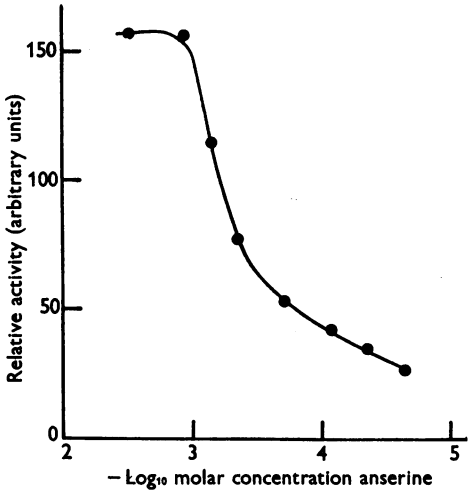

Fig. 8. Relative activities of anserine hydrolysis by acetone-fractionated concentrate at different substrate concentrations. Freeze-dried acetone fraction (ii) (1·58 mg.; 0·1 ml.; N content, 250 μg.) was incubated with different concentrations of anserine in the presence of 10^{-4}M-Zn^{2+} and 40 mM acetate–veronal buffer pH 7·54. Final volume 2 ml.; temp. 15°. 'Relative activities' are arbitrary units based on direct increases in absorptiometer readings over the initial linear region of reaction/time plots.

The preparation of acetone powders from whole muscle, as described in the Experimental section, appears to afford the basis of a method for anserine-cleaving activity determination. Powder (10 mg.) from different codling, suspended in 50 mM acetate–veronal buffer, pH 7·4, and incubated with $10^{-3.4}$M anserine (total volume, 2 ml.) gave varying degrees of hydrolysis (as estimated as β-alanine on P.S.X.-desalted chromatograms) after 16 hr. at 15°.

Examination of other tissues

Extracts of swine intestinal mucosa and rat skeletal muscle were tested for anserine-cleaving activity by the chromatographic spot–elution procedure after incubation with substrate. Tissue (10 g.) was worked up to 50 ml. dialysed extract in each case, with codling muscle as a control. Extracts (1 ml.) were incubated for 6 hr. at 15° with anserine (10^{-3}M), unbuffered at pH 6·9, volume 2 ml. Intestinal mucosa and rat muscle preparations contained no detectable activity. Codling muscle preparation hydrolysed 20% of the substrate.

DISCUSSION

The changes which take place in skeletal muscle of codling during storage in ice under conditions simulating those of the fish trade include increases in free β-alanine and free 1-methylhistidine. These increases are apparent in the earliest stages in storage changes, before bacterial invasion of the tissue, and have been shown to arise from hydrolytic cleavage of muscle anserine. Cleavage results from the action of a factor with the properties of an enzyme, for which the name 'anserinase' is suggested (Jones, 1954*c*).

Anserinase activity has not been reported in skeletal muscle of species other than codling, and indeed may well be absent from mammalian muscle. Rat muscle preparations contained no detectable anserinase when tested under comparable conditions. Rat muscle also lacks carnosinase activity (Hanson & Smith, 1949).

Codling anserinase is active in minces, suspensions and cell-free aqueous extracts as well as in intact muscle. Partially purified stable enzyme powders are readily obtainable from muscle by aqueous extraction, dialysis against water and freeze-drying. Solutions of such preparations, unlike serum glycylglycine dipeptidase (Fleisher, 1953) have a single anserinase pH optimum at pH 7·3 indicating the presence of a single anserine-cleaving enzyme. Anserinase is activated by Zn^{2+}, optimal concentration being $10^{-3.7}$M, above which level heavy metal inhibition effects are progressively encountered. Optimum pH values of non-activated and Zn^{2+}-activated preparations are similar, indicating that the ion may well be a natural activator. Inhibition of dialysed anserinase by cysteine and CN^- is a further indication that the natural system is metal-activated. Unlike carnosinase (Hanson & Smith, 1949) no activation by Mn^{2+} was detected at pH 7·5. This may possibly be due to a marked shift in optimum pH as observed for Mn^{2+}-activated carnosinase. Such a pH shift may also account for the slight anserinase inhibition at a concentration as low as 10^{-4}M.

Some inactive protein has been removed from crude preparations by acetone fractionation. Precise determination of relative activity/N quotients of different preparations has not been attempted. Optimal Zn^{2+} concentration in activated systems would vary with the concentration of inactive protein so that strictly standard conditions would be difficult to achieve. The preparation of non-activated acetone powders from muscle suspension under standard conditions may, however, be a basis for anserinase-activity determinations in different fish within the species.

Anserinase, like carnosinase, appears to differ from the common metal-activated dipeptidases. Swine intestinal mucosa preparations (a good source of the latter enzymes) attack neither dipeptide.

While the present study has shown that β-alanine and 1-methylhistidine may be recirculated into the metabolic pool in the living organism through anserinase action, *in vitro* synthesis of the dipeptide from its constituent amino acids has not been demonstrated. Under certain experimental conditions, almost complete anserine hydrolysis occurred over extended incubation periods. However, the high concentration of anserine in fresh codling muscle, and low free β-alanine and 1-methylhistidine contents, indicate that either anserinase is itself a product of autolysis or that coupling of anserinase with another system results in a reversal of the overall equilibrium. These possibilities are the subject of continued studies.

SUMMARY

1. 1-Methylhistidine and β-alanine are liberated in codling skeletal muscle *post mortem* at 0°.

2. They derive from anserine by the action of a new muscle enzyme 'anserinase'.

3. Partial purification of anserinase from cell-free extracts is obtained by dialysis and acetone-fractionation.

4. Anserinase is activated by Zn^{2+}. High concentrations of Zn^{2+} are inhibitory. Some evidence is available that the enzyme is naturally Zn^{2+}-activated.

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