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The influence of some methodological factors on measurement of tryptophan oxygenase activities in crude homogenates of rat liver

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1. With two different methods for assaying the tryptophan oxygenase activity in rat liver homogenates, the effects of some methodological factors on the activity of the enzyme were studied. 2. In fed, but not in starved, rats a compound(s) absorbing at 365 nm, interfering with the reading of kynurenine absorbance, disappeared gradually during incubation. 3. A correction for this tryptophan-independent reaction was necessary in order to determine correct tryptophan oxygenase activity. 4. Blood remaining in liver tissue *post mortem* can serve as a source of cofactor haem for tryptophan oxygenase, causing spuriously high values for the activity of the holoenzyme form of tryptophan oxygenase. 5. A rapid and progressive activation of tryptophan oxygenase *post mortem* occurs in undisrupted liver tissue, and this activation is temperature-dependent.

Tryptophan oxygenase (L-tryptophan:oxygen 2,3-oxidoreductase, EC 1.13.11.11) is the first and rate-limiting enzyme in the most important pathway for tryptophan degradation, the kynurenine–nicotinic acid pathway in the liver. It is a haem-dependent liver cytosolic enzyme, which exists in haem-free (apoenzyme) and haem-bound (holoenzyme) forms. Its activity has been studied in connection with a variety of diseases characterized by altered tryptophan metabolism (Rose, 1972). Having a short half-life (Feigelson *et al.*, 1959) and being readily inducible by corticosteroids (Knox & Auerbach, 1955; Greengard *et al.*, 1963), it has been used as a marker of rapidly turning-over liver proteins and peripheral steroid-hormone actions (Knox *et al.*, 1966*a*; Kenney, 1970; Litwack & Rosenfield, 1973; Mørland, 1974*a*; Lowitt *et al.*, 1981). Although tryptophan oxygenase has been so widely investigated, there seems to be a lack of standardization of the methodology for its assay. When establishing the methods for our routine assays of tryptophan oxygenase activity in crude liver homogenates, we found that some factors greatly influenced measurements of its activity when at least two different standard methods (Seglen & Jervell, 1969; Badawy & Evans, 1975*a*) were used. These factors were: (*a*) a tryptophan-independent reaction influencing the spectrophotometric reading of the tryptophan oxygenase reaction product, kynurenine, (*b*) an enzyme activation *in vitro* dependent on the blood remaining in the liver at the animal's death and (*c*) another enzyme activation *post mortem* of a progressive type. These phenomena, which have not been studied in any detail previously, can cause serious errors in the measurement of tryptophan oxygenase activities.

Materials and methods

Treatment of animals and liver sampling

Adult male Wistar rats (Møllegaard, Ejby, Denmark) weighing 200–300 g were used; they were exposed to a 12 h-light/12 h-dark schedule with the light being switched off at 19:00 h and on at 07:00 h. The animals were fed *ad libitum* with a standard rat diet (Gesti and Sons, Oslo, Norway) or starved for 20–24 h. All animals were anaesthetized with pentobarbital (Nembutal, 60 mg/kg intraperitoneally) before liver samples were taken. There was no significant difference in tryptophan oxygenase activity between the left, right and median lobes of the liver. Liver samples were frozen in liquid nitrogen exactly 3 min after the beginning of the perfusion, stored at −80°C and analysed within 1 week. No appreciable changes in tryptophan oxygenase activities were found after freezing or during 2 weeks of storage. The detailed procedure of liver sampling for each experiment is explained in the Table or Figure legends.

Chemicals

Haemoglobin and cycloheximide were purchased from Sigma (St. Louis, MO, U.S.A.); L-tryptophan was from Fluka A.G. (Buchs, Switzerland) and Nembutal from Abbot (Saint-Remy-sur-Avre, France). All other chemicals were from Merck (Darmstadt, Germany).

Methods for tryptophan oxygenase assay

Two different methods were used to measure tryptophan oxygenase activities in crude liver homogenates (Seglen & Jervell, 1969; Badawy & Evans, 1975*a*). These methods differ with respect to the source of cofactor haem and to the concentration of liver in the incubation homogenate. In the method of Seglen & Jervell (1969), the activity is measured in a 1% (w/v) liver homogenate, with haemoglobin as a source of haem. For this method, frozen samples of liver tissue were homogenized at 4°C with 99 vol. of ice-cold 0.02 M-sodium phosphate buffer (pH 7.0) containing 2.5 mM-L-tryptophan and 2 μM-haemoglobin for the measurement of total activity, and with the same phosphate buffer containing only 2.5 mM-tryptophan when holoenzyme activity was measured. Samples of the liver homogenates (4 ml) were then incubated in open tubes at 37°C in a shaking water bath. The other method that we used was described by Badawy & Evans (1975*a*), where the activity was measured in a 3.5% (w/v) liver homogenate with haematin hydrochloride as a cofactor for the enzyme. Liver homogenates containing 2.5 mM-tryptophan and 2 μM-haematin hydrochloride for total activity, and 2.5 mM-tryptophan for holoenzyme activity, were incubated at 37°C. We made two modifications to the method described by Badawy & Evans (1975*a*). Firstly, incubation was performed in open flasks instead of stoppered and oxygenated flasks, since atmospheric oxygen was seen to be a sufficient oxygen source for the enzyme reaction. This was demonstrated by a separate control experiment (results not shown) where the same tryptophan oxygenase activities were obtained whether liver homogenates were incubated in open tubes in a shaking water bath or in closed oxygenated Erlenmeyer flasks. The sufficiency of atmospheric oxygen for tryptophan oxygenase is also supported by the similarity of both basal and induced activities obtained by Mørland (1974*b*), using the method of Seglen & Jervell (1969), and by Badawy & Evans (1975*b*). Secondly, metaphosphoric acid replaced trichloroacetic acid as the deproteinizing agent required to stop the reaction. This change made the neutralization of the supernatants easier.

In both methods, the A_{365} of kynurenine was measured with a Zeiss PM QII/M20 spectrophotometer in samples treated with metaphosphoric acid after different incubation time intervals, centrifuged at 1500 *g* for 10 min and neutralized with 1 M-NaOH. The activities for the enzyme, expressed as μmol of kynurenine/h per g of liver, were calculated from the curve of A_{365} versus time by using $\varepsilon = 4.54 \times 10^3$ for 1 M-kynurenine, after an initial lag period of 20–30 min. In both methods, blank determinations of absorbance changes were run for each liver homogenate by incubating these without substrate in phosphate buffer (the absorbance values of the blank samples did not change in the presence of haemoglobin in the buffer). The significance of these blank determinations is explained in the Results section.

Results

Comparison of the two methods for tryptophan oxygenase assay

To compare the tryptophan oxygenase measurements obtained by the method of Seglen & Jervell (1969) with those obtained by the method of Badawy & Evans (1975*a*), the total enzyme and holoenzyme activities were measured from the same livers by both methods. Some of the animals (three out of nine) were treated with ethanol (3.3 g/kg) intragastrically to increase the enzyme activities above the control value (Mørland, 1974*b*). Values for both total enzyme and holoenzyme activities were about 20% lower with the method of Badawy & Evans (1975*a*) than those obtained with the method of Seglen & Jervell (1969). This difference might be due to the liver concentration of 3.5% in the incubation homogenate in the Badawy & Evans (1975*a*) assay, since a lack of direct proportionality of measured tryptophan oxygenase activity to the amount of enzyme in homogenates more concentrated than 2% has been observed (Seglen & Jervell, 1969). However, the correlation between the results obtained with these two methods was high ($r = 0.884$, $P < 0.01$, $n = 9$, and $r = 0.997$, $P < 0.001$, for total and holoenzyme activity respectively), and all subsequent findings in the present study were repeatable with both methods. Therefore only results obtained with one method (Seglen & Jervell, 1969) are given in the present paper.

Blank correction in the tryptophan oxygenase activity curve

Fig. 1 presents a typical curve of A_{365} versus incubation time in liver homogenates from fed and starved rats killed at 13:00 h. In all samples, the initial lag in activity lasting 20–30 min was seen. The A_{365} decreased during incubation of liver homogenates from fed rats in the absence of tryptophan (blank samples). This decrease occurred during the first 1 h of incubation of liver homogenates from fed rats, after which it reached a plateau (Fig. 1*a*). In starved rats, the changes in A_{365} of the blank samples were insignificant (Fig. 1*b*). For fed rats, the enzyme activities calculated from the time points between 30 and 60 min in the assays containing substrate and corrected for the corresponding decrease in A_{365} in blank samples were the same as those calculated from time points after 1 h. At this time there was no change in the A_{365} of the blank sample (see Fig. 1*a*). It should also be noted that the magnitude of the decrease in A_{365} in blank samples became less

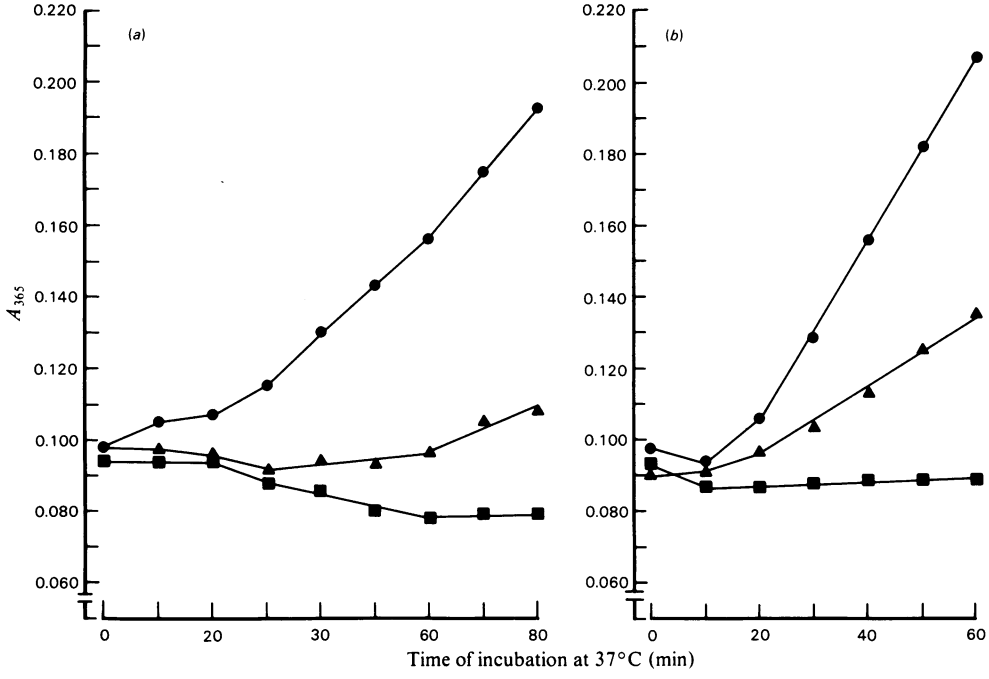

Fig. 1. *A_{365} during the incubation of liver homogenates for tryptophan oxygenase assay*
Liver homogenates from fed (*a*) and starved (*b*) rats were incubated in the presence (total activity, ●) and in the absence (holoenzyme activity, ▲) of added haemoglobin and in the absence of tryptophan (blank sample, ■) as described by Seglen & Jervell (1969). Before assay, livers were purged of blood by perfusion with a saline–heparin mixture as explained in Table 1.

pronounced in fed rats towards the end of the light period of the day, when the fed rats approach a more starved state. The changes in A_{365}, converted into the units of the enzyme activity (μmol of kynurenine/h per g of liver), at 09:00, 11:00, 13:00, 15:00 and 17:00h were respectively (means ± S.D.): −0.89 ± 0.66, −1.22 ± 0.30, −0.95 ± 0.68, −0.78 ± 0.34 and −0.0023 ± 0.0017, whereas the corresponding blank values for starved rats were insignificant and did not change during the day.

The absorption spectra between 200 and 500nm were recorded for blank samples from fed and starved rats before incubation and after incubation at 37°C for 1h (results not shown). No peak was found between 300 and 500nm. Instead, all these blank samples had a high-absorbance plateau between 210 and 280nm, and in fed rats this plateau had a high-absorbance 'tail' reaching the 365nm region. The magnitude of this 'tail' decreased during incubation. Moreover, kynurenine added to the blank samples did not decrease during incubation.

Effect of liver blood content on tryptophan oxygenase activities

Since haemoglobin is known to be a good source of the tryptophan oxygenase cofactor haem (Knox *et al.*, 1966*b*), the effect of liver blood content on the assay of tryptophan oxygenase holoenzyme activity *in vitro* was studied (Table 1). Table 1 shows that the measured holoenzyme activity was critically dependent on the amount of blood associated with the liver, the holoenzyme activities being 2–3 times too high in uncleaned, saline-rinsed and blotted livers, compared with the activities in the liver perfused with saline–heparin (Table 1: $P < 0.01$–0.001). Total enzyme activity was unaffected by any of these treatments.

Activation of tryptophan oxygenase post-mortem

The total tryptophan oxygenase activity increases rapidly in liver tissues kept at room temperature compared with the total activity in a sample from the same liver that was frozen immediately after opening the abdominal cavity of the animal (zero-time sample) (Fig. 2). This increase was statistically significant after 5min or longer (matched-pair *t*-test: $P < 0.01$; for later time points, $P < 0.001$). This activation *post mortem* was similar whether the livers were purged of blood by perfusion with saline–heparin mixture or only rinsed with saline, or whether the livers were from fed or starved rats. Over the whole period *post mortem* studied,

Table 1. *Effect of liver blood content on the activity of the holoenzyme form of tryptophan oxygenase*

The enzyme activities were measured by the method of Seglen & Jervell (1969). Blood was removed from the liver by perfusing *in situ* an ice-cold saline–heparin mixture (1000 units of heparin/100ml of 0.9% NaCl) through the portal vein for 1 min. Liver samples were frozen in liquid N_2 exactly 3 min after opening the abdominal cavity. Other liver samples were rinsed in ice-cold saline or blotted with tissue paper or left uncleaned, then transferred immediately on to ice and frozen in liquid N_2 exactly 3 min after opening the abdominal cavity. Values are means ± S.D. for four to seven starved rats. Significances of differences between the rinsed, blotted and uncleaned livers and the corresponding value of the saline–heparin-perfused livers are denoted by $*P<0.01$ and $**P<0.001$ (Student's *t* test).

Method for cleaning the liver	Tryptophan oxygenase activity (μmol of kynurenine/h per g of liver)		Holoenzyme as a percentage of the total activity
	Total enzyme	Holoenzyme	
Perfused with saline–heparin	2.57 ± 1.11	0.67 ± 0.37	27 ± 12
Rinsed with saline	2.77 ± 0.71	1.31 ± 0.42*	48 ± 11*
Blotted with paper	3.84 ± 1.42	2.10 ± 0.91**	56 ± 9**
Uncleaned	3.42 ± 1.17	1.95 ± 0.51**	59 ± 16**

there was no change in the total protein content of the liver as determined by the biuret method (Gornall *et al.*, 1949) or in wet-weight/dry-weight ratio of the liver samples (results not shown).

When the rats were treated with an inhibitor of protein synthesis, cycloheximide, 1 h before death, the increase in the total tryptophan oxygenase activity *post mortem* was significantly decreased, although not completely abolished (Fig. 2). The total activities at zero time were similar in cycloheximide-treated (means ± S.D.: 3.22 ± 0.78, $n=5$), untreated (3.11 ± 0.50, $n = 10$) or saline-treated (3.32 ± 0.56, $n = 5$) animals. Treatment with saline 1 h before death did not change the increase in the total tryptophan oxygenase *post mortem* from that of the untreated group. The effect of cycloheximide on the increase in the total activity *post mortem* was reproduced with livers purged of blood.

If the liver (perfused briefly with the saline–heparin mixture) was kept on ice for 1 h, there was no increase in either total or holoenzyme activity of tryptophan oxygenase, but the activity was more than doubled at room temperature and more than tripled at 37°C (Table 2). The degree of haem saturation also displayed an increase *post mortem* in

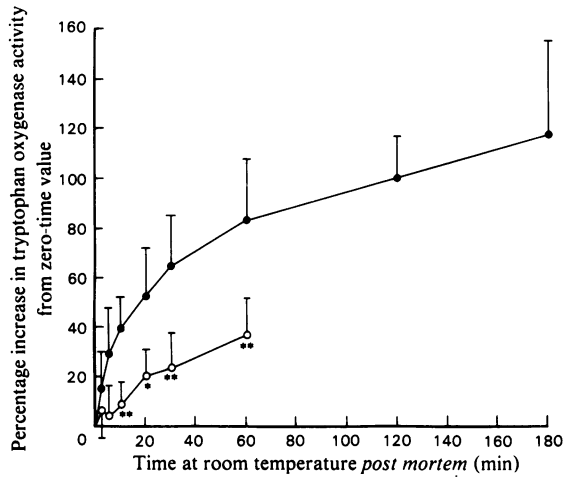

Fig. 2. *Increase in the total activity of liver tryptophan oxygenase post mortem*

The total enzyme activity in untreated (●) and in cycloheximide-treated (○) animals was measured by the method of Seglen & Jervell (1969). Cycloheximide (1.5 mg/kg body wt.) was injected intraperitoneally 1 h before death as a 0.03% solution in saline. Samples of fresh saline-rinsed livers from starved rats were frozen immediately (within 10 s) in liquid N_2 after opening the abdominal cavities of the donor rats (zero-time sample). The remainder of the livers were wrapped in aluminium foil and kept at room temperature (21–23°C) for up to 3 h, during which time additional samples were taken as indicated on the abscissa and frozen for measurements of total tryptophan oxygenase activity. The values on the ordinate represent the increase in the total activity as a percentage of the zero-time value. Each point represents the mean ± S.D. for five animals, except the points from 2 to 60 min in the untreated group, where ten animals were measured. In the control group for cycloheximide treatment, treatment with saline 1 h before death did not change the increase *post mortem* in the total tryptophan oxygenase from that of the untreated group, and therefore the results are not shown. Significances of differences between the values for the cycloheximide-treated group and the corresponding values for the untreated group are denoted by $*P<0.01$ or $**P<0.001$ (Student's *t* test).

a temperature-dependent manner (Table 2). The enzyme was almost fully saturated with haem when the liver (perfused with saline–heparin mixture) was kept at 37°C for 1 h (Table 2). However, if the liver was frozen and thawed three times in order to disrupt the liver cell structure and then kept at room temperature or at 37°C for 1 h, no increase in either total or holoenzyme tryptophan oxygenase activity *post mortem* was seen (results not shown).

Table 2. *Effect of temperature on the increase in the liver tryptophan oxygenase activity post mortem*

The enzyme activities were measured by the method of Seglen & Jervell (1969). The liver from starved rats were purged of blood by perfusion with a saline–heparin mixture as explained in Table 1. The enzyme activities were measured in samples taken immediately after perfusion (zero-time sample) and samples left wrapped in aluminium foil for 1 h on ice, at room temperature (21–23°C) or at 37°C. Each value is the mean ± S.D. for five animals. Significances of differences between post-incubation values and their appropriate zero-time values are denoted by $*P < 0.01$ or $**P < 0.001$ (Student's t test).

Treatment of the liver	Tryptophan oxygenase activity (μmol of kynurenine/h per g of liver)		Holoenzyme as a percentage of the total activity
	Total enzyme	Holoenzyme	
Zero-time sample	1.92 ± 0.58	0.59 ± 0.37	29.4 ± 13.7
1 h on ice	2.31 ± 0.46	0.65 ± 0.30	28.4 ± 10.7
1 h at room temperature	3.96 ± 0.78**	1.61 ± 0.57*	40.2 ± 9.5
1 h at 37°C	5.00 ± 0.56**	4.44 ± 0.49**	84.4 ± 12.4**

Discussion

Our observations, equally demonstrable with the two methods for measuring tryptophan oxygenase activities used in the present study, show that: (*a*) in fed rats the blank correction should be made if the enzyme activities are determined from the first-hour linear portion of the incubation curve, after the initial lag phase of 20–30 min; (*b*) the livers for tryptophan oxygenase holoenzyme assay should be purged of blood; and (*c*) liver samples should be cooled to 0–4°C or frozen immediately, or at least at a fixed time after the killing of the animal, to avoid the effect of the activation *post mortem* on tryptophan oxygenase measurements. If these points are ignored, serious errors may arise. It is obvious from the literature that this might have occurred in several previous studies. We therefore recommend that results obtained without the precautions mentioned above should be interpreted with care.

This applies, for example, to previous studies on fed rats, if the tryptophan oxygenase activities are based on results obtained during the first 1 h of incubation and are not corrected for blank samples. To our knowledge, the correction for blank samples in fed rats has been taken into consideration by only three groups (Knox & Auerbach, 1955; Feigelson & Greengard, 1961; Mørland, 1974*b*). If such a correction is omitted and if tryptophan oxygenase activities are estimated from the first linear portion of the incubation curve (after 20 min), the absorbance decrease of the blank can cover as much as 80% of the holoenzyme activity and 30% of the total activity. The fact that the magnitude of this blank correction in fed rats is highest in the morning and decreases towards the end of the day makes it necessary to standardize the time between the last meal of the animals and their death, if the blank correction is not made. In this context it is worth noting the previous observation of Mørland (1974*b*) that the highest absorbance decrease in blank samples was seen in rats fed with sucrose-containing solution during the last few hours before decapitation. It is therefore possible that inter-laboratory variation in the magnitude of the blank absorbance change might arise as a result of differences in feeding regime and the nature of the rat diet. It also seems advisable to determine whether a blank correction is necessary in other spectrophotometric methods for tryptophan oxygenase activity determination (Metzler *et al.*, 1982).

Previous determinations of holoenzyme activities could also be erroneous in studies in which blood was not removed from livers before measurements of tryptophan oxygenase (e.g. Badawy & Evans, 1975*b*; Badawy *et al.*, 1981*a*,*b*). In such studies, overestimation of the holoenzyme activity is very likely to be due to haemoglobin in blood remaining in the liver tissue, which is not in contact with tryptophan oxygenase *in vivo*, but which after homogenization of the liver tissue can serve as a source of haem cofactor for the enzyme. This overestimation could hide much smaller, but still physiologically important, changes in holoenzyme activity. It is also possible that false differences in holoenzyme activities between groups could be observed if the experimental treatment changes the amount of blood remaining in liver tissue, e.g. by changing the circulation, before or during the death of the animal.

Ignoring the existence of the rapid and progressive activation of tryptophan oxygenase in undisturbed liver tissue *post mortem* constitutes a risk of variability. This activation has been previously mentioned by Schimke *et al.* (1965) and Seglen & Jervell (1969), and, according to our study, is progressive up to at least 3 h. Therefore, if the time interval from killing the animal to freezing or cooling the liver is not strictly controlled, different degrees of activation *post mortem* between livers can cause large variability in the results. Furthermore, it is questionable how well this activated tryptophan oxygenase reflects the activity of the enzyme *in vivo*, especially since the reason for the activation *post mortem* is unknown. It therefore seems uncertain whether a procedure of routinely keeping the samples at room temperature for 5–10 min before freezing (Seglen & Jervell, 1969; Metzler *et al.*, 1982) has any particular advantage, and it appears likely that this treatment might rather increase the

chances of producing intersample variation. The mechanism for this activation of tryptophan oxygenase *post mortem* is still unknown, but the present study indicates that this activation is affected by protein synthesis *in vivo*, and is dependent on temperature and the integrity of the liver cell structure, indicating that enzymic reactions may be involved. On the basis of these observations, it could be suggested that there might be an inactive form of tryptophan oxygenase, which is activated enzymically in liver tissue *post mortem* by a process requiring protein synthesis before death. It would be of interest in further studies to determine whether such an 'inactive precursor' for tryptophan oxygenase does actually exist, and if so, to develop methods for its measurements.

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