Biochemical Pharmacology, Vol. 23, pp. 1173–1180. Pergamon Press, 1974. Printed in Great Britain.

THE USE OF NEW METHODS TO MEASURE: THE EFFECT OF DIET AND INDUCERS OF MICROSOMAL ENZYME SYNTHESIS ON CYTOCHROME P-450 IN LIVER HOMOGENATES, AND ON METABOLISM OF DIMETHYL NITROSAMINE

ANDRÉ E. M. MCLEAN and PAULINE A. DAY

University College Hospital Medical School, University Street, London W.C.1, England

(*Received* 28 *June* 1973; *accepted* 15 *September* 1973)

Abstract—Modified methods for measurement of cytochrome P-450 content of liver homogenates, and of formaldehyde produced in demethylation reactions are described. These methods have been used to measure cytochrome P-450 content, and metabolism of dimethyl nitrosamine in rat liver. Over 80 per cent of cytochrome P-450 present in liver homogenate of phenobarbitone treated rats could be recovered in the microsomal fraction. Feeding a low protein–low fat diet reduced the P-450 content of homogenate, and also reduced the recovery of cytochrome P-450 in the microsomal fraction to 50 per cent or less. The rate of metabolism of dimethyl nitrosamine *in vivo* and *in vitro* was increased by fasting and by phenobarbital treatment, and decreased by feeding low protein diet. Benzo(α)pyrene treatment caused a slight increase in the rate of DMN metabolism *in vivo* and in microsomes. The toxicity of dimethyl nitrosamine is not altered in parallel with changes in the rate of metabolism. It is suggested that the amount of toxic metabolite is more important than the rate at which it is formed.

THE METABOLISM of the alkyl nitrosamines is of interest, because these compounds are toxic, carcinogenic and can be found in the human environment.[1–4]

The damage caused by alkyl nitrosamines, and by many other chemicals that cause cell injury, or are carcinogenic, has been attributed to their activation by the microsomal mixed function oxidase system, centred on cytochrome P-450. The pre-toxin is stable enough to exist in the environment, but its activated reaction products are molecules capable of alkylating cell constituents. This type of mechanism seems involved in the toxicity of CCl_4, paracetamol, benzo(α)pyrene, pyrrolizidine alkaloids and many other compounds.[5–8]

The existence of this mechanism has been taken to imply that an increased rate of metabolism of pre-toxic compound will cause increased amounts of cell damage. But this argument is not logical, for the ultimate damage could be just as great when alkylation proceeds slowly, as when it is rapid.

In a series of recent publications, Arcos,[9–12] and co-workers have claimed that compounds that induce synthesis of microsomal cytochrome P-450, and drug metabolism, have the unusual property of "repressing" demethylation of dimethyl nitrosamine. This conclusion was based on changes of demethylation activity per mg of microsomal protein, but these changes could have been brought about by one of two

mechanisms. Either, the amount of enzyme activity per gram of liver could remain constant while the amount of protein recovered in the microsomal fraction increased, or else there was a true fall in enzyme activity; per cell, per animal, or per gram of liver.

These two related questions, (i.e. is toxicity related to rate of metabolism, and is the rate of metabolism altered by inducers) are of interest in assessing the possible interactions between environmental inducers and environmental toxins.

We have been interested in the relation of diet to toxicity, especially because low protein diets protect rats against the toxic effects of dimethyl nitrosamine, (DMN), while enhancing renal carcinogenesis.[13,14]

There are difficulties in measuring metabolism of DMN because the reaction proceeds slowly, (at a rate of about 1/50 of the demethylation of amino-pyrine), with a high apparent K_m. This means that measurement of the formaldehyde produced is liable to interference from the high blank values, caused by the large amounts of tissue needed. Formation of $^{14}CO_2$ from labelled DMN has been used as an index of DMN metabolism, but has the disadvantage of many intermediate steps from formaldehyde to CO_2, with unknown losses on the way.

Measurement of DMN disappearance is practical *in vivo*,[15] but difficult to apply *in vitro* because the high K_m and low V_{max} for DMN metabolism means that only a small proportion of the initial DMN disappears.

By a modification of the formaldehyde assay of Nash[16] we have been able to measure DMN metabolism satisfactorily and to observe the effects of diet and inducers on the demethylation of DMN in microsomes and post mitochondrial supernatant fractions. By modification of Greim and Remmer's method,[17,18] of measuring cytochrome P-450 in whole liver homogenates, as well as in microsomes, we have been able to assess the losses of cytochrome P-450, during centrifugation steps in preparation of post mitochondrial and microsomal fractions from liver homogenates.

Using these figures for P-450 losses one can make an assessment of the amount of drug metabolising activity present in whole liver from the measured activity found in the microsomal or post mitochondrial fractions. It is then possible to estimate whether a change in specific activity of a microsomal component found after altering the diet of the animal, reflects a real change in that component, or merely an alteration in the ratio of the measured component, to the mixture of proteins in the precipitated microsomes.

MATERIALS AND METHODS

Rats. Male rats of a Wistar strain were bought (from A. Tuck & Son, Rayleigh, Essex, U.K.), and housed in mesh floored cages. Food and water were available *ad lib.* except for the fasted groups.

Phenobarbitone was given as a solution containing 1 mg of sodium phenobarbitone/ml in distilled water as the sole source of drinking water, as previously described.[19]

Benzo(α)pyrene was given as a single intraperitoneal injection of 20 mg/kg body weight, dissolved in corn oil at a concentration of 10 mg/ml. Purified diets were made as previously described,[20] and contained 5% olive oil as their fat source.

Chemicals. Dimethyl nitrosamine was bought from R. Emmanuel & Son. NADP was bought from Boehringer Corp. (London) Ltd., London W.5, U.K., isocitrate and isocitrate dehydrogenase from Sigma Chemical Co. London, and other chemicals from British Drug Houses, Ltd., Poole, Dorset, U.K.

Tissue samples and assays. Rats were killed by exsanguination under ether anaesthesia, the livers removed and rapidly cooled in ice cold saline. A 2 g portion was homogenized in 18 ml 150 mM KCl, containing 20 mM Tris–HCl buffer, pH 7·4, using an Ultra Turrax blender for 8 sec (10% Homogenate).

Metabolism of dimethyl nitrosamine was measured in the supernatant fractions remaining after 7000 *g* maximum centrifugation for 10 min (10% post mitochondrial fraction), and also in the microsomal suspension produced when 8 ml of post mitochondrial fraction was precipitated by centrifugation at 100,000 *g* (maximum) for 60 min in a 10 ml unstoppered polycarbonate tube, and the resulting pellet resuspended to 8 ml (10% microsomes).

Metabolism of dimethyl nitrosamine, in vitro was estimated by measuring the rate of formaldehyde production.

A 5 ml portion of 10% post mitochondrial fraction of liver homogenate, or an equivalent quantity of microsomal suspension, were added to an incubation mixture to give a final vol of 10 ml containing the following: $MgCl_2$, 75 μmoles, Na Isocitrate, 30 μmoles, KH_2PO_4 buffer (adjusted to pH 7·2 with KOH), 500 μmoles, semicarbizide (neutralized to pH 7·2 with NaOH), 40 μmoles, $NADP^+$ 4·5 μmoles. Isocitrate dehydrogenase, (0·1 unit Sigma type 4) were added when microsomal functions were assayed.

The mixture was incubated at 37° in air, in a stoppered 25 ml conical flask, and the reaction started by addition of dimethyl nitrosamine, to give a concentration of 1·4 μmoles/ml. 2·5 ml samples of the incubation mixture were taken out at zero time, and after 10 and 20 min incubation. Protein was precipitated by addition of 1 ml saturated barium hydroxide, and the sample neutralized with 1 ml of a 20% w/v zinc sulphate solution.[21]

Formaldehyde was measured in the supernatant by the method of Nash,[16] with the following modification. 2·5 ml of sample were incubated with 1 ml of double strength Nash reagent at 60° for 30 min, the sample was cooled, and the colour extracted into 1·5 ml amyl alcohol, and the optical absorption at 421 nm measured. The amyl alcohol extraction removed the high and variable blank values that were due to turbidity from glycogen and other interfering substances.

Cytochrome P-450 was measured by developing the method described briefly by Greim,[17] where both cuvettes are gassed with CO and one is reduced with dithionite. This allows the P-450 content of homogenates to be measured as long as certain precautions are observed. In the present experiments the cytochrome P-450 content of homogenates and microsomes was measured using the following criteria for acceptability of any measurement. The homogenate should contain 5–20 mg of tissue/ml in 0·1 M *K*-phosphate buffer. The absolute absorbance of homogenate vs water should be less than 1 at 450 nm. The baseline (homogenate vs homogenate) must be level, with the change of absorbance between 490 and 450 nm less than 0·005, and from 490 to 430 nm less than 0·02.

Reduction should be carried out with a minumum amount of dithionite, (about 1 mg/ml), and should result in a peak due to cytochrome b_5 at 424 nm, separate from the cytochrome P-450 peak at 450 nm.

The Δ absorbance from 490 to 470 should be less than 0·003. The cuvettes should be kept cool throughout, and rapid changes of absorbance with time indicate precipitation of sulphur in the cuvette. The cuvette containing the non-reduced suspension should have air bubbled through it briefly with a pasteur pipette to prevent reduction of cytochrome P-450 by NADPH produced from endogenous substrates.

The P-450 content is calculated from the peak to trough of absorbance (between 490 and 450 nm), using the millimolar extinction coefficient of 91. The peak of absorbance at 450 nm develops in the course of 2 min after addition of dithionite. (In contrast to the immediate maximum found with the addition of CO in the method of Omura and Sato[22]).

With these precautions, satisfactory measurement of cytochrome P-450 can be made using the Unicam SP800 spectrophotometer with external recording amplification, on homogenates of human, or rat liver. A 4 mm wide, 10 mm path length semi-micro cuvette used in the forward position of the spectrophotometer gives satisfactory results, and enables the cytochrome P-450 content of needle biopsy samples of liver to be measured.

Dimethyl nitrosamine in plasma. Dimethyl nitrosamine was injected intra-peritoneally as a solution in saline containing 18 mg/ml DMN. Groups of rats were killed at intervals and blood samples taken from the carotid artery. DMN was then isolated from plasma. One ml of plasma was mixed with 1 ml of distilled water, 1 ml 20% w/v $ZnSO_4$ in 0·25 N H_2SO_4 and 1 ml 0·75 N NaOH. The mixture was centrifuged to bring down the precipitated protein, and 2 ml of the supernatant fluid was mixed with 2 ml distilled water, 0·05 ml 1 N H_2SO_4 and about 400 mg NaCl. The mixture was gently distilled from a 10 ml pear shaped distillation flask, and the first 2 ml of distillate collected. This contained over 80 per cent of added DMN. The DMN was then estimated as nitrite by the method of Daiber and Preussman,[23] after photochemical splitting in a quartz 3 ml spectrophotometer cuvette irradiated for 30 min, 15 cm from a Hanovia photochemical reactor lamp.

RESULTS

The modified method of measuring cytochrome P-450 gives the same results as the method of Omura and Sato,[22] when applied to microsomal suspensions, in addition to which it gave consistent results using liver homogenates.

Table 1 shows that feeding a low protein diet reduces the P-450 content of liver homogenate to one half that found in rats fed stock diet, while phenobarbitone increases cytochrome P-450 by a factor of 3·5.

The amount of cytochrome P-450 recovered in the microsomal fraction varies with the amount originally present. For phenobarbitone treated rats containing large amounts of cytochrome P-450, recovery is 84 per cent, a smaller starting concentration gives a lower percentage yield. The losses during preparation tend to exaggerate the effects of diet and phenobarbitone on the microsomal fraction, in comparison with the changes brought about in the original liver homogenate.[19]

Incubation of DMN with microsomes or post mitochondrial fraction resulted in the formation of formaldehyde. In experiments using low concentrations of DMN, 1 mole of formaldehyde was recovered per mole of DMN disappearing. The other methyl group presumably disappears into methylation reactions.[1]

TABLE 1. MEASUREMENT OF CYTOCHROME P-450 IN RAT LIVER HOMOGENATES AND CELL FRACTIONS

Treatment	Homogenate: P-450 (nmoles/g liver)	Percentage of original cytochrome P-450 from liver homogenate recovered by fractionation procedure	
		Post mitochondrial fraction (%)	Microsomes (%)
Low protein diet	18·2 ± 4·2 (23)	57 ± 12	49 ± 11
Stock pellets (41B)	39·8 ± 8·5 (21)	74 ± 10	64 ± 7
Stock + phenobarbitone	142 ± 35 (11)	83 ± 12	84 ± 5

Male rats weighing 100–200 g were fed stock diet, or 3% casein diet for at least 1 week, and cytochrome P-450 measured as described in the section on Methods. Results are expressed as means ± S.D. Number of determinations each from a separate rat is given in parentheses.

Percentage yield is calculated as: (P-450/ml 10% fraction)/(P-450/ml 10% homogenate) × 100.

The reaction was inhibited by carbon monoxide; (35% inhibition on incubation in 25% CO 75% air), and the reaction proceeds linearly with time and amount of tissue for 20 min, so long as the amount of liver added does not exceed 50 mg/ml incubation medium. The apparent Michaelis constant was approximately 0·25 mM, which does not disagree with the values of 0·22 and 0·53 mM quoted on various occasions by Venkatesan *et. al.*[11,12]

Table 2 shows that fasting causes a great increase in DMN demethylation activity. This increase is present whether the results are expressed per g of tissue, or per mg of microsomal protein, or per rat. In contrast, benzo(α)pyrene treatment caused no significant change in DMN metabolism per gram of liver in either fed or fasted rats. The increase in microsomal protein due to benzo(α)pyrene would however produce an apparent fall in DMN demethylating activity if this were calculated per mg of protein. Low protein diet causes DMN metabolism to fall by about 50 per cent as has previously been shown by other methods.[15]

Comparison of Table 2 with Table 3 shows that for stock rats, the rate of DMN metabolism is higher in the post mitochondrial fraction than in microsomes. However, when the data from Table 1 on recovery of endoplasmic reticulum (as cytochrome P-450), into these fractions, is applied to the results, there is essentially the same

TABLE 2. THE EFFECT OF BENZO(α)PYRENE PRETREATMENT ON DEMETHYLATION OF DIMETHYLNITROSAMINE LIVER MICROSOMES FROM FED, FASTED AND PROTEIN DEPLETED RATS

Treatment	*N*	DMN metabolism (μmoles formaldehyde/g liver/hr)	Liver wt (g/100 g body wt)	Microsomal protein (mg/g liver)
Stock fed	8	1·1 + 0·1	5·0 ± 0·2	25 ± 2
Stock fed + benzo(α)pyrene	4	1·0 ± 0·2	5·0 ± 0·4	33 ± 8
Stock fasted	14	2·9 ± 0·9	3·8 ± 0·3	38 ± 10
Stock fasted + benzo(α)pyrene	7	3·1 ± 0·5	4·4 ± 0·2	44 ± 7
Low protein fed	6	0·49 ± 0·03	5·0 ± 0·5	18 ± 2
Low protein fed + benzo(α)pyrene	6	0·81 ± 0·19	5·6 ± 0·5	22 ± 2

Male rats weighing 100–150 g were fed stock pellets or the protein free diet, for at least 1 week. Benzo(α)pyrene was given as a single intraperitoneal injection 48 hr before the animals were killed. Fasted rats had food removed 18 hr before death. Results are given as the mean ± one standard deviation; *N* is the number of determinations each on a separate rat.

TABLE 3. THE EFFECT OF DIET, BENZO(α)PYRENE AND PHENOBARBITONE ON THE DEMETHYLATION OF DIMETHYLNITROSAMINE BY POST MITOCHONDRIAL FRACTION OF RAT LIVER

Treatment			DMN metabolism (μmoles formaldehyde/g liver/hr)
Stock	Fed	—	1·44 ± 0·21 (12)
Stock	Fed	Phenobarbitone	2·09 ± 0·41 (7)
Low protein	Fed	—	0·67 ± 0·19 (8)
Low protein	Fed	Benzo(α)pyrene	0·66 ± 0·28 (8)
Low protein	Fed	Phenobarbitone	1·30 ± 0·11 (5)
Low protein	Fasted	—	1·69 ± 0·30 (4)
High protein	Fed	—	0·94 ± 0·19 (9)
High protein	Fed	+ 10% Herring oil	1·24 ± 0·40 (4)
High protein	Fed	+ Phenobarbitone	1·57 ± 0·13 (8)

Male rats weighing 100–170 g were fed on stock, low protein (3% casein, 5% olive oil) or high casein (20% casein, 5% olive oil), diets for at least 1 week.

Phenobarbitone was given for 1 week in the drinking water, and benzo(α)pyrene was given as a single intraperitoneal injection 24 or 48 hr before death. Assays were conducted as described in the section on Methods and results are given as means ± one standard deviation. Number of determinations, each on a separate rat is given in parentheses.

activity per g of original liver (when corrected for loss of endoplasmic reticulum), in microsomal and post mitochondrial fractions.

Table 3 also shows that phenobarbitone causes an increase in the rate of DMN metabolism, that is much smaller than the increase in P-450 or microsomal protein caused by this treatment. In the protein depleted rats DMN metabolism was reduced, and could be increased again by fasting or phenobarbitone but not by benzo(α)pyrene.

The rats fed a high protein purified diet (20% casein, 5% olive oil), had lower levels of DMN demethylation activity than stock rats, and giving 10% herring oil, or phenobarbitone, increased this activity. In this respect, DMN demethylation responds to changes of diet in a very similar fashion to cytochrome P-450.[20]

Table 4 shows that the rate at which DMN disappears from the plasma is approximately in agreement with the rate that would be predicted from DMN demethylation activity found *in vitro*. Fasting causes a dramatic reduction in the amount of DMN left after 6 hr, while low protein diet reduces the rate of metabolism. However, there are two discrepancies. Phenobarbitone has little effect on DMN disappearance, benzo(α)pyrene has considerably more, both at 6 and at 24 hr. This is in contrast to the

TABLE 4. THE EFFECT OF DIET, STARVATION AND INDUCERS ON PLASMA LEVELS OF DIMETHYLNITROSAMINE

Diet and treatment	Plasma levels of DMN (nmoles/ml)		
	Time after injection		
	1 hr	6 hr	24 hr
Stock fed	1485 ± 34	1150 ± 121	< 20
Stock fasted		548 ± 214	
Low protein fed	2092 ± 73	1654 ± 117	312 ± 161
Low protein fed + phenobarbitone		1266 ± 94	223 ± 52
Low protein fed + benzo(α)pyrene		1098 ± 160	< 20
Low protein fasted		981 ± 175	< 20

Rats were given a single intraperitoneal injection of DMN at a dosage of 1220 nmoles/g body wt. (90 mg/kg). Blood samples were taken at intervals as described in the section on Methods.

effect on DMN demethylation *in vitro*, where phenobarbitone is by far the more effective inducer of DMN metabolism.

DISCUSSION

From experiments on the effects of phenobarbitone on the toxicity of a number of compounds there has grown up an idea that toxicity depends on the rate of activation of pre-toxin to toxin.

Neither fasting nor phenobarbitone alter the toxicity of dimethyl nitrosamine,[14] although each of them stimulates the rate of metabolism of DMN both *in vivo* and *in vitro*. Benzo(α)pyrene which has less effect on the rate of DMN metabolism, increases the signs of acute hepatotoxicity by DMN.* One is forced to the conclusion that the rate of metabolism of DMN is not critical in the development of toxic effects.

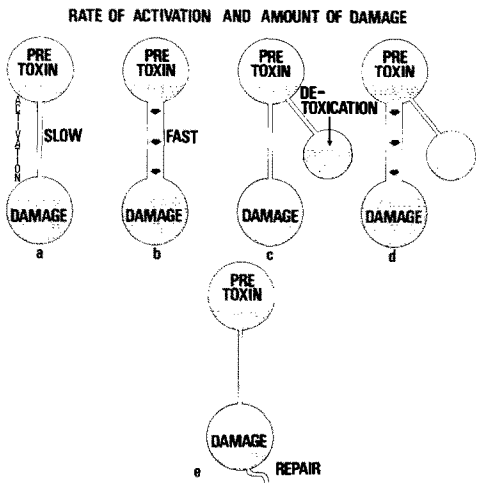

FIG. 1.

In the case of carbon tetrachloride, the inhibition of metabolism produced by feeding low protein diets, allows unaltered CCl_4 to disappear harmlessly through the lungs.[24] So phenobarbitone increases CCl_4 toxicity not because it increases the rate of metabolism, but because it increases the amount of CCl_4 trapped into the toxic pathway of the two alternative paths available.

For DMN it seems likely that no such major alternative route of excretion exists.[25] That means that practically all the DMN injected will be metabolized, either slowly or rapidly.

Heath[25] found that two doses of DMN separated by a few hours, caused almost the same damage as a single dose. So it seems likely that the damage caused by alkylation is cumulative over at least a few hours. Another important influence is that of repair and it is possible that repair processes can catch up if alkylation is slow enough. This may be important for DMN lethality since the damage to central veins in the liver lobule could be compensated for by opening of collateral venous channels.[27]

* A. E. M. McLean, unpublished observation.

The animal fed a protein free diet is protected in spite of the fact that all the DMN is eventually metabolised. There are two main possibilities, either DMN is metabolised into some harmless derivative,[26] or alternatively DMN is still transformed into an alkylating agent, but this now attacks some inessential cell component, such as glycogen, and does no damage.

Figure 1 illustrates these concepts. In cases (a) and (b) the amount of damage is independent of the rate at which pre-toxin is activated, because there is no alternative route for pre-toxin to go into, and there is zero rate of repair.

In case (c) and (d) where there is an alternative route for the pre-toxin to escape by, the rate of activation becomes the critical factor in determining the amount of damage.

In case (e) where there is a significant rate of repair, the rate of activation is again critical, since there will be no damage unless activation can cause damage at a rate greater than the repair process. For paracetamol (acetaminophen), the activation by P-450 is followed by "repair" in the sense of trapping of the activated molecule by glutathione, and the rate of activation is critical.[7]

The claims by Arcos and his co-workers, that inducers "repress the dimethyl nitrosamine demethylase" are not borne out by the present experiments.

It seems likely that the supposed repression is in fact due to greatly increased yield of microsomal protein in the presence of smaller increases of DMN demethylation activity.

Acknowledgements This work was supported by grants from Cancer Research Campaign and Medical Research Council.

REFERENCES

1. P. N. Magee and J. M. Barnes, *Advan. Cancer Res.* **10**, 163 (1967).
2. W. Lijinsky and S. S. Epstein, *Nature, Lond.* **225**, 21 (1970).
3. J. Sander, in *Transplacental Carcinogenesis* (Eds. L. Tomatis and U. Mohr), pp. 159–163 (1973).
4. N. T. Crosby, J. K. Foreman, J. F. Palframan and R. Sawyer, *Nature, Lond.* **238**, 342 (1972).
5. A. E. M. McLean and E. K. McLean, *Br. med. Bull.* **25**, 278 (1969).
6. B. B. Brodie, *Chem. Biol. Interactions* **3**, 247, (1972).
7. D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter, J. R. Mitchell, S. R. Gillette and B. B. Brodie, *Fed. Proc. Am. Soc. exp. Biol.* **32**, 305 (1973).
8. J. A. Miller, *Cancer Res.* **30**, 559 (1970).
9. J. C. Arcos, M. F. Argus and N. P. Buu-Hoi, *Fed. Proc. Am. Soc. exp. Biol.* **32**, 702. Abs. No. 2756 (1973).
10. N. Venkatesan, J. C. Arcos and M. F. Argus, *Life Sci.* **7**, pt i, 1111 (1968).
11. N. Venkatesan, M. F. Argus and J. C. Arcos, *Cancer Res.* **30**, 2556 (1970a).
12. N. Venkatesan, J. C. Arcos and M. F. Argus, *Cancer Res.* **30**, 2563 (1970b).
13. A. E. M. McLean and P. N. Magee, *Br. J. exp. Path.* **51**, 587 (1970).
14. A. E. M. McLean and H. G. Verschuuren, *Br. J. exp. Path.* **50**, 22 (1969).
15. P. F. Swann and A. E. M. McLean, *Biochem J.* **124**, 283 (1971).
16. T. Nash, *Biochem J.* **55**, 416 (1953).
17. H. Greim, *Arch. Pharmak.* **266**, 261 (1970).
18. B. Schoene, R. A. Fleischmann, H. Remmer and H. F. von Oldershausen, *Eur. J. Clin. Pharmac.* **4**, 65 (1972).
19. W. J. Marshall and A. E. M. McLean, *Biochem. Pharmac.* **18**, 153 (1969).
20. W. J. Marshall and A. E. M. McLean, *Biochem. J.* **122**, 569 (1971).
21. R. E. Stitzel, F. E. Greene, R. Furner and H. Conaway, *Biochem. Pharmac.* **15**, 1001 (1966).
22. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
23. D. Daiber and R. Preussmann, *Z. Anal. Chem.* **206**, 344 (1964).
24. A. A. Seawright and A. E. M. McLean, *Biochem. J.* **105**, 1055 (1967).
25. D. F. Heath, *Biochem J.* **85**, 72 (1962).
26. A. Somogyi, A. H. Conney, R. Kuntzmann and B. Solymoss, *Nature*, New Biol. **237**, 61 (1972).
27. E. K. McLean and K. R. Hill, *Br. J. exp. Path.* **50**, 37 (1969).