Biochemical Pharmacology, Vol. 19, pp. 2343–2351. Pergamon Press. 1970. Printed in Great Britain

METABOLISM OF DRUGS DURING RAT LIVER REGENERATION

P. TH. HENDERSON and K. J. KERSTEN

Institute of Pharmacology, Faculty of Medicine, University of Nijmegen, Nijmegen, Netherlands

(*Received* 15 *January* 1970; *accepted* 10 *February* 1970)

Abstract—Enzymatic activities for the *p*-hydroxylation of aniline, the *N*-demethylation of aminopyrine, and the UDPglucuronyl conjugation of *p*-nitrophenol of regenerating rat liver have been measured *in vitro* at different intervals following partial hepatectomy. The *p*-hydroxylating and *N*-demethylating activities decreased during the period of rapid cellular proliferation and subsequently rose to about 100 and 80 per cent respectively of their initial values within 7 days postoperatively. The UDPglucuronyltransferase activity, measured in ultrasonicated homogenates, however was not reduced during the regeneration process. Pretreatment of the rats with phenobarbital resulted in a considerable increase of the drug-oxidizing enzymes even during the period of rapid growth. The possibility that the changes of drug-oxidizing capacities of regenerating liver are related to the rate of liver growth is considered.

MAMMALIAN liver, which ordinarily undergoes very poor proliferation, compensates for the loss of cells after partial removal by a rapid growth.[1, 2] In the course of this regeneration the structure and chemical composition of the endoplasmic reticulum become modified in a characteristic way,[3, 4] accompanied by numerous biochemical events.[5–7]

Von der Decken *et al.*[3] and Fouts *et al.*[8] have reported decreased activities of some oxidative and reductive enzymes involved in the microsomal biotransformation of drugs after partial hepatectomy. It is noteworthy that liver tumors, fetal livers and livers of newborn mammals, all of which show a relatively rapid proliferation, have only a rather small capacity for metabolizing drugs.[9–11]

The present paper describes enzymatic activities of the *N*-demethylation of aminopyrine and the *p*-hydroxylation of aniline measured at different intervals during regeneration following partial hepatectomy. The aim of this study was to establish whether there is any relationship between the change in activity of these drug-oxidizing enzymes and the rate of liver growth presented by the increase of liver weight, mitotic index, and the amount of DNA. The influence of liver regeneration on the responsiveness of the drug-oxidizing enzymes to pretreatment with phenobarbital was studied during the period of rapid cellular proliferation. Further it was examined whether the decrease in activity, as is observed for the microsomal oxidations, holds too for the *in vitro* activity of the conjugating enzyme *p*-nitrophenol glucuronyltransferase (UDPglucuronate glucuronyltransferase, EC 2.4.1.17).

EXPERIMENTAL PROCEDURE

Animals

The animals used were adult male Wistar rats about 3 months old, weighing 150–

180 g. Partial hepatectomy was carried out under ether anaesthesia according to the technique of Higgins and Anderson[12] and about 70 per cent (median plus left lateral lobes) of the total liver was removed. In the sham-operated animals only the peritoneum was opened, and the wound closed as in the hepatectomized rats.

In induction experiments laparotomized and hepatectomized animals received two injections of phenobarbital sodium (75 mg/kg, i.p.), 3 hr before and 12 hr after the operation. Control animals were injected with 0·5 ml 0·9% NaCl.

Tissue preparations

The animals were decapitated under light ether anaesthesia and the livers were rapidly excised. Portions of liver were weighed, minced and transferred into 4 vol. of ice-cold 0·25 M isotonic sucrose solution containing 10^{-3} M sodium ethylenediamine tetraacetate (EDTA-Na) and 5×10^{-2} M tris(hydroxymethyl)aminomethane-HCl buffer (pH 7·4). Homogenates were prepared using a Teflon-glass homogenizer in an ice-bath. Crude microsomal fractions were prepared from the homogenates by centrifugation at 9000 *g* for 20 min at 2°. The supernatant was used in the enzymatic assays for aminopyrine *N*-demethylation and aniline *p*-hydroxylation. The enzyme extracts employed in the *in vitro* estimation of the glucuronidation of *p*-nitrophenol were liver homogenates treated in an ultrasonic disintegrator (MSE-100 Watt) for 1 min.[13]

Enzyme assays

The *N*-demethylation of aminopyrine was measured as previously described by Dewaide and Henderson.[14] The assay mixture containing 5×10^{-2} M Tris-HCl (pH 8·0), 5×10^{-3} M semicarbazide, 8×10^{-4} M $MgCl_2$, 8×10^{-6} M $MnCl_2$, 5×10^{-3} M sodium isocitrate (Sigma), 10 μg isocitric dehydrogenase/ml (Sigma type 4, capable of reducing $5{\cdot}7 \times 10^{-3}$ m-moles NADP/min/mg), $8{\cdot}8 \times 10^{-5}$ M NADP and a saturating level of aminopyrine ($16{\cdot}7 \times 10^{-3}$ M), was preincubated for 10 min at 37° to ensure reduction of all NADP. The reaction was started by the addition of 9000 *g* supernatant fraction equivalent to 80 mg fresh liver. The total volume of the reaction mixture was 3 ml. After 10 min incubation at 37° the reaction was stopped by the addition of 0·5 ml 25% $ZnSO_4 \cdot 7H_2O$ and 0·5 ml of a saturated $Ba(OH)_2$ solution. After centrifugation the amount of formaldehyde was determined in the deproteinized supernatant following the method of Nash[15] as modified by Cochin and Axelrod.[16]

The incubation mixture for the assay of the *p*-hydroxylation of aniline had almost the same composition as the medium mentioned above, except for the substrate and coenzyme concentration which were for aniline 24×10^{-3} M and for NADP 13×10^{-5} M. Semicarbazide was omitted. After incubation at 37° for 20 min the reaction was stopped by the addition of 2 ml ethylacetate. *p*-Aminophenol was extracted with ethylacetate and determined following the phenol indophenol method.[17]

For the measurement of the activity of UDPglucuronyltransferase *p*-nitrophenol was used as substrate. The ultrasonicated homogenates were incubated at 37° for 15 min in a mixture consisting of Tris-HCl buffer (pH 7·4) (5×10^{-2} M), $MgCl_2$ ($3{\cdot}3 \times 10^{-3}$ M), uridine-5-diphosphoglucuronate disodium salt (3×10^{-3} M), *p*-nitrophenol ($1{\cdot}4 \times 10^{-3}$ M) and saccharo-1,4-lactone (10^{-3} M). The total volume was 1·5 ml. The reaction was stopped by addition of 1 ml ethanol and the mixture centrifuged for 15 min at 5000 *g*. The amount of conjugated product was measured indirectly by

determining the disappearance of *p*-nitrophenol. Aliquots of the deproteinized supernatant were diluted with 0·1 N NaOH and assayed spectrophotometrically at 398 mμ.

For all enzyme assays standard solutions and blanks were carried through the whole procedure and were used to quantify the enzymatic activities.

DNA and protein assay

DNA was determined by the diphenylamine test according to Burton[18] after a pre-extraction following Wanka.[19] Calf thymus DNA (British Drug Houses) was employed as a reference standard.

Protein contents of liver homogenates were determined following the method of Lowry *et al.*[20] Bovine serum albumin (Sigma) was used as a reference standard in the calculation of the amounts of protein.

Histological preparations

Liver tissue (about 0·5 g) was fixed for 48 hr by 3·5% formaldehyde (neutralized by excess of $CaCO_3$) in 10% $CaCl_2$. After fixation the tissue was embedded in paraffin and histological sections, 5–10 μ thick, were made, and stained with hematoxylin and eosin. The occurence of mitotic parenchymal cells was determined microscopically under oil immersion (× 1250). The mitotic indices were expressed as percentages of all nuclei of parenchymal cells.

RESULTS

Liver regeneration

Simultaneously with the activities of the drug-metabolizing enzymes the liver growth was followed at the different stages during the regeneration process. As can be seen from Fig. 1a the compensatory growth (weight increase) of the liver remnant is almost completed within 7 days. After a short latency period there is a rapid increase of the liver weight during the second and third day following the partial hepatectomy, which is mainly associated with a cellular hyperplasia.

Most important events in this stage of rapid cellular growth are DNA synthesis and mitosis. The increase of total DNA content, expressed in Fig. 1b per g liver dry weight, precedes the cellular division shown in Fig. 1c. Cell mitosis first appeared after 20 hr. Then the mitotic index rapidly rose to 4·5 per cent at 28 hr. This was followed by a gradual decrease and a second smaller mitotic wave between 40 and 60 hr. One week after the operation only few parenchymal cells in division were observed. These observations concerning the course of liver regeneration are in accordance with the more detailed analysis recently reported by Fabrikant.[2]

p-*Hydroxylation of aniline and* N-*demethylation of aminopyrine*

The *in vitro* activities of the *p*-hydroxylation of aniline and the *N*-demethylation of aminopyrine have been measured in 9000 *g* supernatants at different intervals after partial hepatectomy. In Fig. 2 it is shown that the rates of conversion of aniline and aminopyrine levelled down between 20 and 60 hr postoperatively to a minimum corresponding with the period of rapid growth of the liver, which has been indicated in Fig. 1. Thereupon an increase of the enzymatic activities could be observed concurring with the decline of the cellular proliferation. For the *p*-hydroxylation of aniline an about 100 per cent recovery of the activity was reached within 1 week after the

partial hepatectomy. However, the *N*-demethylation of aminopyrine was only about 80 per cent of the initial value after 1 week.

In order to avoid an interference by the increase of water content of the liver remnant, which was found to occur mainly during the first hours after partial hepatectomy, the enzymatic activities are presented here on the basis of liver dry weight.

No remarkable changes in the amount of total liver protein per gram dry tissue were observed at the different intervals of regeneration.

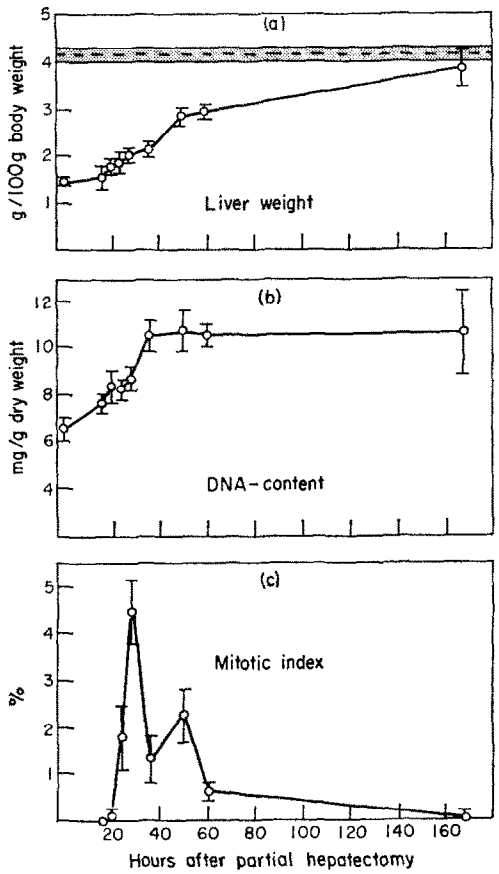

FIG. 1. (a) Growth of the remnant liver after partial hepatectomy. Rat liver weight is expressed per 100 g body weight. The normal value is presented by the dotted line.
(b) The amount of liver DNA at different intervals expressed as milligrams per gram liver dry weight.
(c) Temporal pattern of mitoses during rat liver regeneration. The mitotic index is expressed as percentage of all nuclei of parenchymal cells. Each point indicates the mean value (± 2 S.E.) for six rats.

Sham operation did not significantly affect the rate of *N*-demethylation of aminopyrine (Table 1), nor the mitotic activity in the livers of the control animals. The question arose whether the low activities mentioned above are due to the presence of an inhibitor acting on the level of the enzymatic conversion or to a decrease in the enzyme concentrations during the liver regeneration. The results of enzyme assays in

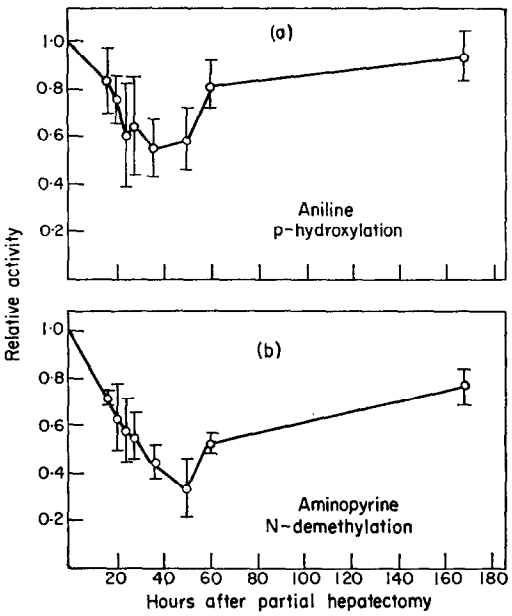

FIG. 2a, b. Hepatic *p*-hydroxylating and *N*-demethylating activities, measured in 9000 *g* supernatants, at different intervals during rat liver regeneration after partial hepatectomy. The mean enzymatic activities on the basis of liver dry weight are given here relative to their own initial values determined in the removed liver lobes. Each point indicates the mean (± 2 S.E.) for six animals.

TABLE 1. *N*-DEMETHYLATION OF AMINOPYRINE AFTER SHAM OPERATION

Time of killing after laparotomy	*N*-demethylating activity*	Percentage of the mean initial value
5 min	14·1–14·4 (14·3)†	100
22 hr	12·5–13·0 (12·8)	90
30 hr	14·3–14·5 (14·4)	101
50 hr	13·4–13·8 (13·6)	95
170 hr	13·6–13·2 (13·4)	94

* Expressed as μmoles formaldehyde produced per hour per gram liver.
† The mean activity is given in parentheses.

which mixtures of 9000 *g* supernatants from regenerating and from normal rat livers were incubated (Table 2) indicate that no inhibitor is involved in the lower rate of drug oxidation, since the combined enzyme preparations show *N*-demethylating and *p*-hydroxylating activities nearly equal to the sum of the individual activities.

In the induction experiments laparotomized and partially hepatectomized rats were injected twice with phenobarbital. The animals were sacrificed during the period of rapid regeneration, at 40 hr postoperatively, and the *in vitro* activities of hepatic *N*-demethylation and *p*-hydroxylation were measured in 9000 *g* supernatants. The enzymatic activities appeared to be enhanced in both laparotomized and partially hepatectomized animals (Fig. 3). However, the response to phenobarbital is more pronounced in the regenerating than in the sham-operated rats.

TABLE 2. *N*-DEMETHYLATING AND *p*-HYDROXYLATING ACTIVITIES OF COMBINED 9000 *g* SUPERNATANTS FROM REGENERATING* AND NORMAL RAT LIVERS*

Exp. number	Formaldehyde production† per: 0·2 ml 9000 *g* supernatant from normal liver (A)	0·2 ml 9000 *g* supernatant from regenerating liver (B)	0·4 ml mixture of 0·2 ml A + 0·2 ml B (C)	(C)/(A)+(B)
1	160	46	219	1·06
2	160	77	251	1·06
3	160	69	231	1·01
	p-Aminophenol production‡ per: 0·1 ml 9000 *g* supernatant from normal liver (A)	0·1 ml 9000 *g* supernatant from regenerating liver (B)	0·2 ml mixture of 0·1 ml A + 0·1 ml B (C)	(C)/(A)+(B)
4	19·5	8·1	26·4	0·96
5	19·5	8·9	27·9	0·98
6	19·5	10·4	29·0	0·97

* The animals were killed 40 hr after partial hepatectomy.
† Expressed in mμmoles, measured after 10 min incubation.
‡ Expressed in mμmoles, measured after 20 min incubation.

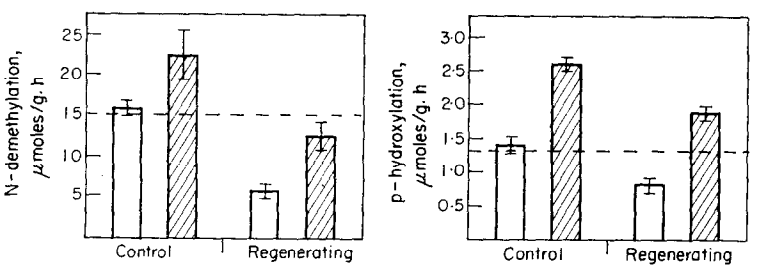

FIG. 3. Effect of pretreatment with phenobarbital on the *N*-demethylation of aminopyrine and the *p*-hydroxylation of aniline by 9000 *g* liver supernatants from laparotomized (control) and partially hepatectomized (regenerating) rats. Mean activities of *N*-demethylation and *p*-hydroxylation are expressed here as micromoles formaldehyde and *p*-aminophenol respectively, produced per gram liver per hour. The broken lines indicate non-induced enzyme levels of unoperated rats. □ treated with saline; ▨ treated with phenobarbital.

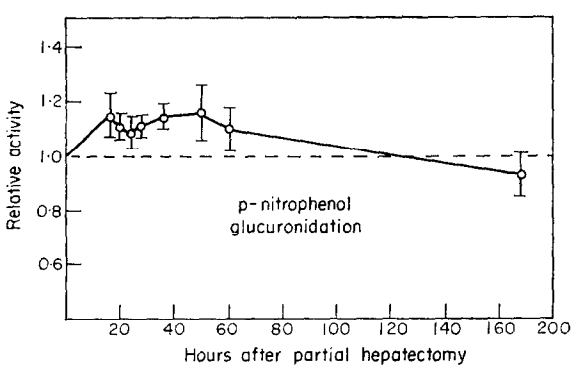

FIG. 4. Pattern of UDPglucuronyltransferase activity of ultrasonicated homogenates during rat liver regeneration after partial hepatectomy. The mean rates of conversion calculated on the basis of liver dry weight are expressed relative to the initial values measured in the removed liver lobes. Each point indicates the mean (± 2 S.E.) for six rats.

UDPglucuronyltransferase

Figure 4 shows the *in vitro* activity of UDPglucuronyltransferase determined at different intervals during rat liver regeneration. Unexpectedly no decrease but even a little enhancement of the conjugating activity is manifested during the period of rapid proliferation.

In order to exclude the possibility that the conjugating capacity is limited by permeability barriers between substrate or cosubstrate and the microsomal UDPglucuronyltransferase, these *in vitro* studies have been carried out with liver homogenates which were further disintegrated by ultrasonication.[13] The influence of change in membrane permeability on the glucuronidating capacity has been reported previously.[21]

DISCUSSION

From the present results it becomes evident that there is a considerable loss in *p*-hydroxylating and *N*-demethylating activity of the liver from 20 to 60 hr after partial hepatectomy. This decrease, however, cannot be directly related to the rate of division of the parenchymal cells, since already at 16 hr postoperatively the activities of these oxidative drug enzymes were significantly reduced, while cells in mitosis first appeared at 20 hr. Increase of the nuclear DNA content has been observed also at 16 hr (Fig. 1b), which is in agreement with the findings of Fabrikant,[2] who reported that DNA synthesis starts at about 12 hr after partial hepatectomy. Most likely all metabolic alterations during the early stages of liver regeneration are consequences of a common factor, which triggers dormant differentiated cells to entering an active phase of generative cycle. It has been supposed by Swann[22] that while preparing for mitosis cells have to synthesize a number of specialized proteins, e.g. contractile proteins of the mitotic spindle. Since the total amount of protein per gram liver has not remarkably changed during the regeneration, it might be reasonable to assume that the protein-synthesizing apparatus for the most part is serving the cellular proliferation at that stage. This implies that the synthesis of proteins involved in more organ-specific functions like oxidative drug metabolism is falling off during this period of rapid proliferation, resulting in lower concentrations of these drug-oxidizing enzymes. This idea is supported by the finding that the lower enzymatic rates have to be ascribed to reduced concentrations of the catalytic proteins rather than to the presence of an inhibiting agent (Table 2). In addition, enzyme levels are also dependent on the rate of enzyme degradation. It cannot be excluded here that the observed lower *p*-hydroxylating and *N*-demethylating activities are caused by an increase of proteolytic enzyme activity. In this connection the numerous autophagic vacuoles, which have been reported[23, 24] to appear in the cytoplasm of the parenchymal liver cells during the early stages of liver regeneration, may play a part.

From the induction experiments (Fig. 3) it can be concluded that drug metabolizing activities can be enhanced by phenobarbital pretreatment even during a period of rapid liver growth. Recently the responsiveness of regenerating rat liver to inducers of drug metabolism has been studied, too, by Gram *et al.*[25] They reported an increase of drug metabolizing activities in regenerating livers from phenobarbital treated rats at day 6 after partial hepatectomy. It remains to be seen, however, whether that time is representative for the period of rapid regeneration, since—as appears from our results (Fig. 1)—the regeneration process has almost been completed within 6 days.

In contrast to the above decrease of enzymatic activities involved in oxidative drug metabolism during the period of rapid growth, which decrease has been demonstrated previously[5, 8] for other drug-oxidizing enzymes, the conjugating enzyme UDP-glucurontransferase measured with *p*-nitrophenol is not lowered but even a little enhanced during the liver regeneration. This is surprising, since it has been reported that in other types of developing mammalian liver such as fetal and newborn liver[26, 27] UDPglucuronyltransferase activity is much lower than in the adult preparations. In some preliminary investigations, however, we found that in ultrasonicated homogenates from fetal rat liver *p*-nitrophenol UDPglucuronyltransferase activity is equal to that measured in ultrasonicated liver homogenates from adults. This affirms the supposition of Dutton,[26] that it will not be possible to prove that deficient glucuronide synthesis in fetal liver is principally due to deficiency of the catalytic protein UDP-glucuronyltransferase, until developmental studies are done with the solubilized enzyme. Since the relatively low rates of glucuronide synthesis in developing livers, as reported previously, are assayed either *in vivo* or in liver slices or in native liver homogenates, it seems acceptable that the relatively low values are attributable to a reduction of accessibility to the UDPglucuronyltransferase of the substrates. The enhanced activity in later stages may be caused by a change in the intracellular environment of the enzyme during the developmental process.

Acknowledgements—The authors are indebted to Professor Dr. E. J. Ariëns, Institute of Pharmacology, and to Professor Dr. Ch. M. A. Kuyper, Laboratory of Chemical Cytology, University of Nijmegen, for helpful suggestions and criticism in this study. We gratefully acknowledge the technical assistance of Miss D. C. A. van der Linden and Miss C. G. Reynen.

REFERENCES

1. N. L. R. BUCHER, *Intern. Rev. Cytol.* **15**, 245 (1963).
2. J. I. FABRIKANT, *J. Cell Biol.* **36**, 551 (1968).
3. A. VON DER DECKEN and T. HULTIN, *Expl Cell Res.* **14**, 88 (1958).
4. W. BERNHARD and C. ROUILLER, *J. Biophys. Biochem. Cytol.* Suppl. **2**, 73 (1956).
5. A. VON DER DECKEN and T. HULTIN, *Expl Cell Res.* **19**, 591 (1960).
6. C. D. KING and J. L. VAN LANCKER, *Archs Biochem. Biophys.* **129**, 603 (1969).
7. N. FAUSTO, *Biochim. biophys. Acta*, **190**, 193 (1969).
8. J. R. FOUTS, R. L. DIXON and R. W. SHULTICE, *Biochem. Pharmac.* **7**, 265 (1961).
9. R. H. ADAMSON and J. R. FOUTS, *Cancer Res.* **21**, 667 (1961).
10. L. G. HART, R. H. ADAMSON, H. P. MORRIS and J. R. FOUTS, *J. Pharmac. exp. Ther.* **149**, 7 (1965).
11. W. R. JONDORF, R. P. MAICKEL and B. B. BRODIE, *Biochem. Pharmac.* **1**, 352 (1959).
12. G. M. HIGGINS and R. M. ANDERSON, *Archs Path.* **12**, 186 (1931).
13. P. TH. HENDERSON, *Life Sci.* **9**, 511 (1970).
14. J. H. DEWAIDE and P. TH. HENDERSON, *Biochem. Pharmac.* **17**, 1901 (1968).
15. T. NASH, *Biochem. J.* **55**, 416 (1953).
16. J. COCHIN and J. AXELROD, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
17. B. B. BRODIE and J. AXELROD, *J. Pharmac. exp. Ther.* **94**, 22 (1948).
18. K. BURTON, *Biochem. J.* **62**, 315 (1956).
19. F. WANKA, *Planta* **58**, 594 (1962).
20. O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
21. P. TH. HENDERSON and J. H. DEWAIDE, *Biochem. Pharmac.* **18**, 2087 (1969).
22. M. M. SWANN, *Cancer Res.* **18**, 1118 (1958).

23. A. VORBRODT, *Folia Histochem. Cytochem.* **5**, 239 (1967).
24. J. DONIACH and K. WEINBREN, *Br. J. Exp. Path.* **32**, 499 (1953).
25. T. E. GRAM, A. M. GUARINO, F. E. GREENE, P. L. GIGON and J. R. GILLETTE, *Biochem. Pharmac.* **17**, 1769 (1968).
26. G. J. DUTTON in: *Glucuronic Acid* (Ed. G. J. DUTTON) p. 185, Academic Press, New York (1966).
27. G. H. LATHE and M. WALKER, *Biochem. J.* **70**, 705 (1958).