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# The Estimation of Anserinase Activity by a Low-Temperature Ninhydrin Reaction

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The properties of an enzyme which cleaves anserine into 1-methylhistidine and β-alanine have been reported previously (Jones, 1955, 1956). In the latter communication, reference was made to an improved procedure for the assay of anserinase. The present paper describes the experimental basis of this procedure.

In the earlier study, enzyme activity was measured by either the chromatographic estimation of liberated β-alanine or the analysis of deproteinized reaction mixtures for increase in amino nitrogen by a ninhydrin colorimetric procedure (Moore & Stein, 1948), the degree of hydrolysis being calculated according to Fleisher (1953) and Schwartz & Engel (1950). The chromatographic procedure is lengthy, but can be used to estimate small amounts of anserine hydrolysis with considerable accuracy. The Schwartz & Engel procedure is relatively rapid but less sensitive. This low sensitivity, which is a particular disadvantage in the measurement of initial velocity constants, is due to the high colour yields given by unhydrolysed anserine relative to those given by small quantities of liberated amino acids at a reaction temperature of 100°.

During the course of an examination of the free amino compounds of fish muscle it was noticed that anserine gave a weak yellow–orange spot on ninhydrin-sprayed chromatograms developed at room temperature, whereas its constituent amino acids gave more typical purplish colorations. It seemed possible, therefore, that similar effects might occur in solution and allow the direct colorimetric determination of anserinase by a method with low or zero anserine 'blanks' and consequent higher potential sensitivity at low levels of hydrolysis.

## EXPERIMENTAL

### *Materials*

*Anserine.* This was isolated from frozen pike (*Esox lucius*) by ion-exchange chromatography on sulphonated-polystyrene and Dowex 2 resins (Jones, 1955). After being recrystallized twice, its m.p. was 240°, decomp. (corr.). Linneweh & Linneweh (1930) record m.p. 238–239° (Found: N, 23·2. Calc. for $C_{10}H_{16}O_3N_4$: N, 23·3%).

DL-1-*Methylhistidine.* Anserine (3 g.) was hydrolysed in barium hydroxide (14%, w/v) for 8 hr. at 120°. The barium was removed as the sulphate and the hydrolysate was displaced from a column of Dowex 2 (OH) resin (15 cm. × 1·8 cm., 80–100 mesh) with 0·02N-HCl through successive columns (12 cm. × 1 cm., 100–200 mesh and 10 cm. × 0·5 cm., 200–400 mesh). The rate of flow from the last column was 0·2 ml./min. Uncontaminated DL-1-methylhistidine fractions were obtained. These were concentrated and, after recrystallizing from water, gave 1·4 g. of the amino acid, m.p. 251·5° decomp. (corr.). Linneweh & Linneweh (1930) reported m.p. 248–252° (Found: N, 22·6. Calc. for $C_7H_{11}O_2N,H_2O$: N, 22·5%).

*β-Alanine.* This was obtained in uncontaminated fractions during the separation of the hydrolysate of anserine on Dowex 2. The fractions were concentrated and after being recrystallized from aqueous ethanol gave 800 mg. of the amino acid, m.p. 200·5° (corr.). Holm (1905) reported m.p. 200° (Found: N, 15·6. Calc. for $C_3H_7O_2N$: N, 15·7%).

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

### *Methods*

*Standard solutions of anserine and 1-methylhistidine plus β-alanine.* Under the usual conditions of dipeptidase assay 0·2 μmole of dipeptide or an equivalent hydrolysis mixture is allowed to react with 1 ml. of ninhydrin reagent, the enzyme being removed first by precipitation with picric acid. Accordingly, standard solutions of (*a*) mM-anserine and (*b*) mM-β-alanine plus mM-1-methylhistidine in 0·5% (w/v) picric acid were made up to represent 0 and 100% hydrolysis. Mixtures of these solutions represented intermediate stages.

*Reaction with ninhydrin.* Standard solutions (0·2 ml.) were mixed with 1 ml. of ninhydrin reagent as described by Moore & Stein (1948). Colour was developed at 35° and 60° in constant-temperature baths and at 100° in a boiling-water bath. Periodically, tubes were removed and chilled in an ice bath and the contents were diluted to 10 ml. with aqueous 50% (v/v) propan-1-ol for colour measurement at 570 mμ in a Hilger Spekker photoelectric absorptiometer. Reagent blanks were treated similarly and subtracted from experimental values. In some later experiments the volume of simulated hydrolysis mixture was increased. The volume of reagent was increased in proportion and the reaction mixture diluted to 10 ml. as above.

## RESULTS

Only traces of colour were detectable with anserine at 35° at the time at which reaction with the equivalent amino acid mixture had levelled off (Fig. 1). The maximum colour yield with the amino acids after 180 min. reaction was 37% of that of a comparable part of the reaction curve (after 17 min.) at 100° and 78% of that at 60° (after 60 min.). At these times the colour yield of equivalent amounts of anserine was 7·3% at 60° and 27% at 100°. After the standard 20 min. at 100° used in the Moore & Stein (1948) procedure, the proportion had increased to 31%. It appears that the rate of reaction between ninhydrin and anserine is negligible at 35°, rises slowly to 60° and thereafter rises steeply to 100°.

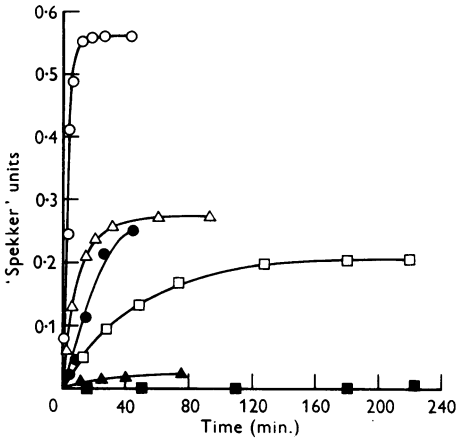


Fig. 1. Reaction of the ninhydrin reagent with anserine and an equivalent amount of 1-methylhistidine + β-alanine at different temperatures. Reagent (1 ml.) and anserine or amino acids (0·2 μmole in 0·2 ml.) were allowed to react at 35°, 60° and 100° respectively. The mixture was chilled rapidly to 0° and diluted to 10 ml. for evaluation. At 100°: ○, combined amino acids; ●, anserine. At 60°: △, combined amino acids; ▲, anserine. At 35°: □, combined amino acids; ■, anserine.

From these data it follows that the potential sensitivity of the ninhydrin method for the measurement of hydrolysis is higher at 35° than at 100°. When the volume of reactants was increased eightfold for final dilution to 10 ml. the colour yield increased linearly at 35° from 0·002 unit at 0% hydrolysis to 0·163 unit at 10% hydrolysis. Under these conditions of concentration, at 100° the 0% hydrolysis colour value was beyond the range of measurement of the instrument. Under the standard conditions of reaction for 20 min. at 100° (with 0·2 μmole of anserine or an equivalent hydrolysate in 0·2 ml.) the colour yield read against reagent 'blanks' increased from 0·180 to 0·211 unit, a smaller rise than that with the higher concentration at 35° and over a less-sensitive region of the instrument's scale.

## DISCUSSION

The colour yield ($D$) in the complete reaction of a partial hydrolysate of anserine with ninhydrin may be represented thus, where Beer's law applies: $D = k_1(1-x) + k_2 x + k_3 x$, where $x$ is the fraction of anserine hydrolysed and $k_1$, $k_2$ and $k_3$ are proportionality factors based on the molar colour yields of anserine, β-alanine and 1-methylhistidine respectively. Under these conditions a plot of colour yield against extent of hydrolysis would give a straight-line relation with an intercept on the ordinate. When the colour yields of the hydrolysate are evaluated against equivalent unhydrolysed anserine in the measuring instrument, a similar plot would give a straight line with a zero intercept but an identical slope.

Reference to Fig. 1 shows that, at the points where the colour yields due to the amino acids level off, the difference between yields from 0·2 μmole of anserine and the equivalent combined values from β-alanine and 1-methylhistidine was 0·410 unit at 100°, 0·254 unit at 60° and 0·202 unit at 35°. Under the standard 20 min. at 100° (Moore & Stein, 1948) the difference had diminished to 0·395 unit. The reaction between anserine and ninhydrin at these times was so far from complete that the reaction–time curves can, without serious error, be regarded as linear. Consequently, as the rate of reaction under these conditions is directly

proportional to anserine concentration, the equation above still applies (with a different $k_1$ value), and the differences between colour yields will give the slopes of straight lines representing the relationship of colour yield to the extent of hydrolysis.

In practice it is found that reaction at 100° with 0·2 μmole of anserine or an equivalent hydrolysate is satisfactory when the extent of hydrolysis is greater than 30%. Below 10% hydrolysis, accurate assessment is difficult and the limitations of the standard Schwartz & Engel (1950) procedure, as applied to anserinase assay, become apparent. The concentrations of reactants cannot be increased appreciably as the $k_1(1-x)$ component of the equation above would exceed the level beyond which Beer's law does not apply, and increases in colour yield are small compared with the remaining anserine.

With standard reaction mixtures (0·2 μmole of dipeptide or an equivalent hydrolysate), the sensitivity of the ninhydrin–amino acid reaction at 35° is about half that at 100°, but interference from anserine is reduced to negligible proportions. By keeping the proportion of enzyme reaction mixture to ninhydrin reagent constant, the volume of hydrolysis mixture has been increased from 0·2 to 1·6 ml. for reaction with 8 ml. of reagent at 35° and dilution to 10 ml. This increased the sensitivity some 400% as compared with that of the standard 100° procedure. By the use of cells with longer path-lengths, the sensitivity may be increased further.

## SUMMARY

1. Anserine reacts negligibly slowly with ninhydrin reagent at 35°, whereas its component 1-methylhistidine and β-alanine are reactive.

2. This provides a basis for an improved colorimetric procedure for measuring low levels of hydrolysis of anserine.

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# The Metabolism of Acetaldehyde in Mammalian Tissues

## REACTIONS IN RAT-LIVER SUSPENSIONS UNDER ANAEROBIC CONDITIONS

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Although a large part of the energy metabolism of mammals may be covered through the metabolism of ethanol, the actual pathway followed when this substance is catabolized is only imperfectly known. It is generally accepted that the first product of ethanol oxidation is acetaldehyde. For the subsequent reactions of this substance there are, however, a number of possibilities among which the oxidation to acetate is perhaps the most obvious. This oxidation may proceed in different ways. The presence in the liver of an aldehyde mutase catalysing the dismutation of two molecules of aldehyde to one molecule of alcohol and one of the corresponding acid was assumed for many years. This enzyme system was studied in considerable detail by Dixon & Lutwak-Mann (1937). They showed that diphosphopyridine nucleotide was necessary as a coenzyme. Adler, Euler & Günther (1938) showed that the mutase contains alcohol dehydrogenase, and Reichel & Burkart (1939) found that addition of a flavoprotein changed the dismutation into an oxidation. Racker (1949) prepared a diphosphopyridine nucleotide-requiring aldehyde dehydrogenase from ox liver and pointed out that the mutase activity observed by Dixon & Lutwak-Mann might be caused by a combination of aldehyde dehydrogenase and alcohol dehydrogenase. The presence of a specific mutase beside the two dehydrogenases has, however, not been disproved.

Apart from oxidation of acetaldehyde other possibilities exist. The reaction with pyruvate, resulting in the formation of acetoin with elimination of carbon dioxide, has been demonstrated in brain tissue by Stotz, Westerfeld & Berg (1944) and Berry & Stotz (1954), and has been studied in