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THE STABILITY OF THE DRUG METABOLISING ENZYMES OF LIVER MICROSOMAL PREPARATIONS

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Abstract—The stability of the drug metabolising enzymes of various preparations of rat liver microsomes has been investigated.

Four reactions have been studied: (1) the *N*-demethylation of morphine, (2) the *N*-demethylation of codeine, (3) the *O*-demethylation of codeine and (4) the hydroxylation of diethyl tryptamine.

The rates of all these reactions are influenced by temperature and an absolute requirement for oxygen and the enzyme activity is linear only over a 30 minute period when the incubation mixture is shaken in air at 37·5°C.

The causes of the instability of these enzyme systems are discussed.

THE INTERACTION of a drug or toxic agent with a receptor site is thought by many to be the most important factor in drug action. Recently, however, the metabolism of the drug *in vivo* has been shown to be of very considerable importance because it can produce marked changes in its pharmacological activity. As a result of many studies on drug metabolism it is now clear that the majority of foreign compounds are metabolized by enzymes which are present in the microsomal fraction of the liver cell. Drug metabolism does not take place exclusively in the microsomal fraction but occurs in other parts of the liver cell and in other tissues. However, the liver microsomal enzymes afford a readily available system which metabolises a wide spectrum of foreign compounds and so provide a convenient point from which to attack the problem of drug metabolism.

A study of this microsomal system may lead to results of considerable importance in developing and assessing pharmacologically active compounds. Thus, the demonstration of a relationship between the rate of metabolism of a drug and its pharmacological activity in a number of species would permit the extrapolation of the pharmacological data to man if the rate of its metabolism in man were known. Even the demonstration that a drug was metabolised in the same way in man as in test animals could provide a firmer basis for the extrapolation. Once a correlation could be established between the metabolism and the pharmacological activity of a series of related compounds, a simple biochemical screening test would be available which could substantially reduce the number of compounds in that series which needed to be tested by bioassay.

Before commencing the investigation outlined above it was of importance to determine the stability of the microsomal system. Although the drug metabolising enzymes of liver have been investigated extensively[1, 2] little information is available concerning the stability of the system. Hence the stabilities of those enzymes which *N*-demethylate

morphine,[3] *O*- and *N*-demethylate codeine[3, 4] and hydroxylate diethyl tryptamine[5] have now been studied both in microsomal preparations *in vitro* and in post mortem liver.

Although this drug metabolising system has an absolute requirement for $NADPH_2$ (Reduced Nicotine Adenine Dinucleotide Phosphate) and oxygen the greater part of the published work on this topic has been carried out with the oxidized form, NADP, using the enzymes and substrates of the soluble fraction of the liver cell to reduce the NADP. Therefore the stability of the combined microsomal and soluble fractions was investigated as well as that of isolated microsomes. The preparations were made from liver homogenized either in a Waring Blendor, in which the tissue is broken up by rotating knife blades or in a Potter–Elvehjem homogenizer, in which the tissue is broken up by forcing it through the narrow clearance between a pestle and mortar. These two homogenizing techniques were investigated because they are commonly used and it was possible that they might produce microsomes of differing activity and stability.

The stability of the microsomes *in vitro* has been determined under varying conditions at different temperatures around 37°C and under different conditions of storage at low temperatures.

Since one of the ultimate objects of this programme was to examine the activity of post mortem human tissue, the activity of microsomal preparations made from the livers of animals which had been killed many hours previously and kept under conditions approximating to those normally employed in mortuaries was determined.

METHODS

Preparation of liver microsomes

The preparation of microsomes was based on the method of Axelrod.[3] For the *in vitro* investigations liver microsomes were prepared from 150–180 g male albino rats of the Porton Strain (S.P.F.). The rats were killed instantaneously by breaking the neck, the liver removed, rinsed in 0·10 M Tris–HCl buffer, pH 7·50, and then homogenized in two volumes of buffer. Homogenising was carried out either with a glass Potter–Elvehjem homogeniser, when the livers of eight rats were homogenized individually and the homogenates combined (total volume 250 ml), or in a Waring Blendor, when the livers of fifteen rats were combined and homogenized together for 20 sec at full speed (total volume of homogenate 450 ml). The homogenates were centrifuged at 10,000 *g* for 20 min to spin down the nuclei, mitochondria and unbroken cells. The supernatant containing the microsomal and soluble fractions of the liver cell was used in the *in vitro* studies. A purer preparation was made by centrifuging the Potter–Elvehjem homogenate at 10,000 *g* for 20 min and then recentrifuging the supernatant at 100,000 *g* for one hr to spin down the microsomes. After decanting the supernatant the microsomal pellet was suspended in the original volume of fresh buffer, recentrifuged and then resuspended in buffer.

In all the experiments the microsomes were freshly isolated and in the storage studies the microsomal preparations were kept either (a) at 0°C, or (b) at −40°C (the preparations were placed in a deep freeze where they froze solid within one hr), or (c) as a freeze-dried powder at −40°C. The frozen material was stored in 5 ml lots so that each assay was carried out with microsomes which had been frozen and thawed

only once and the freeze-dried preparation was reconstituted by the addition of a suitable amount of ice-cold water to a weighed amount of powder.

In the experiments involving post mortem changes the microsomes were prepared from the livers of 1·5 to 2 kg male Old English rabbits. The animals were killed by breaking the neck and placed in a cold room at 0–3°C. At appropriate times a piece of liver weighing about 5 *g* was removed and the microsomes plus soluble fraction prepared using a Potter–Elvehjem homogenizer. Where several preparations were made from the same liver, tissue damaged by the previous removal of liver, was carefully avoided. The cut abdominal wall was clipped up between sampling.

Assay of microsomal enzyme activity

The *N*-demethylation of morphine was measured by the formaldehyde produced in the reaction. The formaldehyde was established colorimetrically as diacetyl-dihydrolutidene.[6]

The *O*-demethylation of codeine was assayed by estimating the unmasked phenolic group with silicomolybdate.[7]

The *N*-demethylation of codeine was followed by measuring the total *N* and *O* demethylation by the production of formaldehyde and subtracting the *O*-demethylation.

The metabolism of diethyl tryptamine was assayed colorimetrically by the dye produced by its 6-hydroxy derivative and diazotized sulphanilic acid in acid solution.[8]

Incubation conditions

Preparations of microsomes containing the soluble fraction were incubated in the following solution:

Microsomes plus soluble fraction	0·05 *g* liver/ml
Substrate	5×10^{-4} M
Nicotinamide	10^{-2} M
$MgCl_2$	5×10^{-3} M
NADP	4×10^{-5} M
Tris–HCl Buffer, pH 7·50	5×10^{-2} M

Total volume 5·0 ml.

When formaldehyde was produced in the reaction, semicarbazide–HCl, adjusted to pH 7·50, was added to a final concentration of 2×10^{-3} M. When washed microsome preparations were used the NADP was replaced by 5×10^{-4} M $NADPH_2$ since in these preparations the enzymes and substrates of the soluble fraction required to reduce NADP had been removed.

Except where it is stated otherwise, the incubations were carried out over a 30 min period in 50 ml tubes shaken at 37.5° C and open to the air. The results were expressed in μmoles metabolite produced per gram of liver per hour (μM/*g*/hr).

Protein assay

Protein was determined by a modification of the Folin–Ciocalteu method.[8]

RESULTS

The stability of rat liver microsomes at 37°C

In the preliminary experiments the incubation of the substrates with the microsomes was carried on for up to two hr. However, the various reactions studied were found to

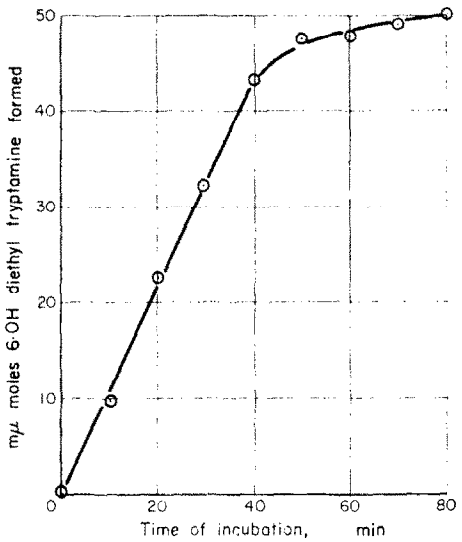

FIG. 1. The activity–time curve for the hydroxylation of diethyl tryptamine by rat liver microsomes. Diethyl tryptamine (5×10^{-4} M) was incubated with the combined microsome plus soluble fraction from rat liver under the standard conditions (see text) and the amount of 6-hydroxy diethyl tryptamine formed was determined at the times indicated.

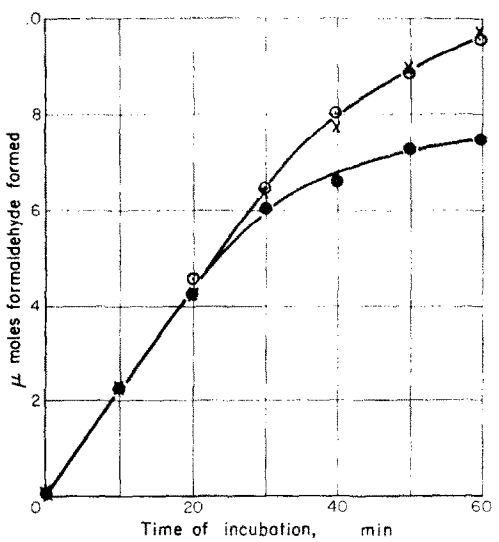

FIG. 2. The effect of shaking and aeration on the demethylation of codeine by rat liver microsomes. Codeine (5×10^{-4} M) was incubated with the combined microsomal plus soluble fraction from rat liver in the standard incubation mixture (see text) at 37·5°C. With shaking in air (○—○), with aeration by bubbling air through the incubation mixture (×—×) and without shaking or aeration(●—●). The combined *O*- and *N*-demethylation of codeine was measured at the times indicated by the amount of formaldehyde formed.

proceed linearily only over the first 30–40 min, after which the rate of metabolism decreased rapidly (Fig. 1). This inactivation occurred with both the microsomal plus soluble fraction preparations and the washed microsomes. It was not prevented by the addition of $NADPH_2$ (final concentration $0{\cdot}5 \times 10^{-3}$ M) to either of the preparations 30 min after the start of the incubation. Another possibility was that inactivation might occur by shaking during the period of incubation but the data, illustrated in Fig. 2, showed that the microsomal metabolism was linear for a longer period when the suspension was aerated either by shaking or by bubbling air through it than when the incubation was carried out under undisturbed conditions.

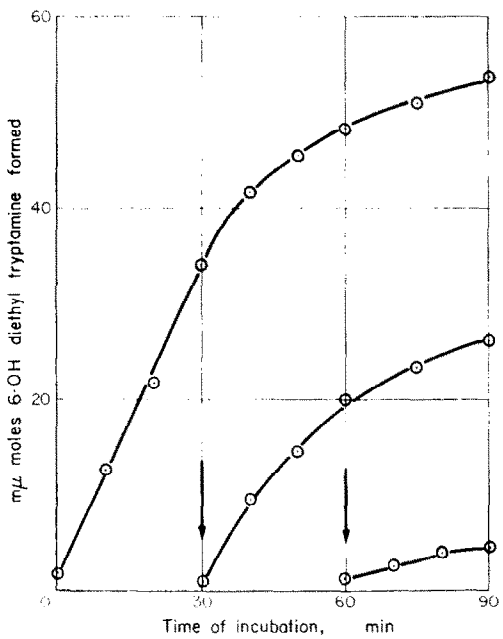

FIG. 3. The effect of preincubation on the diethyl tryptamine hydroxylating activity of rat liver microsomes. The combined microsome plus soluble fractions from rat liver was incubated under the standard conditions (see text) but the diethyl tryptamine was added at 0, 30 and 60 min (arrowed) after the commencement of the incubation. The amount of 6-hydroxy diethyl tryptamine formed was determined at the times indicated.

That the loss of activity was due to the inactivation of the microsomal enzymes and not to breakdown of substrate was shown by the fact that the amounts of diethyl tryptamine hydroxylated during the periods of 30–60 min and 60–90 min were approximately the same irrespective of whether the substrate was added after 0, 30 or 60 min (Fig. 3). In fact the longer the reaction mixture was incubated prior to the addition of substrate the greater was the loss of enzyme activity. Thus when the substrate was added after 30 min preincubation the enzyme activity was only 53 per cent of its value when the microsomes and substrate were mixed without prior incubation. When the enzyme was preincubated for 60 min the activity fell by 88 per cent.

The effect of varying the temperature between 30–45°C upon the activity of microsomal enzyme preparations is shown in Fig. 4. From 30 to 35°C there was a small but definite increase in activity: from 40 to 45°C there was a marked loss of activity; the optimum temperature lying between 35 and 40°C.

As a result of these observations a standard technique for the estimation of enzyme activity was employed in the remainder of the investigation. All incubations were carried out for 30 min, the incubation mixture was shaken in air at a temperature of 37·5° C and the enzyme activity determined from the slope of a time–activity curve.

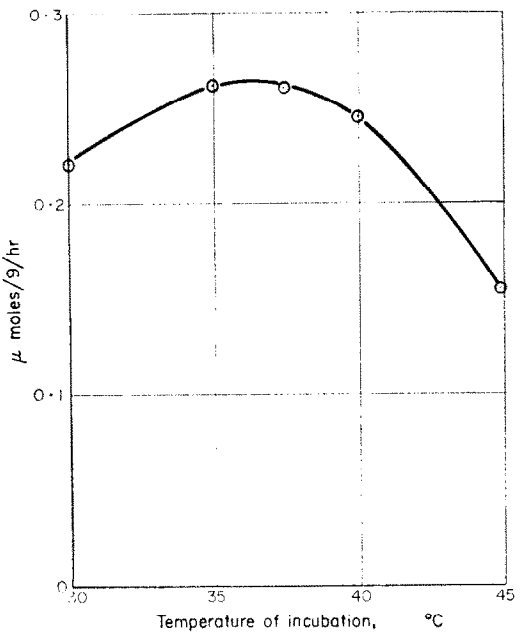

FIG. 4. The effect of the temperature of incubation on the hydroxylation of diethyl tryptamine by rat liver microsomes. The combined microsome plus soluble fraction from rat liver was incubated with diethyl tryptamine under the standard conditions (see text) at various temperatures. Each point represents the rate of hydroxylation of diethyl tryptamine determined from the slope of an activity–time curve.

The stability of rat liver microsomes in vitro

The technique of homogenizing the liver appears to influence the stability of the microsomal enzyme systems. Thus irrespective of how the preparations were stored, homogenizing the liver with a Waring Blendor resulted in a more stable preparation than homogenizing with a Potter–Elvehjem glass homogeniser. This is shown in Table 1 where the percentage activity remaining after storage for 30 days is tabulated for the enzymes which *N*-demethylate morphine and codeine, *O*-demethylate codeine and hydroxylate diethyl tryptamine.

In Fig. 5 the rates of loss of activity of the same enzymes stored at −40°C, either frozen or freeze-dried, and at 0°C, in suspension, are compared. The stability of a washed microsomal preparation is also shown. Homogenizing with a Waring Blendor

TABLE 1. THE EFFECTS OF THE METHOD OF PREPARATION AND THE CONDITIONS OF STORAGE ON THE STABILITY OF MICROSOMAL ENZYMES

Storage conditions	Method of preparation of the Microsomes	Percentage of original enzymic activity remaining after storage for 30 days			
		Hydroxylation diethyl tryptamine	*N*-Demethylation morphine	*N*-Demethylation codeine	*O*-Demethylation codeine
Frozen at −40°C	Waring Blendor	98	99	100	40
	Potter–Elvehjem	72	84	86	52
Freeze-dried at −40°C	Waring Blendor	75	79	88	33
	Potter–Elvehjem	63	78	64	52

The microsomes were made from rat liver homogenized either with a Waring Blendor or a Potter–Elvehjem glass homogenizer and were stored at −40°C either in frozen suspension or freeze-dried. The enzymic activity of the preparations was determined after various times of storage and the percentage activity remaining after 30 days storage was estimated from the graph of activity against time of storage.

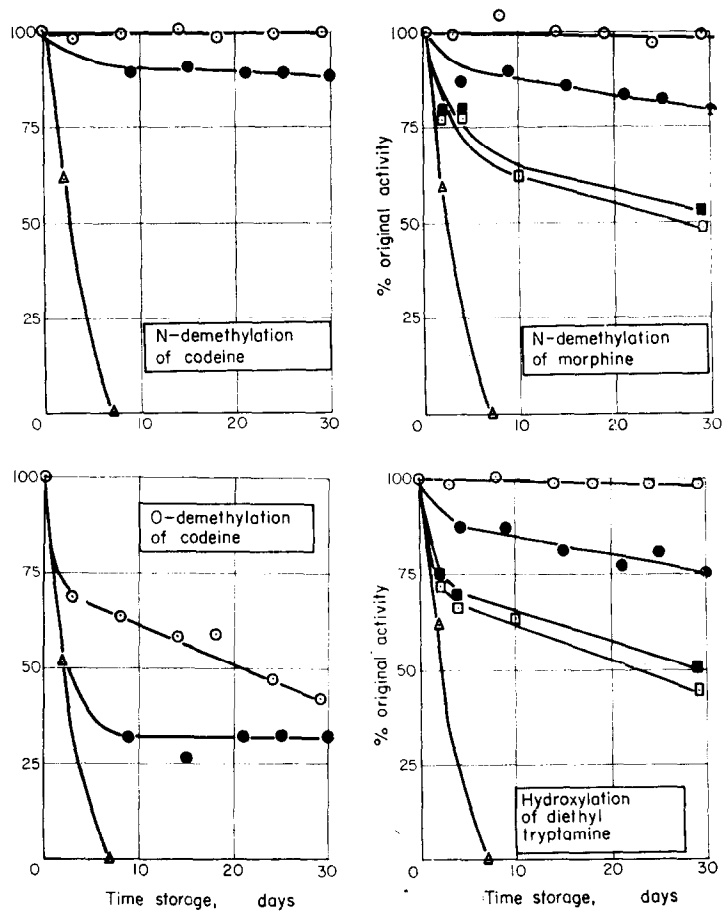

FIG. 5. The stability of various rat liver microsomal preparations of drug metabolising enzymes under different conditions of storage. The microsomes were prepared and the drug metabolising activity assayed as described in the text. The microsomal preparations studied were (1) combined microsome plus soluble fraction: stored at 0°C (△—△); frozen and stored at −40°C (○—○); freeze-dried and stored at −40°C (●—●) and (2) washed microsomes: frozen and stored at −40°C (□—□) and freeze-dried and stored at −40°C (■—■).

and storage at −40° C frozen, gave the most stable preparation. Under these circumstances no loss of activity occurred in three of the four reactions examined over a period of 30 days. The freeze-dried preparation was less stable, between 10–25 per cent activity being lost during the same period. The washed microsomal preparations were markedly less stable, between 20–25 per cent activity being lost in 3–4 days and about 50 per cent within 30 days. There was little difference between the stabilities of these preparations when stored either frozen or freeze-dried at −40°C. Storage at 0°C was very unsatisfactory since all the enzymic activity disappeared within 7 days.

The enzymes which *N*-demethylate morphine and codeine and hydroxylate diethyl tryptamine have similar stabilities whereas that which *O*-demethylates codeine is considerably more unstable. Between 50 and 70 per cent of the microsomal codeine *O*-demethylating activity was lost within 30 days irrespective of how the microsomes were prepared or whether they were stored frozen or freeze-dried at −40° C.

The Waring Blendor preparations were always more active than the Potter–Elvehejm preparations. This was found to be related to the microsomal protein content of the preparations which is shown in Table 2. The Waring Blendor preparations contained more microsomal protein than the Potter–Elvehjem but the specific microsomal activities (i.e. the activity referred to 1 mg of microsomal protein) were essentially the same.

TABLE 2. THE DISTRIBUTION OF PROTEIN IN POTTER-ELVEHJEM AND WARING BLENDOR PREPARATIONS OF RAT LIVER MICROSOMES

Cell fraction	Potter–Elvehjem Preparation			Waring Blendor Preparation		
	Protein content (mg/ml)	Diethyl tryptamine hydroxylating activity		Protein content (mg/ml)	Diethyl tryptamine hydroxylating activity	
		μ moles/gm liver/hr	* Specific activity		μ moles/gm liver/hr	* Specific activity
Microsome plus soluble fraction	34·6	0·224	17·5	41·4	0·315	17·1
Microsomes	6·4	0·211	16·5	9·2	0·300	16·3
Soluble faction	28·7			30·1		

* Specific Activity—mμ M/mg microsomal protein/hour.

The livers from six rats were pooled and microsomes were prepared from Waring Blendor and Potter–Elvehjem homogenates of the tissue. The microsomes were separated from the soluble fraction by centrifuging at 100,000 *g* for 60 min. The protein content of each of the three fractions and the diethyl tryptamine hydroxylating activities of the microsome plus soluble fraction and of the isolated microsomes were determined.

The post mortem stability of rabbit liver microsomes

In the initial series of experiments the post mortem stability of liver microsomes from a single rabbit was determined, as described in the Methods Section. The results of one such experiment are shown in Fig. 6. The microsomal activity fell off very rapidly to only 15–40 per cent of the original level in the first twenty-four hours. The subsequent rate of inactivation was much lower and forty-eight hours after death 15–30 per cent of the activity still remained.

To check that opening the abdomen to remove portions of liver was not accelerating the loss of microsomal activity a number of rabbits were killed, placed in the cold room and a single liver microsomal preparation made from each animal at various

times after death. The results are shown in Table 3, and were essentially the same as those found in the previous series of experiments: a large loss of activity in the first twenty-four hours followed by a much smaller loss over the subsequent twenty-four hours. Two rabbits were stored for five days post mortem and the liver microsomes still retained measurable activity towards codeine and diethyl tryptamine.

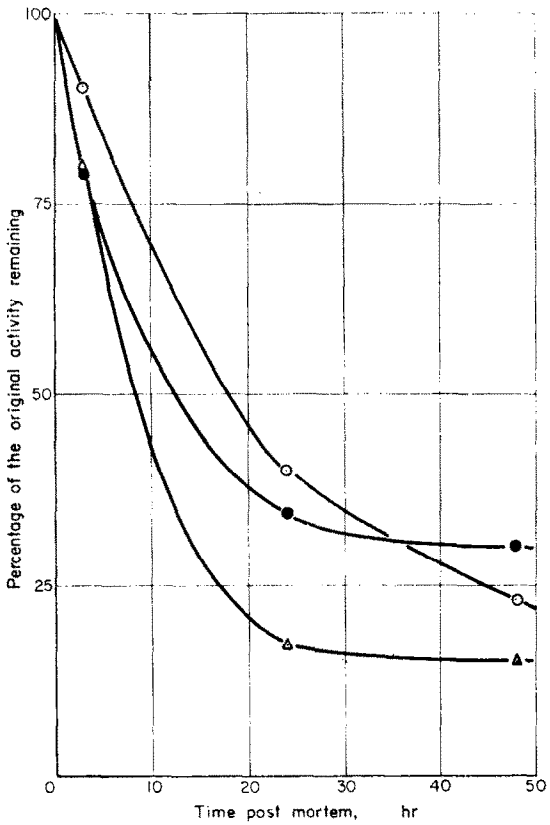

FIG. 6. The post mortem stability of the drug metabolising enzymes of rabbit liver microsomes. The rabbit was killed and placed in a cold room at 0–4°C. At various times portions of the liver were removed, the combined microsome plus soluble fraction prepared and the activity of the preparation determined in the hydroxylation of diethyl tryptamine (○—○), N-demethylation of morphine (●—●) and combined *O*- and *N*-demethylation of codeine (△—△). The results are expressed as the percentage of the enzymic activity of the microsomes prepared immediately after death.

Since loss of activity might have been due to the inactivation of the NADP reducing system of the soluble fraction and not to inactivation of the microsomal drug metabolising enzymes $NADPH_2$ was added to two of the twenty-four hour post mortem preparations (rabbits 5 and 6) to a final concentration of 5×10^{-4} M in the incubation mixture but did not increase the rate of hydroxylation of diethyl tryptamine.

DISCUSSION

In studies of the drug metabolising activity microsomes reported in the literature the period of incubation has varied from 15 min to 3 h.[4–7, 10, 11] However, the present

work has clearly shown that, for the reactions studied, the time–activity curve is only linear over the first 30–40 min of the incubation. The fact that the addition of $NADPH_2$ had no effect on the decrease in activity suggested that the limiting factor was the denaturation of the microsomal enzymes and not the breakdown of the NADP or $NADPH_2$ or the inactivation of the soluble fraction enzymes required to reduce the coenzyme. Nor was the loss of activity due to breakdown of the substrate. These results are essentially the same as those obtained by Creaven and Williams[11] on the stability of the aromatic hydroxylating microsomal enzymes in excised liver tissue at 37° C. They found that about 60 per cent of the activity was lost within 2 hr and concluded that the loss of activity "was due to deterioration of both the soluble and microsomal fractions but the deterioration was somewhat greater with the microsomes" i.e. the inactivation of the microsomal enzymes was the factor determining the activity of the preparation.

TABLE 3. THE POST MORTEM STABILITY OF THE DRUG METABOLISING ACTIVITY OF RABBIT LIVER MICROSOMES

Time post mortem (hr)	Rabbit No.	Rate of microsomal metabolism (μM/g/hr) Codeine *O* and *N*-Demethylation	Diethyl tryptamine hydroxylation
	1*	2·22	0·76
0	2	3·50	1·17
	3	1·02	0·50
	4	3·38	1·05
	Mean	2·53	0·84
	5*	0·66	0·15
24	6	0·09	0·03
	7	1·74	0·33
	8	0·00	0·03
	Mean	0·62	0·14
	9*	0·24	0·11
48	10	0·38	0·13
	11	0·13	0·05
	12	0·45	0·12
	Mean	0·30	0·10

* These animals were exsanguinated after breaking the neck.
The rabbits were placed in a cold room at 0–4°C immediately after death. Liver microsomes were prepared either as soon after death as possible or after 24 or 48 hr and the enzymic activity of the preparations determined.

The rate of inactivation of these drug metabolising enzymes was the same whether the incubation mixture was aerated by shaking or by bubbling air through it (Fig. 2). If the mixture was not aerated the loss of enzymic activity occurred sooner and was more marked. In the latter case the inactivation was probably due to the loss of the dissolved oxygen in the incubation solution as this enzyme system has an absolute requirement for oxygen.[1] Thus while the aeration may well have accelerated the inactivation of the enzyme system it could not be dispensed with because a supply of oxygen was essential to obtain a maximum rate of metabolism. However, it is noteworthy that over a period of 20 min the rate of metabolism was virtually constant irrespective of whether the solution was aerated or not.

It is clear from the storage experiments that the method of preparing and storing the microsomal preparations can modify the stability of the enzyme systems. The reasons for this are not clear. Thus loss of activity during storage was not due to breakdown of NADP or $NADPH_2$ nor to the loss of the enzyme system in the soluble fraction used to reduce NADP since in the experiments with washed microsomes $NADPH_2$ was added to the incubation mixture. It is more likely that the loss of activity may be due to mechanical damage to the enzyme system during its preparation. Each method of preparation involves a different manipulative treatment and may result in varying degrees of damage to the microsomes making them more susceptible to inactivation during storage. Indeed, the washed microsomal preparations which are subjected to considerable mechanical stress by centrifuging three times at 100,000 *g* for 60 min are considerably less stable than the microsome plus soluble fraction preparations from which they are isolated. However the manipulative procedures themselves do not affect the activity of the preparations since in preliminary experiments it was shown that repeated freezing and thawing up to five times did not reduce enzymic activity of the microsomes; neither did the process of freeze drying and redissolving.

The instability of the washed microsome preparations suggests another possibility, namely that washing the microsomes may result in the removal of a stabilising factor or activator present in the soluble fraction.

The instability of the microsomal enzymes *O*-demethylating codeine is very striking when compared with the stability of the enzymes with *N*-demethylate the same compound. This obviously suggests that the *O*-demethylation of codeine is effected by a different enzyme system from that catalysing its *N*-demethylation.

The data on the post mortem stability of rabbit liver microsomes showed that up to 40 per cent of the original activity remained twenty-four hours after death. If it is assumed that the microsomal enzymes of human and rabbit liver are inactivated at a similar rate under similar post mortem conditions then a study of 24–48 hr post mortem human liver would yield the qualitative pattern of the metabolic activity of the human drug metabolising enzyme system. Preliminary studies on five samples of human post mortem liver reported by Creaven and Williams[11] confirmed that the drug metabolising activity of the liver microsomal system can be measured up to forty-eight hours after death.

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