# A NEW METHOD FOR THE ASSAY OF TRYPTOPHAN 2,3-DIOXYGENASE

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## 1. Introduction

Tryptophan 2,3-dioxygenase (tryptophan pyrrolase, L-tryptophan: oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11) catalyses the first reaction of tryptophan catabolism through the kynurenine pathway to acetyl CoA:

L-tryptophan + $O_2$ → *N*-formylkynurenine

The enzyme is found in many organisms, but in mammals is restricted to the liver. As a frequent with regulatory enzymes in liver, tryptophan 2,3-dioxygenase concentrations change markedly with variations in dietary and hormonal status [1]. Basal activities are, however, very low and have presented problems in assaying. A number of procedures have been used for quantitating the enzyme, but are open to criticism on practical grounds. The formation of kynurenine (produced by the cleavage of formylkynurenine by an active formylase) can be followed spectrophotometrically in both continuous [2] and stopped [3] assays, but the sensitivity is low, and relatively large amounts of tissue are required. The presence of *apo-* and *holo-* forms of the enzyme necessitates a preincubation period with a haem compound, ascorbate and tryptophan before assay [4]. This preincubation increases blank values and the assay is further complicated by absorbance increases attributable to reaction between ascorbate and other components [5].

A radiochemical assay based on D,L-[*ring*-2-$^{14}$C]-tryptophan has been devised [6], but has not been widely used, probably because of the difficulty of preincubation, the need for ion-exchange column chromatography and the use of optically unresolved labelled substrate.

In whole tissues and organisms, the activity of tryptophan 2,3-dioxygenase has been determined both by chromatographic determination of kynurenine [7] and by formation of $^{14}CO_2$ from labelled substrate [8,9], as well as by removal of tryptophan [10]. More recently, this has been re-examined with purified L-[*ring*-2-$^{14}$C]tryptophan and determination of all products [11]. Here, we report the development of this last system to provide an assay procedure, suitable for crude extracts, which is sensitive, reproducible and easy to perform.

## 2. Materials and methods

### 2.1. *Materials*

Norit GSX was from the Norit Clydesdale Co., Glasgow. D,L-[*ring*-2-$^{14}$C]tryptophan was from Schwarz-Mann, Orangeburg, NY, or ICN through Laboratory Impex, Twickenham, and was resolved optically by affinity chromatography on immobilized bovine serum albumin [11,12] (L-[*ring*-2-$^{14}$C]-tryptophan is available from CEA, Gif-sur-Yvette.) Other chemicals, of the purest grade available, were from standard suppliers.

Male Sprague-Dawley rats (150–250 g), were used throughout. In some experiments, enzyme activity was increased by injection i.p. of L-tryptophan (750 mg/kg body wt, in 0.9% (w/v) NaCl, 0.1% (w/v) Tween 80) and triamcinolone (1 mg/kg body wt in 0.9% (w/v) NaCl) every 4 h for a total of 12 h.

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2.2. *Preparation of extracts*

Livers were dispersed, with a mechanical tissue disintegrator (Ystral GmbH), in 3 ml 0.14 M KCl, 0.02 M Na-phosphate (pH 7.0)/g tissue. After centrifugation at 38 000 × *g*, 40 min, 4°C, the supernatants were decanted and stored at 0°C for assay.

2.3. *Assay of tryptophan 2,3-dioxygenase activity*

For comparative purposes, enzyme activity was measured spectrophotometrically after preincubation according to [13].

The standard assay procedure was as follows: Liver extract was preincubated in a medium containing 50 mM Na-phosphate, 30 mM Na-ascorbate, 2.5 mM L-tryptophan and 0.5 mg methaemoglobin (pH 7.0) in 1 ml total vol. at 37°C for 30 min. This ensures that all *apo*-enzyme is converted to the *holo*-form.

Portions of each incubation were then transferred to 1.5 ml plastic conical (Eppendorf) centrifuge tubes, and an equal volume of a suspension of Norit GSX in water (50 mg/ml) was added. After mixing, the charcoal was removed by centrifugation at 12 000 × *g* for 2 min. This procedure quantitatively removes all tryptophan [11,14].

Appropriate volumes of supernatants containing 10 μg methaemoglobin were added to medium whose final composition was 60 mM Na-phosphate, 30 mM Na-ascorbate, 10 μg methaemoglobin and 0.5 mM L-tryptophan (containing 0.06 μCi L-[*ring*-2-$^{14}$C]-tryptophan) (pH 7.0); final vol. 0.1 ml, in 1.5 ml 'Eppendorf' tubes (without stoppers). The tubes were preincubated at 30°C for 5 min before the addition of tryptophan, and were then placed in 15 ml conical glass centrifuge tubes. These were closed with a rubber stopper through which was suspended a disposable polypropylene centre well (Kontes, NJ) containing a small piece of folded filter paper. Incubations were at 30°C in triplicate, and were terminated at 60 min (or other times; section 3) by the injection (through the stoppers) of 50 μl 0.6 M $HClO_4$ into the reaction mixture. Immediately afterwards, 0.2 ml phenylethylamine/methanol (1:1, v/v) was injected similarly into the centre wells. All tubes were then gently shaken (75 osc./min) at room temperature for 60 min to ensure complete absorption of $^{14}CO_2$. Wells were removed and added directly to scintillation vials containing 5 ml Cocktail T scintillation fluid (Hopkin and Williams, Chadwell Heath, Essex).

The 'Eppendorf' tubes were removed carefully, and 0.1 ml Norit GSX suspension (50 mg/ml in water) was added to each. After thorough mixing, precipitated protein and charcoal were removed by centrifugation (12 000 × *g*, 2 min, room temperature) and 0.1 ml portions of the supernatant were mixed with 1 ml PCS scintillation cocktail (Hopkin and Williams, Chadwell Heath, Essex) in minivials. The first scintillation vial contains the total counts released as $^{14}CO_2$, and the second known fraction of the counts in non-aromatic $^{14}$C-labelled products (very largely formate). Under these conditions the binding of [$^{14}$C]tryptophan is quantitative; [$^{14}$C]formate is not bound to charcoal [14]. Blanks with no enzyme added therefore give counts little above background. The usual blanks performed involve addition of $HClO_4$ at zero time; the total counts so obtained are maximally <0.25% of those added as [$^{14}$C]tryptophan.

## 3. Results and discussion

The product of the tryptophan 2,3-dioxygenase-catalysed reaction is *N*-formylkynurenine. In crude extracts, however, the presence of a high activity formylase ensures that formate and kynurenine are generated [2,3]. Formate may be metabolised in the whole cell either through the tetrahydrofolate-dependent pathway or by direct oxidation involving catalase [15]. In extracts, the latter pathway still persists so that a proportion of the formate generated is oxidised to $CO_2$. Methods involving determination of formate alone [6], therefore, will underestimate true activities. These procedures measure both $CO_2$ and non-aromatic products, so that any conversion of formate to other 'simple' molecules does not affect the validity of the assay. This method is furthermore not dependent on the presence of formylase, since *N*-formylkynurenine hydrolyses rapidly in acid solution [16].

Enzyme activity, as implied above, is calculated from the sum of $^{14}CO_2$ and non-aromatic [$^{14}$C]-products. Appropriate corrections for quenching were made after counting reagent blanks to which known amounts of label were added. Under standard conditions, the counts in $^{14}CO_2$ after 60 min incubations are ~5% of the total counts. Over longer periods, however, this percentage increases. Furthermore, the proportion found as '$CO_2$' increases with the length of exposure to $HClO_4$ (≤100% at 24 h); this may be attributable to direct absorption of free HCOOH by the phenylethylamine.

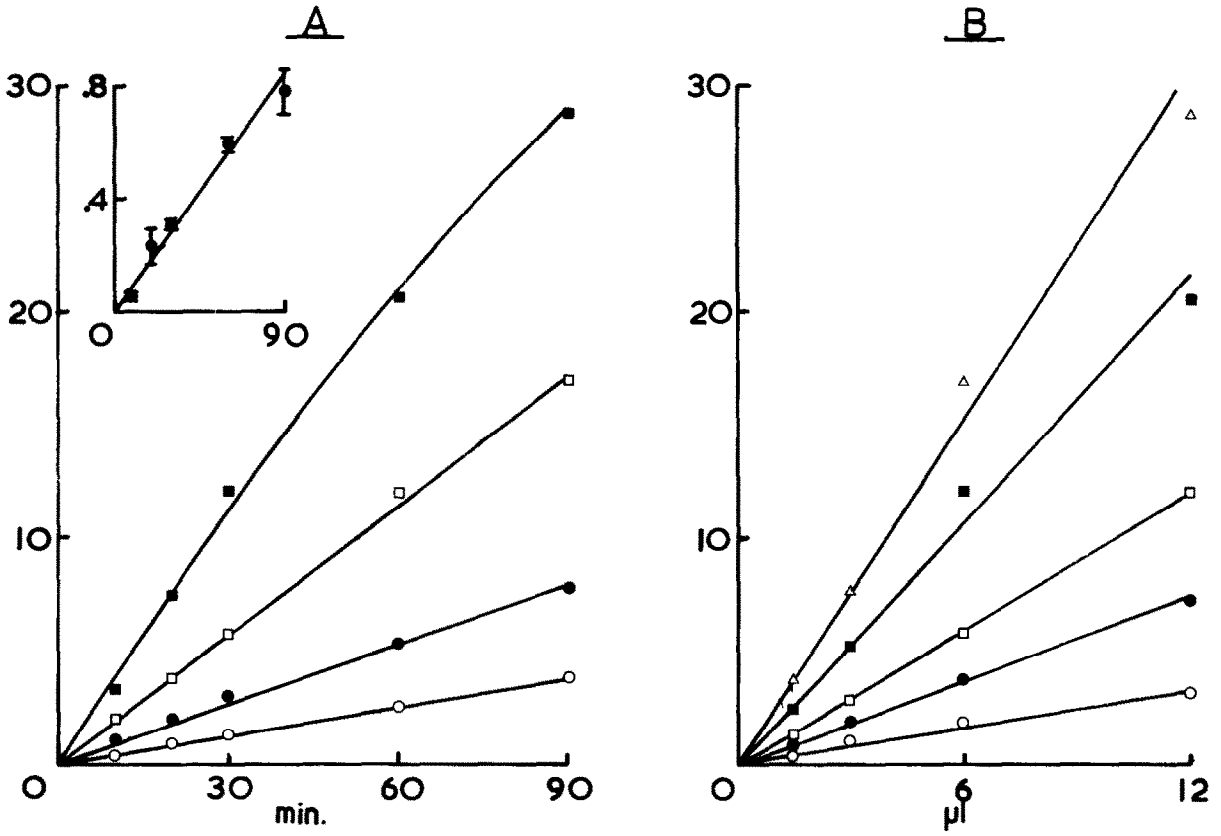


Fig.1. (A) Time courses of tryptophan oxidation with different extract concentrations. The liver extract was from rats previously given tryptophan and triamcinolone; it was 270 mg wet tissue/ml and the tryptophan 2,3-dioxygenase activity 6.87 nmol . mg wet tissue$^{-1}$ . h$^{-1}$. All points are the means of 3 determinations. Volumes of extract: (○) 1.5 µl (●) 3 µl; (□) 6 µl: (■) 12 µl. Insert: Rate with 'non-induced' extract. Values are means ± SD of 3 determinations. (B) Linearity of response to increasing extract concentration. Conditions were as above: (○) 10 min; (●) 20 min; (□) 30 min; (■) 60 min; (△) 90 min. Ordinate: (A,B) nmol L-tryptophan oxidised; (insert) nmol L-tryptophan oxidised/mg wet wt.

The reaction is linear with respect to both time and extract concentration in extracts containing both basal and induced activities (fig.1), provided that <20 nmol substrate are utilized. Simultaneous spectrophotometric assays of liver extracts show that identical results are obtained with this radiometric method and conventional systems.

It was thought theoretically possible that 'activated' enzyme in extracts might revert to the *apo*-form after removal of the activating factors with charcoal (at the end of the preincubation period). It was found, however, that samples can be left for at least 60 min (and possibly longer) after the addition of charcoal without any diminution in the activity with L-[*ring* 2-$^{14}$C]tryptophan with both basal and induced activities, provided that methaemoglobin is present in the final assay medium. Similar results were also obtained when the enzyme was assayed spectrophotometrically.

Enzyme activity increased sigmoidally with increases in tryptophan concentration (fig.2), in agreement with [17]. Calculation of the app. $K_m$ yields 0.35 mM, in the middle of the range reported [18–21].

This procedure employs a concentration of tryptophan (0.5 mM) which is below that giving maximal rates. Higher concentrations can be used as easily, but of course involve more isotope or lower specific activities. Measurements at 2.5 mM (i.e., maximal velocity) and 0.5 mM tryptophan give a ratio of rates of 1.96 ± 0.08 (mean ± SEM, 6 determ.).

With the specific activity used routinely for this work, it is possible to achieve reproducible enzyme activities in as little as 2 mg of liver from control, 'basal', rats. This compares with the difficulty of

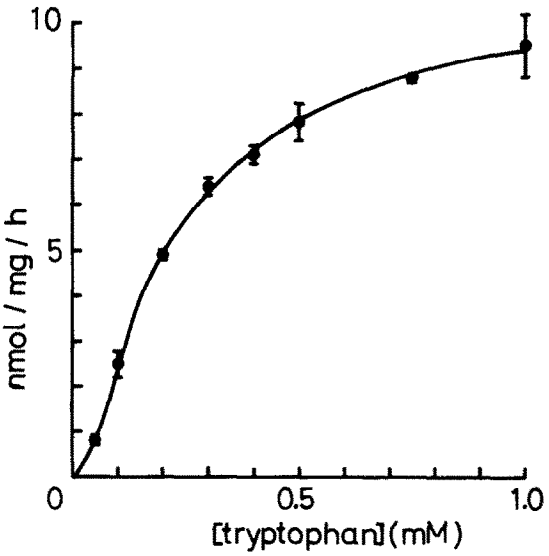


Fig.2. Effect of tryptophan concentration on enzyme activity in a crude extract from the liver of a rat previously given tryptophan and triamcinolone.

obtaining trustworthy values with 20–50 mg tissue using spectrophotometric methods. The sensitivity can be increased appreciably by raising the specific activity of the [$^{14}$C]tryptophan, and the backgrounds are sufficiently low to make this practicable. As described, the cost/assay is ~$0.20 (US).

The only other activity which can interfere with this assay system (as with all others) is that of the less-specific indoleamine 2,3-dioxygenase (EC 1.13.11.17). No example is recorded, however, of the two enzymes occurring in the same source.

This assay requires readily available chemicals with a minimum of purification procedures, is simple, sensitive, reproducible and easy to perform with large numbers of samples. It may further be simply adapted (by omission of the pre-incubation step) for measurements of the proportions of both *apo*- and *halo*-enzyme.

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