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Interference by reactions of kynurenine metabolism in the estimation of tryptophan pyrrolase in rat-liver homogenate

In an investigation of aspects of the mechanism of tryptophan pyrrolase induction using a continuous-perfusion technique[1], the results of which are to be published elsewhere, a tryptophan pyrrolase assay was established using minor modifications of the method outlined by KNOX[2]. The significant modification was the use of a Dounce, hand-operated homogenizer[3] instead of a Waring blendor for dispersion of the tissue. Under these conditions, in contrast to those of previous workers[4,5], it was found that the kynurenine synthesized during incubation of the homogenate with tryptophan was being further metabolized. Therefore, the kynurenine content of the incubation mixture at the end of the incubation period was not a true measure of the tryptophan pyrrolase activity.

The incubation mixture (total volume 3.0 ml and final pH 7.0) contained 9 μmoles L-tryptophan, 150 μmoles sodium phosphate buffer and 125 mg rat liver as an homogenate (12.5%, w/v, in 0.14 M KCl). Incubation was for 2 h at 37°.

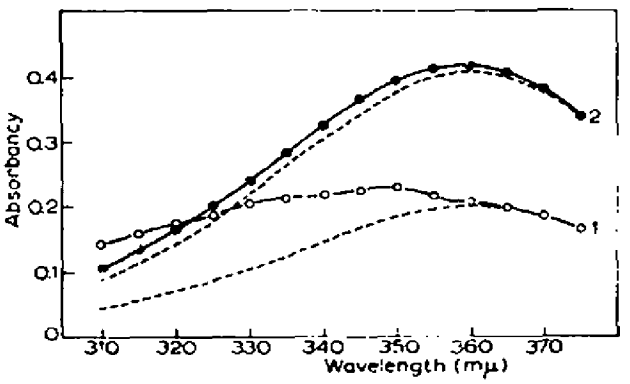

Fig. 1. Absorption spectra of the reaction products of the tryptophan pyrrolase assay using an homogenate freshly prepared with a Dounce homogenizer and using part of the same homogenate after it had been frozen in a dry ice–acetone mixture and immediately thawed. Conditions of assay were as outlined in the text. Values represent the means of three determinations. ○—○, freshly prepared homogenate; ●—●, frozen and thawed homogenate; — — —, authentic L-kynurenine.

After deproteinization and neutralization, the absorbancy of the supernatant was determined at intervals throughout the range of wavelengths 309–375 mμ. No difference in levels of activity was found between mixtures incubated in an atmosphere of air and those incubated in oxygen. All values given were corrected for controls incubated without substrate and each determination was carried out in triplicate.

As shown in Fig. 1, Curve 1, the products of the reaction included other compounds besides kynurenine which showed absorption within this range. These compounds were found to be the products of kynurenine metabolism.

It was found that such further metabolism of kynurenine or "secondary reactions" could be inhibited to a large extent by freezing and thawing the homogenate before incubation with substrate (Fig. 1, Curve 2). Thus, on the basis of the $A_{340\ m\mu}$

TABLE I

EFFECT OF FREEZING AND THAWING ON THE APPARENT LEVEL OF ACTIVITY OF TRYPTOPHAN PYRROLASE IN RAT-LIVER HOMOGENATES

Rat-liver homogenates were prepared using a Dounce, hand-operated homogenizer. Tryptophan pyrrolase assay as outlined in the text was carried out on the homogenates when freshly prepared and after freezing and thawing. The L-kynurenine synthesized was estimated from $A_{340\ m\mu}$ values using a molecular extinction coefficient of $4.53 \cdot 10^3$. Nitrogen was estimated by nesslerization[9] and the determinations were carried out on trichloroacetic acid precipitates of the homogenates. Values represent the means with standard deviations of 5 determinations.

Homogenate	μmoles kynurenine synthesized per h	
	per g liver (wet wt.)	per mg N
Freshly prepared	1.22 ± 0.14	0.05 ± 0.006
Frozen and thawed	2.48 ± 0.2	0.09 ± 0.008

of the reaction products, it seemed that the tryptophan pyrrolase activity of a frozen and thawed homogenate was about twice that shown before freezing (Table I). However, comparison of the absorption spectra (Fig. 1 and Table II) indicated that this was not necessarily so. It is known that rat liver contains relatively high levels of kynurenine transaminase (EC 2.6.1.7) and kynureninase[6] and these could therefore lead to the production of kynurenic acid and anthranilic acid from kynurenine.

TABLE II

ABSORBANCY RATIOS OF THE PRODUCTS OF THE TRYPTOPHAN PYRROLASE ASSAY

Homogenates of normal rat liver were prepared as indicated below and assayed for tryptophan pyrrolase as outlined in the text. The $A_{370\ m\mu}$ is due almost entirely to L-kynurenine.

Preparation used	$A_{325\ m\mu}/A_{370\ m\mu}$	$A_{360\ m\mu}/A_{370\ m\mu}$	$A_{390\ m\mu}/A_{370\ m\mu}$
Dounce homogenate			
Freshly prepared	0.85	1.22	1.24 (9)
Frozen and thawed	0.28	0.78	1.21 (16)
Waring-blendor homogenate			
0.5 min	0.43	0.90	1.22 (2)
2 min	0.29	0.78	1.20 (1)
Authentic L-kynurenine	0.26	0.72	1.20

Assuming the presence of kynurenine, kynurenic acid and anthranilic acid, the values $A_{309\ m\mu}$, $A_{333\ m\mu}$, and $A_{360\ m\mu}$ were resolved using simultaneous equations[7]. The calculated total molar values of kynurenine, kynurenic acid and anthranilic acid from incubation mixtures using freshly prepared homogenates were very close to those of the kynurenine produced using frozen and thawed homogenates.

Homogenates freshly prepared from livers of rats in which a high level of tryptophan pyrrolase had been induced by prior injection of tryptophan[8] also showed further metabolism of the kynurenine synthesized during the assay. Freezing and thawing the homogenates before assay again greatly reduced these "secondary reactions".

Preparation of rat-liver homogenates using a Waring blendor[4,5] for 2 min largely inactivated these "secondary" reactions as shown in Table II, while use of an all-glass, machine-driven, Potter–Elvehjem homogenizer did not. However, in the absence of added tryptophan, tissue dispersion using either a Waring blendor or a Potter–Elvehjem homogenizer caused rapid inactivation of the tryptophan pyrrolase as shown in Table III.

TABLE III

EFFECT OF DIFFERENT METHODS OF TISSUE DISPERSION ON THE ACTIVITY OF TRYPTOPHAN PYRROLASE IN RAT LIVER

Homogenates of normal rat livers were prepared as indicated below and assayed for tryptophan pyrrolase as outlined in the text. During dispersion using the Dounce and the Potter–Elvehjem homogenizers the temperature of the suspension was maintained at 0–2°. Although chilled at frequent intervals, the contents of the Waring blendor rose to 3.5° after 0.5 min, 7.5° after 1 min, 14° after 2 min and 20° after 3 min. A sample of the 0.5-min homogenate was preincubated for 2 min at 20° before addition of substrate. The homogenates prepared with the Dounce and the Potter–Elvehjem homogenizers were frozen and thawed before assay. Activity is expressed as μmoles kynurenine synthesized/g liver (wet wt.)/h.

Type of homogenizer	*Dispersion time (min)*				
	0.5	1	1.5	2	3
Machine driven Potter-Elvehjem	2.2	1.4			1.0
Waring blendor	1.4	2.7	2.5	2.2	1.4
	1.6*	2.4		1.9	1.3
	2.5*§				
Dounce, hand-operated homogenizer	2.4*	2.4	2.3	2.2	2.3

§ Preincubated 2 min at 20°.
* Prepared from the same pool of tissue.

Storage of homogenates at −20° for periods from 2 h up to 5 days did not affect the tryptophan pyrrolase activity provided each sample was frozen and thawed only once. Freezing and thawing homogenates a second and third time caused a decrease in tryptophan pyrrolase activity of 3–4% each time.

In view of these results, homogenates for use in tryptophan pyrrolase assays were routinely frozen and thawed before incubation with substrate. Homogenates were prepared using a Dounce, hand-operated homogenizer kept at 0–2° in an ice-

water bath, and after filtration through four thicknesses of cheese-cloth were rapidly frozen in a dry ice–acetone bath. Frozen homogenates were either stored at −20° for subsequent use or immediately thawed rapidly by intermittent immersion with vigorous shaking in a water bath at 37°. Care was taken not to allow the temperature of the homogenate to rise above 4–5°. Thawed homogenates were immediately transferred to an ice-water bath. When homogenates were used without storage, incubation with substrate was usually begun within 20–30 min after the death of the animal.

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Studies of small intestine during development

II. The intracellular location of intestinal β-galactosidase

Recent studies have indicated that intestinal β-galactosidase (EC 3.2.1.23) can be sedimented with the "nuclear" and microsomal fractions derived from an homogenate of intestinal mucosa[1]. In liver several acid hydrolases are contained within a lysosome, which when disrupted will release the active enzymes[2]. This study was designed to determine more accurately the distribution of β-galactosidase by comparing its cellular location with that of acid phosphatase (EC 3.1.3.2) and β-glucuronidase (EC 3.2.1.31) (typical lysosomal enzymes) and also with alkaline phosphatase (EC 3.1.3.1), an enzyme associated with the brush border[3].

Rats of the Wistar strain, 2–4 days of age were used. The method of preparation of the intestinal homogenate, the centrifugal fractionation (except for the use of a somewhat higher force, 600 × *g*, for sedimentation of the nuclear fraction) and the β-galactosidase assay, using lactose as substrate have been described[1]. Homogenates