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Comparison of Radioimmunoassay and Spectrophotometric Analysis for the Quantitation of Hypoxanthine in Fish Muscle

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ABSTRACT

The level of hypoxanthine in fish muscle either alone, or in conjunction with the levels of other ATP metabolites, is used as an indicator of quality. A radioimmunoassay for hypoxanthine is described, where 6-trichloromethyl purine conjugated to ovalbumin was used to raise a polyclonal antiserum to the analyte. The avidity of the affinity-purified antiserum was such that only a 5 min incubation period was required to form the maximum amount of antigen–antibody complex.

A comparison was made between hypoxanthine levels in fish muscle measured by an established manual xanthine oxidase assay, and the corresponding manual radioimmunoassay values. A linear relationship was established for values determined by both methods. The advantages of the radioimmunoassay are speed, greater specificity and a wider dynamic range.

INTRODUCTION

The accuracy of a determination may be defined as the concordance between it and the true value. There are two ways of determining the accuracy of a new analytical method, the absolute method and the comparative method.

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For the absolute method, synthetic samples containing known amounts of the analyte are used. Where it is impossible to prepare synthetic samples in this way, the comparative method is used. Samples are analysed by one or more supposedly 'accurate' methods of analysis. The agreement between two methods of essentially different character can usually be accepted as indicating the absence of an appreciable error in either.

Validation of a radioimmunoassay for hypoxanthine, therefore, involved finding the correlation between hypoxanthine concentrations in fish muscle as measured by radioimmunoassay and by a conventional assay method. Other approaches to quality assessment include the analysis of hypoxanthine, and in some cases other ATP metabolites also, either by HPLC (Murray *et al.*, 1984), or by means of enzyme based biosensors (Nomoto & Ohno, 1987; Mulchandani *et al.*, 1989) or electrodes (Watanabe *et al.*, 1986; Moody *et al.*, 1987).

For this investigation the most convenient of the established methods for measuring hypoxanthine was the enzymatic method using xanthine oxidase. Neutralised, deproteinised extracts are treated with the enzyme, and the increase in UV absorbance at 290 nm, due to the production of uric acid, is measured (Jones *et al.*, 1964).

Hypoxanthine levels in samples of trout and whitebait measured by radioimmunoassay were compared with measurements made by spectrophotometric analysis. The computational method of Brace (1977) was used to establish a linear relationship for values for hypoxanthine concentration as determined by both methods.

MATERIALS

[G-^{3}H] hypoxanthine (16·2 mCi/mg; 599 MBq/mg) was purchased from Amersham International, Bucks (Amersham code TRA 74). Non-ulcerative Freund's adjuvant was from Guildhay Antisera Ltd., Guildford, Surrey. Hydrochloric acid was obtained from May and Baker Ltd, Dagenham. AH-Sepharose 4B was from Pharmacia Ltd, Milton Keynes.

Trifluoroacetic acid, 6-trichloromethyl purine, sulphuryl chloride and perchloric acid were obtained from Aldrich Chemical Co. Ltd, Gillingham, Dorset. Hypoxanthine, xanthine oxidase from milk (grade IV), uric acid, bovine serum albumin (Cohn fraction V), ovalbumin, 6-methyl purine, tetrahydrofuran, Linde type 4A molecular sieve and Tris (hydroxymethyl) aminomethane were from Sigma Chemical Co. Ltd, Poole, Dorset. All other chemicals and solvents were purchased from BDH Chemicals Ltd, Eastleigh, Hampshire, and were of analytical grade.

METHODS

Preparation of 6-trichloromethyl purine

The method used was adapted from a method describing the conversion of 6-methyl purine into 6-trichloromethyl purine, using either *N*-chlorosuccinimide or sulphuryl chloride (Cohen *et al.*, 1962).

Trifluoroacetic acid and sulphuryl chloride were both dried before use, by standing for 24 h over Linde type 4A molecular sieve. 6-methyl purine (250 mg) was dissolved in 5 ml trifluoroacetic acid. Sulphuryl chloride (1 ml) was added, and the mixture was refluxed for 4 h. The solvent was evaporated by passing nitrogen gas through the solution, resulting in a product of syrupy consistency. The residue was freed from adhering acid by repeated addition of methanol and evaporation (three times). The product was taken up in the minimum volume of methanol and shaken vigorously to ensure thorough mixing. After standing overnight at 4°C, 6-trichloromethyl purine was obtained as a white crystalline material. The crystals were dried with filter paper and stored in a desiccator over silica gel.

Conjugation of 6-trichloromethyl purine with ovalbumin

Some haptens are reactive enough to be coupled to proteins directly, examples being penicillin (Levine & Ovary, 1961; Levine, 1966) and phenylalanine mustard (Burke *et al.*, 1966). 6-trichloromethyl purine was reactive enough to be conjugated with ovalbumin directly, using the method of Butler *et al.* (1962).

Ovalbumin (494 mg) and 6-trichloromethyl purine (170 mg) were dissolved in 4% aqueous tetrahydrofuran (75 ml). The mixture was stirred at room temperature for 3 h, and the pH was maintained throughout at 10·0–10·5 by the addition of sodium hydroxide (1 M). During the first hour, the pH decreased rapidly, so that it was necessary to add sodium hydroxide at intervals of less than a minute, in order to keep the pH within the correct range, but the reaction gradually slowed over the next 2 h. The solution (which became dark brown in colour during the course of the reaction), was dialysed overnight against running tap water.

Upon acidification to pH 4·5 with hydrochloric acid (0·1 M), the conjugated protein precipitated. It was redissolved in sodium bicarbonate solution (25 ml; 0·15 M), dialysed against running tap water, and then freeze-dried.

Antiserum production

A Suffolk Cross sheep was immunised with the hypoxanthine–ovalbumin conjugate (6 mg) intramuscularly into multiple sites in the rear legs, using

non-ulcerative Freund's adjuvant (2 ml) and BCG vaccine (Evans Medical) (0·1 ml). Subsequently, the animal was 'boosted' at intervals of approximately 4 months with 2 mg of the conjugate in Freund's adjuvant without BCG. Blood was collected from the jugular vein (20 ml/bleed) at approximately 2 to 3 week intervals. Larger bleeds (500 ml/bleed) were taken after each boost.

The blood was allowed to clot overnight at room temperature, and serum separated and centrifuged; this was stored at 4°C with the addition of 0·1% w/v sodium azide.

Preparation of immunoadsorbent

In order to prepare an immunoadsorbent for anti-hypoxanthine antibodies, AH-Sepharose 4B was used as the solid support. This matrix has free primary amino groups at the end of 6-carbon spacer arms, so that the most usual coupling reaction involves ligands with free carboxyl groups. However, 6-trichloromethyl purine is reactive enough to be coupled directly with free amino groups. This hypoxanthine derivative was, therefore, reacted with AH-Sepharose 4B, so that the procedure was analogous to that used for linking the hapten to the E-amino groups of lysine residues of ovalbumin, when preparing the immunogen.

AH-Sepharose 4B (5 g) was allowed to swell in an excess of sodium chloride solution (0·5M) for 15 min. The gel was then poured into a sintered glass funnel in a Buchner flask and washed with sodium chloride solution (1 litre, 0·5M) to remove lactose and dextran, followed by distilled water (1 litre) to remove sodium chloride. 6-trichloromethyl purine (47 mg) was dissolved in 4% aqueous tetrahydrofuran (40 ml) and this was added to the AH-Sepharose 4B with slow magnetic stirring. The pH was maintained between 10·0–10·5 for $1\frac{1}{2}$ h by the dropwise addition of sodium hydroxide solution (1M). To remove any excess uncoupled ligand, the adsorbent was washed alternately with high and low pH buffer solutions; carbonate/bicarbonate buffer (0·05M, pH 9·8, 500 ml) was used, followed by acetate buffer (0·2M, pH 4·5, 500 ml), each containing sodium chloride (0·5M). The gel was then washed with distilled water (1 litre) followed by barbitone buffer (0·05M, pH 8·6, 80 ml). The derivatised Sepharose was resuspended in barbitone buffer (20 ml) so that the final concentration was 0·25 g/ml.

Adsorption of antibodies specific for hypoxanthine

Different batches of antiserum were pooled before affinity purification. In a typical experiment for the adsorption of anti-hypoxanthine antibodies, derivatised Sepharose suspended in barbitone buffer (5 ml, 0·25 g/ml) was

added to neat antiserum (10 ml) in a Sovril tube. In order to maximise the interaction between the immunoadsorbent and the antiserum, the Sovril tube was rotated end over end for 24 h at 4°C rather than passing the antiserum straight down a column.

After 24 h, the contents of each Sovril tube were poured into a 3 × 8 cm sintered glass column. In order to check that all anti-hypoxanthine antibodies had been removed by the immunoadsorbent, an antiserum dilution curve was carried out on the effluent, which was thus shown to have no capacity for binding radiolabelled hypoxanthine.

In order to remove non-specifically bound proteins, the column was washed extensively with barbitone buffer (0·05M, pH 8·6) containing sodium chloride (0·5M). The presence of protein in the washings was monitored by measuring the absorbance of the effluent at 280 nm, and washing was continued until the absorbance caused by eluted protein was $<0{\cdot}04$. It was found that after the addition of 30 ml of buffer, there was no further decrease in the absorbance at 280 nm.

Elution of affinity-purified anti-hypoxanthine antibodies

Sodium hydroxide solution (0·05M, 6 ml) was added to each column, and the antibodies were eluted into Tris/HCl buffer (1M, pH 7·0, 1 ml) followed by dialysis against Tris/HCl buffer (5 mM, pH 7·4) with five changes of buffer during a period of 24 h. After dialysis, the antibodies were concentrated by ultrafiltration using a concentration cell with a PM 10 Diaflo ultrafiltration membrane (Amicon Ltd) and a pressure of 30 psi, and freeze-dried in aliquots (2 ml) for long term storage.

Dilution of [G-^{3}H hypoxanthine

[G-^{3}H] hypoxanthine (1 mCi) (specific activity 81·4 G Bq/mmol, 599 M Bq/mg) was dissolved in hydrochloric acid (8·8 ml; 0·1M) to give a solution of concentration 7 μg/ml. For long-term storage, 0·5 ml aliquots of this solution were placed in vials, freeze-dried and stored at -20°C to minimise decomposition of the tritiated label. As required, the contents of a vial were dissolved in distilled water (50 ml) (1:100 dilution) and stored at -20°C in 1 ml aliquots as a stock concentration (114 n Ci/ml; 70 ng/ml). Each 1 ml aliquot was thawed, then diluted 1/10 with buffer immediately before use, so that 5·1 p moles (700 pg) of [G-^{3}H] hypoxanthine were added per tube. This gave approximately 4000 cpm in the total counts tube when later measured by liquid scintillation counting after using ammonium sulphate for phase separation.

Preparation of neutralised perchloric acid extracts of fish muscle

A protein-free extract of hypoxanthine was obtained from samples using perchloric acid as a protein precipitant, by a modification of the method of Jones *et al.* (1964).

Whole whitebait were obtained frozen from a local fishmonger and were of indeterminate age, having been stored in a commercial freezer, with unknown history prior to freezing. A rainbow trout was obtained freshly killed from a local fish farm, so that samples were prepared approximately 1 h *post mortem*, by dissecting out 5 g blocks of muscle from the skin and bone.

The whole whitebait were likewise cut into 5 g samples, but without any sample pretreatment other than that which had been carried out commercially. Samples were incubated in petri dishes at 4°C over periods of time ranging from 0–9 days, to give samples of varying degrees of freshness. Each sample was homogenised for 1 min in a Waring blender with perchloric acid (50 ml, 0·6M). Following homogenisation, the extract was centrifuged at 0°C at 2000 rpm for 5 min in a Beckman J6B refrigerated centrifuge in order to remove the precipitated proteins. The supernatant, which was the protein-free extract of fish muscle containing hypoxanthine, was decanted, and 30% w/v potassium hydroxide was added to bring the pH to 6·5–7·0. Upon the addition of potassium hydroxide solution, potassium perchlorate was precipitated. The volume was made up to 75 ml with distilled water and the precipitate of potassium perchlorate was removed by centrifugation at 0°C at 2000 rpm for 10 min.

Estimation of hypoxanthine

1. By xanthine oxidase

The method was adapted from that described by Jones *et al.* (1964). Xanthine oxidase from milk, grade IV (Sigma) (88 mg protein/ml; specific activity 0·14 units/mg) was used for the oxidation of hypoxanthine. The enzyme solution was stored in concentrated form at 4°C, and a solution of the appropriate dilution was freshly prepared before each assay. For dilution of the enzyme, an aliquot of the commercial preparation (250 μl) was made up to 50 ml with phosphate buffer (0·1M, pH 7·6), giving a solution of specific activity of 0·06 units/ml. The dilute enzyme solution was kept in ice throughout the assay.

A solution of hypoxanthine (125 nmole/ml) in phosphate buffer (0·1M, pH 7·6) was diluted with the same buffer to give hypoxanthine standards ranging from 10·4–62·5 nmole/ml. Aliquots of the diluted xanthine oxidase solution (0·5 ml; 0·06 units/ml) were added to each of the hypoxanthine

standards (2·5 ml). Incubation was for 30 min in a 37°C water bath, followed by determination of the absorbance at 290 nm against a blank consisting of phosphate buffer.

Xanthine oxidase is subject to inhibition at high substrate concentrations. Neutralised perchloric acid extracts of whitebait (which contained high concentrations of hypoxanthine) were diluted 1/20 with phosphate buffer (0·1 M, pH 7·6). Trout samples (which contained lower levels of hypoxanthine) were diluted 1/5. The blank in each case consisted of the sample at the appropriate dilution incubated in the absence of enzyme. Results obtained were multiplied by the dilution factor to give results in μmole/g fish muscle.

Ultra violet spectrophotometric measurements were carried out on an Ultrospec 4050 LKB fixed wavelength spectrophotometer using quartz cuvettes with a 1 cm light path.

2. By radioimmunoassay

All radioimmunoassay procedures were performed in barbitone buffer (0·05 M, pH 8·6) containing 0·1% bovine serum albumin. This buffer was used for dilution of antisera, standards, [G-^{3}H] hypoxanthine, and also for dilution of samples. Affinity-purified antibodies, freeze-dried in 2 ml aliquots, were reconstituted as required by adding 4 ml barbitone buffer. A solution of hypoxanthine (544 μg/ml; 4 μmole/ml) was double diluted with barbitone buffer to give standards ranging in concentration from 15·6–1000 nmole/ml. Standards were freshly prepared before each assay. All measurements were made in duplicate. Each assay was set up with tubes standing in iced water. The reagents (in the order indicated in Table 1) were added to LP3 plastic tubes (Luckham Ltd, Sussex). The contents were mixed by vortexing after the addition of [G-^{3}H] hypoxanthine, and again after the addition of saturated ammonium sulphate.

TABLE 1
Measurement of [G-^{3}H] Hypoxanthine by Radioimmunoassay
(Volume of reagents added in μl)

Reagent	*Total counts tube*	*Zero standard tube*	*Standard or sample tube*
Diluent buffer	200	100	—
Hypoxanthine standard/sample	—	—	100
Affinity purified antibodies	—	100	100
[G-^{3}H] hypoxanthine	100	100	100
Saturated ammonium sulphate	150	150	150

Ammonium sulphate was used for phase separation, aliquots of a saturated solution being used to give 33% saturation after addition to all tubes, including the total counts tubes. Ammonium sulphate was added to all assay tubes in order to ensure equal quenching. It precipitates antibodies and antibody–antigen complexes, so that free hypoxanthine remains in the supernatant.

The tubes were centrifuged at 3000 rpm for 10 min at 4°C in a Beckman J6B refrigerated centrifuge. An aliquot of the supernatant (250 μl) was taken from each tube and placed in LSC Minivials (LKB) containing scintillation cocktail Optiphase Safe (LKB) (4 ml). When ammonium sulphate is used for phase separation and aliquots of the supernatant are added to liquid scintillant, eventually the aqueous layer settles out from the organic solvent layer. Such phase separation can cause problems in liquid scintillation counting, so vials were counted before and after separation in order to establish that there was no such effect. The amount of [G-^{3}H] hypoxanthine in each vial was determined using an LKB Rackbeta (1212) liquid scintillation counter, with external standard and counting efficiency of the order of 60%. Each vial was counted for 300 sec. All measurements were made in duplicate.

Counts per minute (CPM) values were computed as follows:

$$\%\ \text{free}\ [\text{G-}^3\text{H}]\ \text{hypoxanthine} = \frac{\text{CPM specific binding tube}}{\text{CPM total counts tube}} \times 100$$

$$\%\ \text{bound}\ [\text{G-}^3\text{H}]\ \text{hypoxanthine} = 100\text{-}\%\ \text{free}\ [\text{G-}^3\text{H}]\ \text{hypoxanthine}$$

RESULTS

Calibration curve

In order to construct a calibration curve (Fig. 1), [G-^{3}H] hypoxanthine bound/total radioactivity added × 100 was plotted against hypoxanthine concentration, and a classical sigmoidal curve was obtained. The calibration points in the assay ranged from 15·6–1000 nmole/ml, with an effective working range between 15 nmole/ml and 300 nmole/ml. This gave a greater dynamic range than the spectrophotometric assay where the working range was between 10 nmole/ml and 120 nmole/ml (Fig. 2).

Cross-reactivity

The procedure for testing for cross-reactivity of the antibodies with other purines and nucleosides analogous to hypoxanthine was identical to that

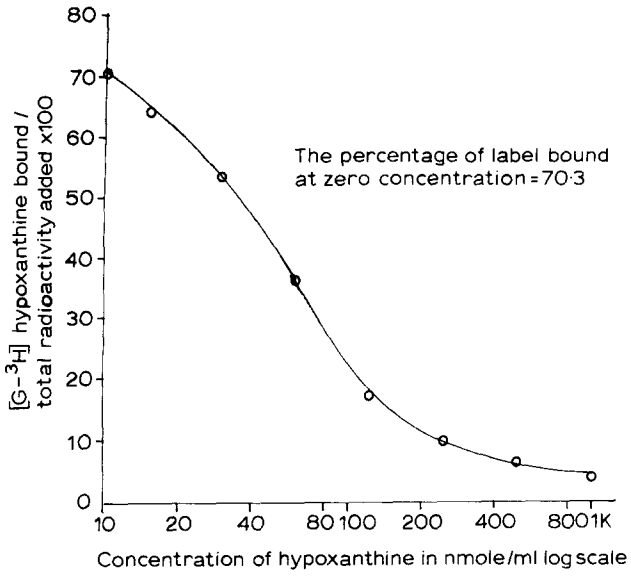

Fig. 1. Representative standard curve for the measurement of hypoxanthine by radioimmunoassay.

used to obtain a standard curve, except that putative cross-reacting substances were used instead of the standards, at concentrations up to 10 μmole/ml. If there is zero cross-reaction the label is bound to the same degree, and if there is cross-reaction, less label is bound by the antibodies.

The percentage cross-reaction was calculated as the ratio × 100 of the weights of antigen and cross-reactant required to reduce the binding of

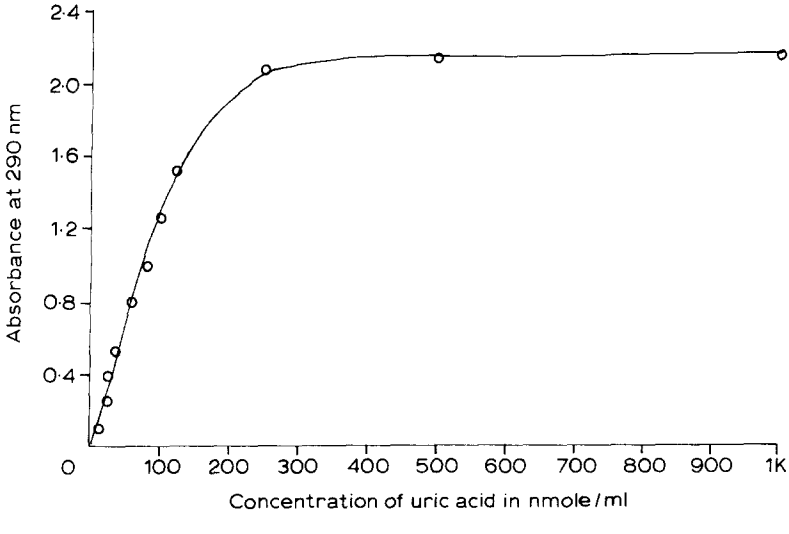

Fig. 2. Uric acid standard curve.

radiolabelled antigen present to 50% of maximum. Adenine gave a 1·4% cross-reactivity. Cross-reactivity was too small to be measured in the cases of inosine, guanine, uric acid, adenosine and xanthine.

Precision

The coefficient of variation was calculated for five values obtained on five different days for [G-^{3}H] hypoxanthine bound/total radioactivity added × 100, for each of the data points forming the radioimmunoassay standard curve. At hypoxanthine concentrations not greater than 125 nmole/ml this was 5·90 ± 1·49 (mean ± SEM) ($n = 5$). However, at hypoxanthine concentrations > 125 nmole/ml, there was a general increase in coefficients of variation for the data points of the standard curve, indicating less accuracy at higher concentrations of hypoxanthine. This inaccuracy is inherent in the nature of the radioimmunoassay standard curve, which is not linear. The sigmoidal shape of the curve (Fig. 3), means that very small differences in the value of [G-^{3}H] hypoxanthine bound/total radioactivity added × 100 (y axis), result in very large differences for analyte concentration (x axis) being read from the standard curve at high analyte concentrations.

In order to evaluate inter-assay precision, five samples containing 65, 134, 263, 336 and 401 nmole/ml hypoxanthine were measured over a period of 5

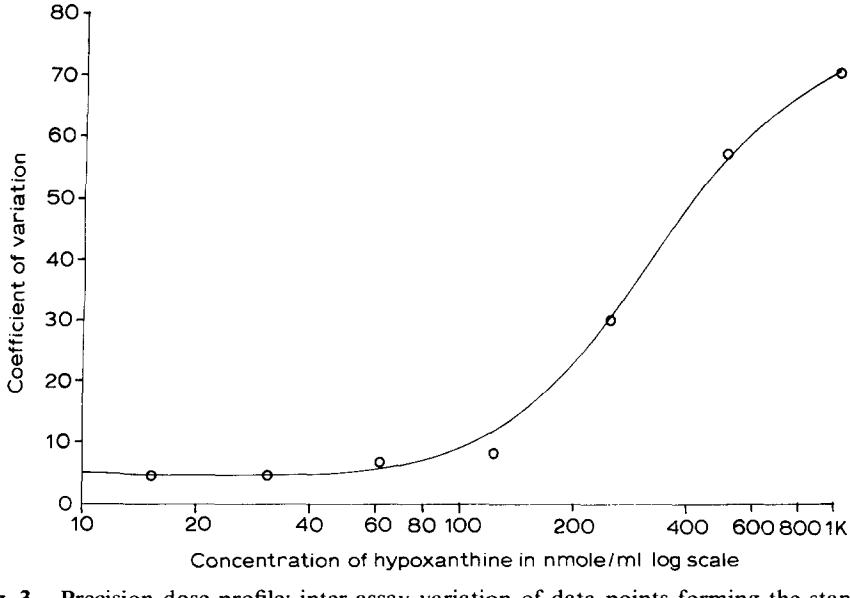

Fig. 3. Precision dose profile: inter-assay variation of data points forming the standard curve for the measurement of hypoxanthine by radioimmunoassay. Each value of coefficient of variation is derived from five determinations of the standards.

TABLE 2
Precision of the Radioimmunoassay for Hypoxanthine

Concentration of hypoxanthine nmole/ml undiluted fish extract (five determinations)	*Coefficient of variation*
65	7·6
134	14·5
263	12·6
336	5·0
401	7·3

days. The values for coefficient of variation were 7·6, 14·5, 12·6, 5·0 and 7·3%, respectively (Table 2).

Correlation

Hypoxanthine was assayed in 45 whitebait samples by radioimmunoassay and by spectrophotometric analysis. The values for hypoxanthine concentration as determined by radioimmunoassay were plotted against those determined by the spectrophotometric method, and the line of best fit was computed. The linear regression line obtained had the following characteristics:

y (radioimmunoassay) = 0·993 x (spectrophotometry) + 0·476
where $r = 0{\cdot}845$ (Fig. 4).

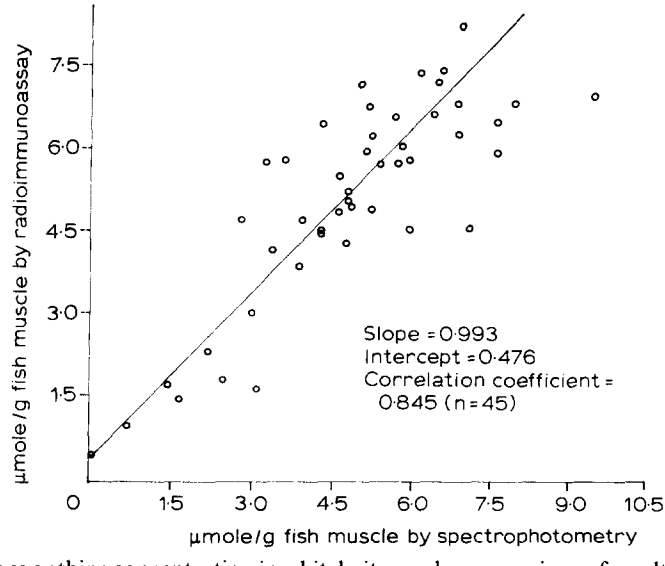

Fig. 4. Hypoxanthine concentration in whitebait muscle: comparison of results obtained by radioimmunoassay and enzymic analysis.

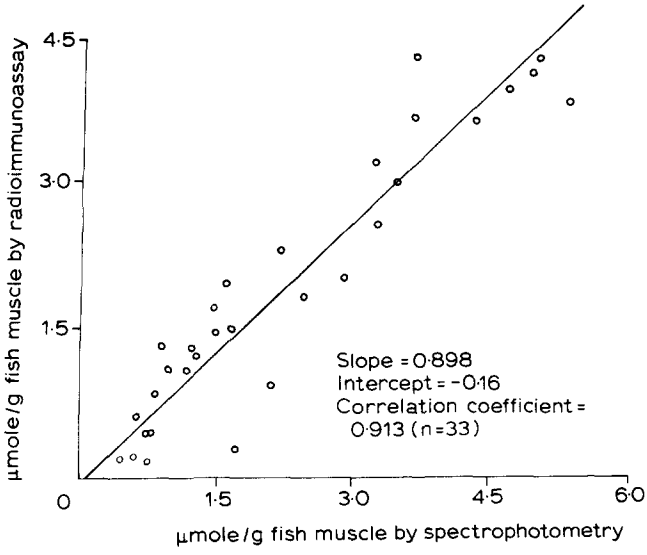

Fig. 5. Hypoxanthine concentration in trout muscle: comparison of results obtained by radioimmunoassay and enzymic analysis.

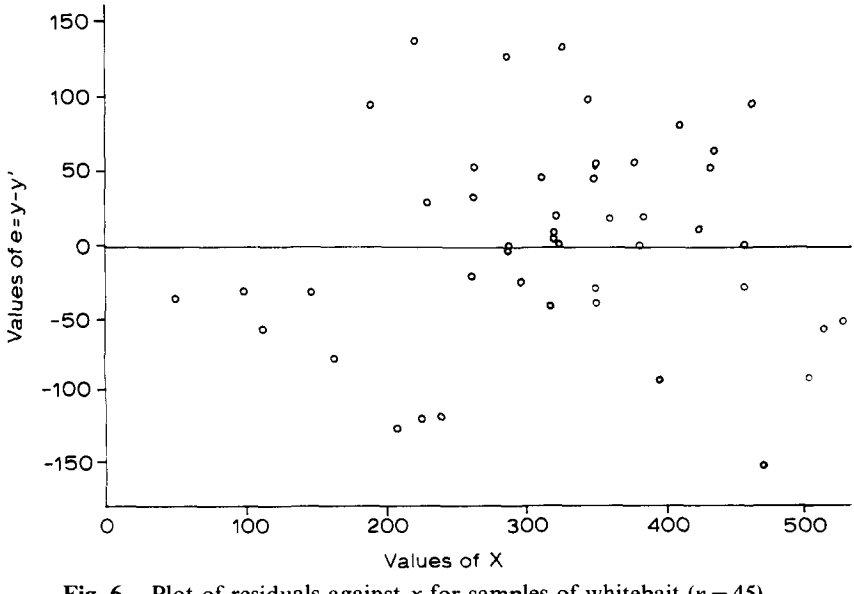

Fig. 6. Plot of residuals against x for samples of whitebait ($n = 45$).

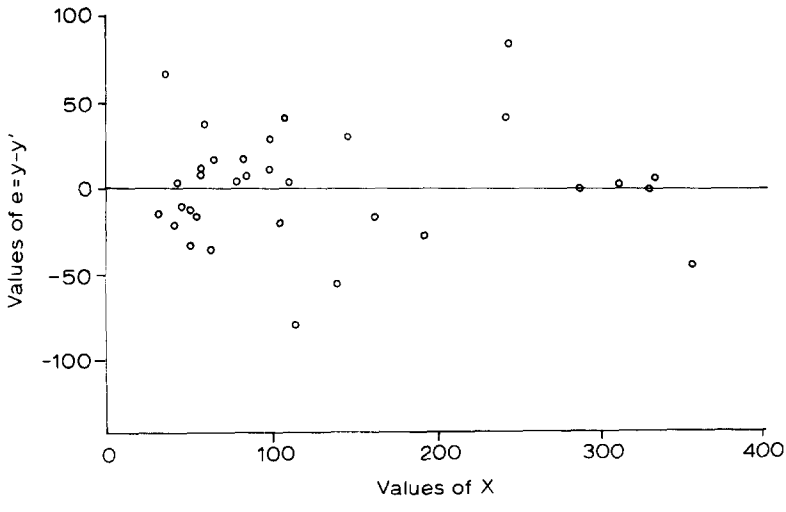

Fig. 7. Plot of residuals against x for samples of trout ($n = 33$).

Hypoxanthine was also assayed in 33 trout samples by both methods, and the regression characteristics were:

y (radioimmunoassay) = 0·898 x (spectrophotometry) − 0·16
where $r = 0{\cdot}933$ (Fig. 5).

The individual residuals of y, δy, are a measure of the extent to which the experimental values deviate from the best fit. $\delta y = y\text{-}a\text{-}bx$, where a and b are the linear least-squares fit parameters. If the various assumptions of the linear regression model are satisfied, a plot of residuals, $e = y\text{-}y^1$, against each of the x variables should show no discernible trends or patterns, as was shown to be the case in Fig. 6 and 7.

DISCUSSION

The method presented here gave a good correlation when compared with a conventional assay method, and the radioimmunoassay has the advantages of speed and a wide dynamic range. Most small molecules react very rapidly with high affinity antibodies, equilibrium usually being reached in less than a minute. The radioimmunoassay for hypoxanthine is carried out as a continuous operation, with no time set aside for incubation, other than the time taken to add reagents to all tubes in the assay and mix by vortexing.

Correlations between the present method and the spectrophotometric method gave acceptable correlation coefficients. With standard least-squares regression analysis, the value of the slope and intercept depends heavily on the choice as to which variable is treated as independent, as the

method treats the data as if all errors are in the y variable, and no errors are present in the x variable. However, when x and y values represent two different experimental methods for determining the same quantity, errors exist in both methods, although the exact value of these errors is unknown. Under this condition, standard least-squares regression analysis underestimates the value of the slope. Therefore, the computation used to establish a linear relation for values of hypoxanthine concentration as determined by both methods, was that of Brace (1977), where the slope found by the conventional least-square regression analysis (with x the independent variable) is divided by the absolute value of the correlation coefficient. The intercept is then found from $b = y\text{-}ax$.

Assay precision was acceptable for a manual assay, being between 5% and 14·5% for between assay runs.

The radioimmunoassay was specific for hypoxanthine, the affinity purified antibodies only having a 1·4% cross-reaction with adenine, the principal cross-reactant. Other compounds tested had no cross-reactivity, even at levels which grossly exceeded their likely relative concentrations in food samples (Roberts *et al.*, 1988). Xanthine oxidase, on the other hand, is an enzyme of broad specificity. Formaldehyde and acetaldehyde are known substrates of the enzyme, and also components of fish muscle (Ota, 1958; Amano *et al.*, 1963; Amano & Yamada, 1964; Greenlee & Handler, 1964), although their oxidation would not lead to an increase in optical density at 290 nm, the wavelength used to monitor uric acid production from hypoxanthine. However, xanthine, if present, would be oxidised to uric acid and estimated as hypoxanthine Kassemsarm *et al.* (1963), detected traces of xanthine ($< 0{\cdot}1$ μmole/g) after 14 days of chill storage in plaice and haddock, and xanthine was also detectable in lemon sole at 12 and 23 days. The absence of xanthine from samples, therefore, needs to be established before measurements can be accepted as those of hypoxanthine alone when using the enzymatic method, whereas no interference from xanthine occurred in the radioimmunoassay.

Another possible source of error can occur with the enzymatic method if there is too great a contamination of xanthine oxidase by other enzymes, which may lead to estimate of inosine and other nucleoside derivatives as hypoxanthine (Jones *et al.*, 1964). Again, with the radioimmunoassay there was no cross-reactivity with inosine, nor with other nucleoside derivatives tested (Roberts *et al.*, 1988)'

Pre-dilution of samples was necessary to adapt the system to a useful range, but lower dilution could be performed if more sensitivity was needed. One advantage of radioimmunoassay is that the range of concentrations of the analyte encompassed by the standard curve is much greater than for the spectrophotometric method. Ideally, a standard curve should encompass the

range of values of the analyte likely to be found in samples, so that no adjustment of sample concentration is necessary prior to analysis. However, the concentration range over which a linear relationship was observed between hypoxanthine concentration and absorbance at 290 nm had an upper limit of 120 nmole/ml (Fig. 2), which proved to be considerably lower than the concentration of hypoxanthine in neutralised perchloric acid extracts of fish, prepared as described.

The extraction method was an adaptation of that used by Jones *et al.* (1964) where muscle from the anterio-dorsal portion of cod fillets was homogenised with perchloric acid. In the method described here, trout and whitebait samples were prepared using the same volume of perchloric acid, but using a smaller weight of sample in order to give less concentrated extracts of hypoxanthine. However, the concentration of the analyte was still too high for values to be read directly from the standard curve without sample dilution.

Rejection limits of 1·7 μmole/g have been suggested for rainbow trout (Murray *et al.*, 1984), 5 μmole/g for winter flounder (Jahns *et al.*, 1976), and a more tentative value of 4–8 μmole/g of muscle has been put forward as indicative of incipient spoilage in shrimp (Flick & Lovell, 1972). Following the perchloric acid procedure described, trout and flounder containing these amounts of hypoxanthine would result in solutions of 113 and 333 nmole/ml concentration, respectively, and shrimp muscle would give an extract of 267–533 nmole/ml. Dilution of the more concentrated extracts would, therefore, be necessary in order to give values lying on the most sensitive part of the radioimmunoassay standard curve. One unexplored possibility is the addition of a larger volume of water after the neutralisation stage, when all samples are made up to the same volume. This modification to the extraction procedure would give a less concentrated solution of hypoxanthine for analysis.

The most serious drawback of the enzymatic method is that xanthine oxidase exhibits enzyme inhibition at high substrate concentrations. Burt *et al.* (1968) obtained maximum responses with hypoxanthine standards of about 125 μg/ml. Higher levels gave deflections that, with increasing concentration, decreased to the point that 500 μg/ml gave the same response as 100 μg/ml. Although with the radioimmunoassay hypoxanthine determinations become less accurate at concentrations > 125 nmole/ml, this method does not have the serious drawback of giving low readings for hypoxanthine concentrations when, in reality, concentration is high, as can happen with the spectrophotometric method. In situations where precise determination of concentration is unnecessary and all that is needed is to decide a suitable cut off point above which hypoxanthine concentration is unacceptably high, radioimmunoassay would, therefore, be the method of choice.

Although it was not convenient in the present study to compare the radioimmunoassay with an established HPLC method, generally radioimmunoassays achieve more rapid throughput of extracts, although the HPLC methods usually quantify other ATP metabolites also.

Neither was it possible to compare the radioimmunoassay with the enzyme electrode and biosensor methods, briefly referred to in the introduction, since none were commercially available.

Under the circumstances the radioimmunoassay described here deserves serious consideration when a specific method is required for hypoxanthine determination in fish muscle.

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