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# Indoleamine 2,3-dioxygenase

## A NEW, RAPID, SENSITIVE RADIOMETRIC ASSAY AND ITS APPLICATION TO THE STUDY OF THE ENZYME IN RAT TISSUES

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A simple and convenient assay for indoleamine 2,3-dioxygenase has been developed. This depends on the conversion of D-[*ring*-2-$^{14}$C]tryptophan to [$^{14}$C]formate, excess substrate is removed by adsorption onto charcoal. This assay, which is 20-fold more sensitive than previous procedures, is applicable both to crude extracts and to large numbers of samples. Activity in rat tissues is very much lower than in those of the rabbit; measureable activity is found only in the stomach, spleen, intestine and kidney. Enzyme activity in the rat intestine was increased by 50% in rats pretreated with L-tryptophan.

Indoleamine 2,3-dioxygenase [indole–oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.17] is a haem-containing enzyme capable of catalysing the oxidative cleavage of the indole moiety of a number of substituted and unsubstituted indoleamines, such as D- and L-tryptophan, 5-hydroxytryptophan, tryptamine, melatonin and 5-hydroxytryptamine (Hirata *et al.*, 1974). Studies on the enzyme purified from rabbit small intestine have revealed that, unlike the classical hepatic L-tryptophan 2,3-dioxygenase [pyrrolase; L-tryptophan–oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11] it appears to use the superoxide anion $O_2^-$, rather than molecular oxygen as the oxygenating agent (Hirata & Hayaishi, 1975; Ohnishi *et al.*, 1977). In addition, indoleamine 2,3-dioxygenase differs from L-tryptophan 2,3-dioxygenase in that it is found in a number of tissues in the rabbit, including the intestine, lung, stomach, spleen, brain and possibly liver (Hirata & Hayaishi, 1975).

Further study of indoleamine 2,3-dioxygenase is of considerable interest, since its function *in vivo* and the nature of the physiological substrate(s) are still unclear. It has been suggested that it may be involved in the regulation of serotonin levels in the brain and small intestine (Hirata *et al.*, 1974, 1977) and in the degradation of melatonin (Hirata & Hayaishi, 1975).

The measurement of indoleamine 2,3-dioxygenase activity in small tissue samples is limited by the sensitivity of currently available spectrophotometric methods (Yamamoto & Hayaishi, 1967; Hirata & Hayaishi, 1975), although a more sensitive radiometric procedure, in which enzyme activity is determined by isolation and quantification of [$^{14}$C]-formate released from DL-[*ring*-2-$^{14}$C]tryptophan (by ion-exchange chromatography), has been applied to highly purified enzyme preparations (Ohnishi *et al.*, 1977). However, this method is slow and its use is restricted to the purified enzyme or to crude tissue extracts that are known to be free of L-tryptophan 2,3-dioxygenase activity. Further, interpretation of results obtained with DL-[*ring*-2-$^{14}$C]tryptophan as substrate may be difficult, since indoleamine 2,3-dioxygenase displays greatly different catalytic properties with respect to the two optical isomers of tryptophan (Shimizu *et al.*, 1978).

In the present study, we report a new radiometric assay procedure for indoleamine 2,3-dioxygenase which overcomes these problems by (i) using D-[*ring*-2-$^{14}$C]tryptophan as substrate, and (ii) replacing the ion-exchange chromatographic separation of [$^{14}$C]formate from [$^{14}$C]tryptophan with a charcoal-adsorption procedure. Using this radiometric procedure, which is at least 20-fold more sensitive than comparable spectrophotometric assays and which is applicable to large numbers of crude tissue samples, the distribution of indoleamine 2,3-dioxygenase in rat tissues and its response to L-tryptophan and corticosteroid administration have been determined. The results are discussed in relation to the possible physiological role of the enzyme.

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## Materials and methods

### *Animals*

Male Sprague–Dawley rats (CD/ASH strain; 200–250g) and male New Zealand white rabbits (2–3kg) were used throughout. All animals received food [no. 1 maintenance diet (rats) from Cooper Nutritional Products, Witham, Essex, U.K., or SGI diet (rabbits) from Grain Harvesters, The Old Collier, Wingham, Canterbury, Kent, U.K.] and water *ad libitum*. In some experiments rats were injected intraperitoneally with L-tryptophan (750mg/kg body wt.) or triamcinolone (5mg/kg body wt.) as described previously (Pogson & Smith, 1975; Smith & Pogson, 1977).

### *Chemicals and biochemicals*

D- and L-tryptophan were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. PCS scintillator cocktail was from Hopkin and Williams, Chadwell Heath, Essex, U.K. and Norit GSX charcoal was purchased from Norit Clydesdale Co., Glasgow, Scotland, U.K. Triamcinolone (9$\alpha$-fluoro-16$\alpha$,17$\alpha$-isopropylidenedioxy-1-dehydrocorticosterone) acetonide was a gift from E. R. Squibb and Sons, Moreton, Liverpool, U.K. DL-[*ring*-2-$^{14}$C]-Tryptophan (sp. radioactivity 35Ci/mol) came from Schwarz–Mann, Orangeburg, NY, U.S.A. [$^{14}$C]-Formate (specific radioactivity 50Ci/mol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals and biochemicals were of the purest grade available from standard suppliers.

### *Purification of* D-[*ring*-2-$^{14}$C]*tryptophan*

DL-[*ring*-2-$^{14}$C]Tryptophan was resolved optically by affinity chromatography on bovine serum albumin–Sepharose 4B as described by Stewart & Doherty (1973). This procedure is dependent upon the stereospecific binding of L-tryptophan to bovine serum albumin; D-tryptophan remains unbound and is eluted from the column in the sample application buffer (25mM-sodium borate, pH9.2). D-[*ring*-2-$^{14}$C]Tryptophan was extracted from borate buffer by adsorption onto a column (50mm × 15mm) of Amberlite XAD-2 ion-exchange resin, pre-equilibrated with water. After washing with 2 column volumes of water, D-[*ring*-2-$^{14}$C]tryptophan was eluted with 10ml of methanol. The methanol was removed under a current of $N_2$ at 60°C and the residue was redissolved in a small volume of distilled water to give a stock solution of D-[*ring*-2-$^{14}$C]-tryptophan of specific radioactivity 100$\mu$Ci/ml. The overall recovery of D-[*ring*-2-$^{14}$C]tryptophan was 85%.

### *Enzyme preparation and assay procedures*

Animals were killed by cervical dislocation and exsanguination. The gastrointestinal tract was rapidly removed, placed on ice and divided into anatomical regions. The contents of the individual gut regions were flushed out with ice-cold 0.9% (w/v) NaCl. Tissue samples were finely minced with scissors, washed with 0.9% NaCl, blotted gently and then extracted in 50mM-potassium phosphate, pH6.0 (2ml/g) by using a Teflon/glass homogenizer. Homogenates were centrifuged (5min, 12000*g*) at 4°C, and the resultant supernatants were used as the crude enzyme preparation.

(*i*) *Spectrophotometric assay.* Indoleamine 2,3-dioxygenase was assayed by a modification of the method of Yamamoto & Hayaishi (1967). Incubations (37°C) were performed in 0.5ml Beckman centrifuge tubes containing, in a final vol. of 0.2ml, pH7.5; 100mM-potassium phosphate, 0.2mM-Methylene Blue, 10mM-sodium ascorbate, 50$\mu$g of catalase, 5mM-D-tryptophan and enzyme preparation. Incubations, in triplicate, were terminated by addition of 0.02ml of 20% (w/v) $HClO_4$. After centrifugation (5min, 12000*g*) to remove precipitated protein, the absorbance at 360nm of 0.2ml of the supernatant was determined after mixing with 1ml of 1M-Tris/HCl, pH7.0. Enzyme activities were calculated assuming a molar absorbance coefficient for kynurenine of $4500\,M^{-1}\cdot cm^{-1}$ (Higuchi & Hayaishi, 1967). Blank determinations, in which D-tryptophan was either omitted or added after $HClO_4$, were carried through the entire assay procedure.

(*ii*) *Radiometric procedure: ion-exchange method.* Incubation conditions were identical with those described for the spectrophotometric assay except that the concentration of D-tryptophan (containing 0.1$\mu$Ci of D-[*ring*-2-$^{14}$C]tryptophan) was decreased to 0.5mM. After acidification with 0.02ml of 20% $HClO_4$ and centrifugation to remove precipitated protein, samples were left at room temperature (approx. 20°C) for 1h to ensure complete hydrolysis of *N*-[$^{14}$C]formyl-D-kynurenine (Ohnishi *et al.*, 1977). Portions (0.2ml) of supernatant were applied to columns (2cm × 0.5cm) of Amberlite CG-120 ($H^+$ form) ion-exchange resin contained in disposable Pasteur pipettes. Unlabelled carrier sodium formate (5$\mu$mol) was added to each sample and [$^{14}$C]formate was eluted from the columns with 1.8ml of water and counted for $^{14}$C radioactivity in 10ml of PCS scintillator cocktail. Under these conditions [$^{14}$C]tryptophan was quantitatively adsorbed onto the column.

(*iii*) *Radiometric procedure: charcoal adsorption method.* Incubation conditions were as described in (ii) above. After addition of $HClO_4$, 0.1ml of an aqueous suspension (50mg/ml) of Norit GSX charcoal was added to each tube. After thorough mixing, charcoal and precipitated protein were removed by centrifugation (5min, 12000*g*). A

sample (0.25 ml) of the supernatant was counted for $^{14}C$ radioactivity in 2 ml of PCS scintillator cocktail. Under these conditions binding of unmetabolized D-[*ring*-2-$^{14}C$]tryptophan is quantitative; there is no detectable binding of [$^{14}C$]formate (Smith & Pogson, 1980). Blank determinations, in which radiolabelled substrate was added after $HClO_4$ addition, were included in each experiment. All radioactive measurements were corrected for quenching by internal standardization.

The protein concentrations of tissue extracts were determined either by the biuret (Gornall *et al.*, 1949) or Lowry (Lowry *et al.*, 1951) methods.

*Expression of results*

Enzyme activities are expressed as nmol of *N*-formylkynurenine formed/min per mg of protein at 37°C (mU/mg). All determinations were performed in triplicate; for each, standard deviations were always less than 2% and have been omitted from the text.

## Results and discussion

*Development of a radiometric assay for indoleamine 2,3-dioxygenase*

For the development of a radiometric assay for indoleamine 2,3-dioxygenase, a crude enzyme preparation from the distal 25 cm of rabbit small intestine was used. For comparative purposes, the activity of indoleamine 2,3-dioxygenase in these gut extracts was routinely determined by using a spectrophotometric assay (Higuchi & Hayaishi, 1967). The rate of formation of D-kynurenine was linear both with incubation time (up to 90 min) and enzyme concentration (up to 6 mU). With this assay procedure enzymic activity could not be detected in extracts of rabbit ileum containing less than 0.25 mU of indoleamine 2,3-dioxygenase. Indoleamine 2,3-dioxygenase activity of rabbit small intestine (Table 1) was somewhat lower than reported earlier (Higuchi & Hayaishi, 1967; Shimizu *et al.*, 1978); this is probably attributable to large (10–20-fold), seasonal variations in the activity of the enzyme derived from this source.

The radiometric assay procedure with [*ring*-2-$^{14}C$]tryptophan as substrate is a modification of that of Ohnishi *et al.* (1977), based on the principle originally described by Peterkofsky (1968) for hepatic tryptophan 2,3-dioxygenase. Indoleamine 2,3-dioxygenase catalyses the oxidative cleavage of the pyrrole moiety of [*ring*-2-$^{14}C$]tryptophan; radioactive carbon at position 2 appears in the formyl group of *N*-formylkynurenine. Determination of enzymic activity is dependent upon the isolation and subsequent quantification of [$^{14}C$]formate, after acid-catalysed cleavage of *N*-[$^{14}C$]formylkynurenine.

The incubation conditions for the radiometric assay procedure were identical with those of the spectrophotometric assay with the exception that the concentration of D-tryptophan was decreased 10-fold to 0.5 mM. In preliminary experiments [$^{14}C$]-formate was separated from unmetabolized D-[*ring*-2-$^{14}C$]tryptophan by ion-exchange chromatography as described earlier (Peterkofsky, 1968; Ohnishi *et al.*, 1977). However, this method is tedious and yields column eluates of large volume, thus necessitating the use of correspondingly large volumes of scintillant. To overcome these disadvantages, resolution of [$^{14}C$]formate and D-[*ring*-2-$^{14}C$]tryptophan was effected by direct addition of 5 mg of Norit GSX charcoal to acidified incubation tubes. After centrifugation to remove precipitated protein and charcoal, small volumes of supernatant are counted for [$^{14}C$]formate radioactivity. Under these experimental conditions, binding of D-[*ring*-2-$^{14}C$]tryptophan by charcoal is quantitative (determined in incubations in which radiolabelled substrate was added after $HClO_4$).

The rate of [$^{14}C$]formate release from D-[*ring*-2-$^{14}C$]tryptophan was linear with time (up to 60 min) and with enzyme concentration (up to 6 mU); Fig. 1. Under the conditions specified, oxidation of formate to $CO_2$ was less than 5%. Table 1 shows that the two radiometric assays for indoleamine 2,3-dioxygenase yield equivalent results. However, comparison of the specific activities of indoleamine 2,3-dioxygenase in rabbit ileum extracts (Table 1) shows that the spectrophotometric assay gave higher values than

Table 1. *Comparison of spectrophotometric and radiometric assays for indoleamine 2,3-dioxygenase from rabbit small intestine*

Assays, in triplicate, were performed as described in the Materials and methods section. Enzyme activities are expressed as the means ± S.E.M. for three separate tissue preparations.

| Assay method | [Tryptophan] (mM) | Indoleamine 2,3-dioxygenase activity (nmol of *N*-formylkynurenine formed/min per mg of protein) |
|---|---|---|
| Spectrophotometric | 5.0 | 1.220 ± 0.050 |
| Radiometric (ion-exchange) | 0.5 | 0.754 ± 0.030 |
| Radiometric (charcoal) | 0.5 | 0.814 ± 0.023 |

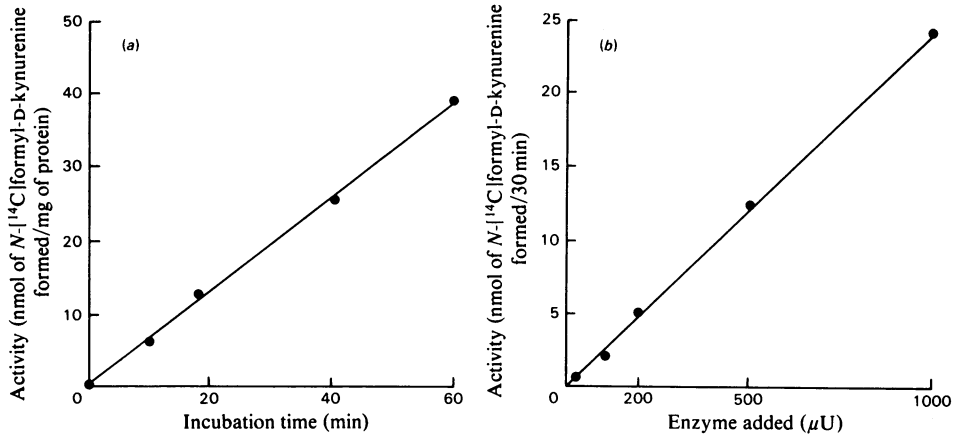


Fig. 1. *Radiometric assay of indoleamine 2,3-dioxygenase: linearity with respect to (a) time and (b) enzyme concentration*
Assays were performed at 37°C with an enzyme preparation from rabbit ileum under the conditions decribed in the Materials and methods section. [$^{14}$C]Formate was separated from unmetabolized D-[*ring*-2-$^{14}$C]tryptophan by charcoal absorption.

Table 2. *Distribution of indoleamine 2,3-dioxygenase activity in rat small intestine*
Enzyme activity was determined in extracts prepared from sections of rat small intestine by using the charcoal–radiometric assay procedure. The values are taken from a representative distribution study of one animal, and are given ± S.D.

| Distance from stomach (cm) | $10^3$ × Indoleamine 2,3-dioxygenase activity (nmol of *N*-formylkynurenine formed/min per mg of protein) |
|---|---|
| 0–2 | 13.30 ± 4.66 |
| 2–10 | 6.35 ± 0.63 |
| 10–20 | 5.28 ± 0.48 |
| 20–30 | 2.99 ± 0.27 |
| 30–40 | 2.95 ± 0.21 |
| 40–50 | 2.31 ± 0.21 |
| 50–60 | 2.36 ± 0.42 |
| 60–70 | 2.33 ± 0.28 |
| 70–80 | 2.17 ± 0.09 |
| 80–90 | 4.49 ± 0.49 |
| 90–100 | 5.11 ± 0.51 |
| 100–120 | 4.77 ± 0.53 |

the radiometric methods. This observation is directly attributable to the different concentrations of D-tryptophan used. The $K_m$ for D-tryptophan of indoleamine 2,3-dioxygenase has been reported to be 0.3 mM (Shimizu *et al.*, 1978) and we have confirmed this value for rat intestinal extracts (results not shown); in the spectrophotometric assay the enzyme is virtually substrate-saturated (D-tryptophan concentration, 5 mM) whereas in the radiometric assay the substrate concentration is sub-optimal (0.5 mM).

Direct comparison of the sensitivity of the charcoal–radiometric assay with the spectrophotometric method reveals that the former is at least 20-fold more sensitive; enzyme activity could routinely be detected in tissue extracts containing as little as 10 μU of indoleamine 2,3-dioxygenase.

*Indoleamine 2,3-dioxygenase in rat tissues*

Indoleamine 2,3-dioxygenase activity was not measurable in any gut region of the rat by using the standard spectrophotometric assay procedure. However, enzymic activity was readily detectable with the charcoal absorption–radiometric method. Unlike the rabbit, in which enzymic activity is associated with the distal 25 cm of the small intestine, indoleamine 2,3-dioxygenase shows a different pattern of distribution in the rat gut. The highest activity was found in the 2 cm distal to the stomach, with a relatively constant low activity along the remainder of the small intestine (Table 2). The specific activity of indoleamine 2,3-dioxygenase in rat gut extracts was less than 2% of that in similar rabbit intestinal preparations.

Other rat tissues were examined for the presence of indoleamine 2,3-dioxygenase activity. The activities in extracts prepared from stomach, spleen and kidney were $2.33 \times 10^{-3}$, $1.17 \times 10^{-3}$ and $0.08 \times 10^{-3}$ nmol of *N*-formylkynurenine formed/min per mg of protein respectively.

No activity was detectable in the oesophagus, colon, rectum, heart, lung or brain. Again, these findings are in direct contrast with the rabbit (Hirata & Hayaishi, 1975) in which tissues such as lung, colon and rectum contain appreciable amounts of indoleamine 2,3-dioxygenase. The physiological importance of these different patterns of tissue distribution is unclear.

*Effects of* L-*tryptophan and corticosteroids on indoleamine 2,3-dioxygenase activity in rat small intestine*

Administration either of L-tryptophan or of adrenal corticostroids to rats increases the activity of hepatic tryptophan 2,3-dioxygenase (Schimke *et al.*, 1965). It was of interest to determine whether the activity of intestinal indoleamine 2,3-dioxygenase is influenced similarly. Table 3 shows the indoleamine 2,3-dioxygenase activity of extracts prepared from the 2cm of small intestine distal to the stomach of rats 6h after treatment either with L-tryptophan or triamcinolone. Triamcinolone was without effect, but L-tryptophan produced a 50% increase in activity.

## General discussion

In the present report, we describe the development of a new, specific radiometric assay procedure for indoleamine 2,3-dioxygenase that offers a number of advantages over earlier methods, and that is applicable to crude tissue preparations. Previous methods have used a racemic mixture of D- and L-[*ring*-2-$^{14}$C]tryptophan as substrate coupled with ion-exchange chromatographic procedures for the separation of [$^{14}$C]formate from unmetabolized substrate. Use of D-[*ring*-2-$^{14}$C]tryptophan eliminates the kinetic problems inherent with DL-[*ring*-2-$^{14}$C]tryptophan and the introduction of the specific charcoal-adsorption step enables sample volumes required for radioisotope determination to be kept to a minimum. The radiometric procedure described above confers at least a 20-fold increase in sensitivity over comparable spectrophotometric methods; this may be enhanced by increasing the substrate specific radioactivity.

To our knowledge, indoleamine 2,3-dioxygenase has not previously been quantified in the rat. However, the presence of the enzyme in rat tissues was suggested following the isolation and identification of 5-hydroxykynurenine, the oxidative ring cleavage product of 5-hydroxytryptophan, from rat urine (Konishi *et al.*, 1971). The precise physiological role of indoleamine 2,3-dioxygenase is still unclear. Hayaishi and co-workers (Hirata *et al.*, 1974, 1977; Fujiwara *et al.*, 1979) suggest that the enzyme is involved in the regulation of serotonin levels in the small intestine. Serotoninergic mechanisms may be involved in the control of gut motility. Human patients with the carcinoid syndrome, a disease characterized by excessive intestinal 5-hydroxytryptamine synthesis, are subject to chronic dysentery (Thorsen, 1958). It is of interest to note that indoleamine 2,3-dioxygenase from rabbit ileum displays considerable seasonal variations; periods of low enzyme activity (and possibly, therefore, high gut serotonin concentrations) and dysentery are closely correlated (Shimizu *et al.*, 1978).

The concentration of indoleamine 2,3-dioxygenase within the short section of small intestine adjacent to the stomach of the rat may indicate a possible role for this enzyme in the regulation of gastric emptying in this species. The sensitivity of the enzyme to tryptophan administration may be solely a pharmacological response; further work is required to elucidate the function and regulation of indoleamine 2,3-dioxygenase activity under physiological conditions.

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Table 3. *Effect of* L-*tryptophan and triamcinolone on indoleamine 2,3-dioxygenase activity in rat small intestine*
Rats were given triamcinolone (5mg/kg body wt. intraperitoneally) or L-tryptophan (750mg/kg body wt. intraperitoneally) and were killed 6h later. Enzyme activities are means ± S.E.M. for three observations. *Significance of differences of means between control and tryptophan-treated groups <0.05 (by Student's *t* test).

| Treatment | $10^3 \times$ Indoleamine 2,3-dioxygenase activity (nmol of *N*-formylkynurenine formed/min per mg of protein) |
|---|---|
| None | 2.98 ± 0.12 |
| Triamcinolone | 3.43 ± 0.24 |
| L-Tryptophan | 4.51 ± 0.45* |

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