Biochemical Pharmacology, Vol. 23, pp. 1809–1816. Pergamon Press, 1974. Printed in Great Britain.

STUDIES OF GLUCURONIDATION AND SULFATION IN TUMOR-BEARING RATS

T. GESSNER

Department of Experimental Therapeutics and J. T. Grace Jr. Cancer Drug Center, Roswell Park Memorial Institute, New York State Department of Health, Buffalo, N.Y. 14203, U.S.A.

(*Received* 2 *July* 1973; *accepted* 30 *November* 1973)

Abstract—Rats bearing subcutaneous Walker 256 carcinoma or intramuscular Reuber H-35 hepatoma were tested for their ability to metabolize ^{14}C-*p*-nitrophenol into its sulfate and glucuronide. Amounts of *p*-nitrophenyl glucuronide (PNPGA) and *p*-nitrophenyl sulfate (PNPS) excreted by the Walker hosts did not differ significantly from those excreted by the controls. Hepatoma hosts, compared with controls, excreted comparable amounts of PNPS but significantly higher amounts of PNPGA. To clarify the basis for the changes in the conjugate levels in urine of the hepatoma hosts, sulfotransferase and glucuronyl transferase activities of the hepatoma and of host and control livers were assayed. The hepatoma possessed significantly higher glucuronyl transferase activity than either type of liver, but had a very low sulfotransferase activity. Microsomal glucuronyl transferase of the hepatoma obeyed Michaelis–Menten kinetics; K_m value for *p*-nitrophenol is 0·22 mM. Liver transferase(s) gave different and irregular kinetics, suggesting qualitative differences between the transferases of the hepatoma and liver.

KATO *et al.*[1] and Wilson[2] observed that the activity of several drug-metabolizing enzymes is depressed in the liver of tumor-bearing rats. As a consequence, the action of drugs was prolonged (e.g. that of pentobarbital or zoxazolamine) in animals bearing Walker 256 carcinoma,[3,4] Sarcoma 45, Flexner–Jobling carcinoma or T_s Guerin tumor.[5] Both prolongation of drug action and decrease in drug metabolism progressed with the increase in weights of Walker 256.[3] The enzymes affected included pentobarbital oxidase, carisoprodol oxidase, hexobarbital hydroxylase, aminopyrine *N*-demethylase, aniline hydroxylase and nitro reductase.[6]

Detailed studies have revealed that liver microsomal oxidase activity and the amount of hepatic cytochrome CO-binding pigment (P-450) are decreased in rats bearing such neoplasms as pituitary mammotropic tumor,[7] Walker 256 carcinoma,[6] Morris hepatoma 9618A-2, 7800, 7795 or 7787.[8] Additionally, microsomal drug oxidations have been reported to be depressed in the presence of such tumors as Hilf adenocarcinoma[9] and a fibrosarcoma.[10] Besides decreased levels of cytochrome P-450 in tumor-bearing rats, decreased interaction of the cytochrome with such substrates as testosterone, progesterone,[11] hexobarbital, aminopyrine or aniline has been observed.[12]

Recent studies by Boulos *et al.*[10] with parabiotic rats showed that a factor present in the circulation may be responsible for decreased drug metabolism in rats bearing a fibrosarcoma. Also, Bartosek *et al.*[13] have found that blood from Walker 256 tumor-bearing rats could impair pentobarbital metabolism in perfused liver from a normal rat.

Besides the hepatic mixed-function oxidases, other hepatic enzymes are affected by the presence of extrahepatic tumors. For example, lowered liver alanine transaminase activity was observed in rats bearing large (30–100 g) Walker 256 carcinoma tumors.[14] More recently, many liver enzymes of intermediary carbohydrate and amino acid metabolism have been found to be affected.[15]

In view of the profound effects of extrahepatic tumors on the hepatic metabolism of the host, it was of interest to examine the possible influence of such tumors on glucuronidation and sulfation, these being important drug detoxification pathways. In this study, rats bearing subcutaneous Walker 256 tumors or intramuscular hepatoma Reuber H-35 were tested for their ability to metabolize ^{14}C-*p*-nitrophenol into its sulfate and glucuronide.

MATERIALS AND METHODS

Materials

p-Nitrophenol-2,6-^{14}C (16·7 mM) was from ICN Chemical and Radioisotope Division, Irvine, Calif. Uridine diphosphoglucuronic acid (UDPGA), *p*-nitrophenyl glucuronide (PNPGA) and *p*-nitrophenyl sulfate (PNPS) were from Sigma Chemical Co., St. Louis, Mo.

Animals

Male Sprague–Dawley rats, from A. R. Schmidt, Madison, Wis., were used for the experiments with Walker carcinoma 256. This tumor was implanted subcutaneously into the right flank of rats weighing about 180 g and the animals were used for experiments within 2–3 weeks after transplantation, when the weight of the Walker tumor ranged between 33 and 168 g. The Walker tumor line was initially obtained from Dr. K. Sugiura of the Sloan–Kettering Institute in 1958 and, since then, it has been maintained in this Department by subcutaneous passage in Sprague–Dawley rats. The rats bearing Walker tumor used in the experiments reported herein were kindly provided by Dr. Fred Rosen of this Department.

Male ACI rats, from Laboratory Supply Co., Indianapolis, Ind., were used for the experiments with hepatoma. The Reuber H-35 line was obtained from Dr. Bernacki of this Department. It was transplanted by mincing freshly excised tumor in 0·9% NaCl and injecting the 20% mince bilaterally, 0·25 ml into each flank, i.m. The tumor was allowed to grow for about 4–8 weeks before the animals were used for the experiments; tumor weight ranged between 8 and 79 g.

Groups of tumor-bearing rats (hosts) and non-tumor-bearing animals (controls) were maintained under identical conditions. In experiments both *in vivo* and *in vitro* pairs of control and tumor-bearing rats of the same age, strain and lot were tested concurrently.

Experiments in vivo

^{14}C-*p*-nitrophenol (50 mg/kg, 4–20 μCi/rat) was injected i.p. as the Na salt in 0·9% NaCl, in an injection volume equivalent to 1 per cent of body weight. The animals were placed in glass metabolic cages with drinking water *ad. lib.* Urine samples and 24-hr feces samples were collected as indicated.

Assays in vitro

Freshly excised tissue (liver or hepatoma) was homogenized with 3 vol. of 0·154 M KCl in a Teflon–glass homogenizer and the volume was adjusted with KCl to give a 20% (w/v) preparation. To obtain microsomal fractions, the homogenate was centrifuged for 15 min at 15,000 *g*, the supernatant was then centrifuged at 105,000 *g* for 1 hr, and the microsomal pellet was washed by mashing it in KCl (0·154 M) and respinning for 1 hr at 105,000 *g*. The final microsomal fraction was suspended in a volume of 0·154 M KCl equal to the volume of homogenate from which it was derived.

Sulfotransferase. The method of Schneider and Lewbart[16] was modified as follows. The 5′-phosphoadenosyl-3′-phosphosulfate (PAPS) generating system (consisting of 0·014 M ATP, 0·014 M $MgCl_2$, 0·008 M K_2SO_4, 1 mM ascorbic acid, 0·1 M phosphate buffer, pH 6, and 10% liver homogenate) was either incubated concurrently with *p*-nitrophenol or was incubated for 30 min at 37° prior to the addition of 0·5 mM ^{14}C-*p*-nitrophenol. The reaction was allowed to proceed for 30–60 min and was stopped by the addition of 3 vol. of 90% ethanol containing carrier amounts of *p*-nitrophenyl sulfate, followed by freezing. A control and a host liver, and the hepatoma were assayed concurrently.

Glucuronyl transferase. Homogenates or microsomes (equivalent to 0·1 g tissue/ml of incubation medium) were incubated with 0·1 to 1·5 mM ^{14}C-*p*-nitrophenol, 2 mM uridinediphosphoglucuronic acid (UDPGA) and 0·2 M phosphate buffer at pH 7·4. Incubations were carried out for 15 min at 37° and were stopped with 3 vol. ethanol containing some unlabeled *p*-nitrophenylglucuronide as carrier, followed by freezing. A control and a host liver, and the hepatoma were assayed concurrently.

Metabolite detection and estimation

Freshly collected urine was pooled with cage rinsings and adjusted to known volumes (10–20 ml). Samples of the diluted urine were mixed with 3 vol. ethanol containing carrier amounts of *p*-nitrophenol glucuronide and sulfate, and were centrifuged. Aliquots (0·5 ml) of the supernatants were counted in a Packard Tri-Carb liquid scintillation spectrophotometer using a toluene scintillation mixture [4 g 2,5-diphenyloxazole and 0·5 g 1,4-bis-2-(5-phenyloxazolyl)-benzene/liter of toluene and containing 15% of Beckman Bio-Solv formula BBS-3]. Portions of the supernatants were chromatographed on Whatman No. 1 paper, using *n*-butanol–pyridine–water (14:3:3) for separation of the metabolites. Metabolite peaks were detected with the aid of a 4 Pi Tracerlab chromatogram scanner and were identified by coincidence with the ultraviolet-quenching spots of reference compounds. These were cut out and counted in the toluene mixture (without Bio-Solv BBS-3). The counting efficiency for ^{14}C on the immersed paper strips was found to be 43 per cent.

Samples of the ethanolic supernatants of urine were also examined for the presence of any unchanged *p*-nitrophenol using the thin-layer chromatography (TLC) method described earlier.[17]

Supernatants of incubation mixtures were analyzed chromatographically and quantitated as described above for the urine extracts. Extracts of feces were examined similarly after homogenization with ethanol containing carriers and filtration of the mixtures.

RESULTS AND DISCUSSION

In agreement with the increase in host liver size observed by others,[6] significantly increased ratios of liver weight to body weight of rats bearing Walker 256 carcinoma were also noted in the present study (Table 1). In contrast with the profound effects of this tumor on many enzymes of host liver, no significant differences were observed in the amounts of *p*-nitrophenyl glucuronide or sulfate excreted by the host as compared to the control group. *p*-Nitrophenol was rapidly metabolized by all rats, up to 70 per cent of the dose being excreted in urine within the first 3 hr, and up to 95 per cent within 12 hr. No appreciable amounts of unchanged *p*-nitrophenol were excreted; feces contained very little additional radioactivity, hence their metabolite content was not examined. *p*-Nitrophenyl glucuronide was the main urinary metabolite of *p*-nitrophenol. It is interesting that the extent of individual variation in the glucuronide excretion was significantly greater ($P < 0.05$) among the host than the control animals, and this was especially noticeable in the early urine samples (Table 1). This effect cannot be explained at this time, but it raises the possibility that distribution of *p*-nitrophenol or the glucuronide or of both was affected by the presence of the tumor (though no proportionality to tumor weight was discernible). There was no such variability in the sulfate excretion. An unidentified urinary metabolite (X) of ^{14}C-*p*-nitrophenol was detected in both control and host urine. The metabolite was most prominent in the late urine samples, at which time the control samples were seen to contain significantly higher amounts of it than the host samples (Table 1). Excretion of X by the hosts appeared to decrease progressively with increasing tumor

TABLE 1. COMPARISON OF URINARY EXCRETION OF *p*-NITROPHENOL GLUCURONIDE (PNPGA), *p*-NITROPHENOL SULFATE (PNPS) AND AN UNIDENTIFIED METABOLITE X BY NORMAL AND WALKER 256-BEARING RATS GIVEN 50 mg/kg OF ^{14}C-*p*-NITROPHENOL*

	Control	Host	P value†
No of rats	4	4	
Tumor wt range (g)	0	95 ± 65	
Body wt range (g)	254·5 ± 17·4	234·5 ± 16·1	
Liver wt range (g)	11·3 ± 0·9	15·9 ± 3·6	
Ratio liver wt/body wt	0·044 ± 0·001	0·067 ± 0·013	<0·05
PNPGA (% dose)			
0–3 hr urine	41·0 ± 1·6	34·0 ± 21·1	NS
0–6 hr urine	43·0 ± 3·4	47·4 ± 10·8	
0–24 hr urine	49·7 ± 3·9	52·4 ± 9·4	NS
PNPS (% dose)			
0–3 hr urine	22·2 ± 5·7	14·5 ± 3·0	NS
0–6 hr urine	19·1 ± 7·0	20·0 ± 3·5	
0–24 hr urine	21·9 ± 5·2	24·2 ± 3·0	NS
Metabolite X (% dose)			
0–3 hr urine	1·3 ± 0·2	0·7 ± 0·6	NS
0–6 hr urine	5·6 ± 3·9	1·7 ± 0·4	
0–24 hr urine	15·5 ± 2·4	7·3 ± 2·0	<0·002
Ratio PNPGA/PNPS in 0–24 hr urine	2·39 ± 0·66	2·18 ± 0·45	NS
Total radioactivity in 0–24 hr urine (% dose)	92·0 ± 5·0	90·7 ± 9·4	

* Values are means ± S.D.

† P values were computed by Student's *t*-test; control and experimental groups were not significantly different from each other (NS) when P values were >0·05.

weight. *p*-Aminophenol conjugates, amounting to 18 per cent of a dose, have been previously detected in urines of *p*-nitrophenol-treated animals.[18] It is conceivable that X is such a conjugate; the metabolite traveled on chromatograms between *p*-nitrophenyl sulfate and *p*-nitrophenyl glucuronide. Nitro reduction is known to be depressed in Walker 256-bearing rats[6] and this could explain the behavior of X. In any event, X can be regarded as monitoring another pathway of biotransformation of *p*-nitrophenol, one which was clearly influenced by the tumor.

TABLE 2. COMPARISON OF URINARY EXCRETION OF *p*-NITROPHENOL GLUCURONIDE (PNPGA), *p*-NITROPHENOL SULFATE (PNPS) AND AN UNIDENTIFIED METABOLITE X BY CONTROL AND REUBER H-35 HEPATOMA-BEARING RATS GIVEN 50 mg/kg OF ^{14}C-*p*-NITROPHENOL*

	Control	Hosts	P value*
No. of rats	5	5	
Tumor wt range (g)	0	46 ± 30	
Body wt range (g)	204 ± 35	196 ± 55	
Liver wt range (g)	8·5 ± 1·5	9·1 ± 2·4	
Ratio liver wt/body wt	0·041 ± 0·001	0·061 ± 0·009	<0·002
PNPGA (% dose)			
0–3 hr urine	34·2 ± 4·3	56·3 ± 13·4	<0·01‡
0–6 hr urine	45·1 ± 6·2	67·2 ± 4·9	<0·002‡
0–24 hr urine	52·8 ± 5·8	67·6 ± 7·1	<0·01
PNPS (% dose)			
0–3 hr urine	12·0 ± 0·8	12·5 ± 1·0	
0–6 hr urine	16·5 ± 4·5	14·5 ± 2·0	
0–24 hr urine	19·1 ± 4·1	16·3 ± 2·7	NS
Metabolite X (% dose)			
0–3 hr urine	3·4 ± 2·5	0·4 ± 0·1	NS‡
0–6 hr urine	14·3 ± 3·8	1·0 ± 0·4	<0·01‡
0–24 hr urine	18·3 ± 4·6	2·0 ± 0·9	<0·002
Ratio PNPGA/PNPS in 0–24 hr urine	2·83 ± 0·40	4·29 ± 1·05	<0·02
Total radioactivity in 0–24 hr urine (% dose)	91·7 ± 5·9	95·0 ± 5·2	

* Values are means ± S.D.

† P values were computed by Student's *t*-test; control and experimental groups were not significantly different from each other when P values were >0·05.

‡ These values were computed excluding the data for the host of the 8 g tumor.

Reuber hepatoma H-35-bearing rats showed a significantly increased glucuronide excretion in comparison with the corresponding controls (Table 2), without significant changes in the excretion of the sulfate conjugate. The increase in glucuronide excretion was very pronounced, and highly significant, in the case of hosts of 21–81 g tumors; an 8 g tumor produced only a slight increase, but there was no direct proportionality between the tumor weights and the urinary glucuronide levels. Urine of the hepatoma hosts contained much less of the metabolite X than did control urine (Table 2). Again, there was no direct proportionality between this effect and tumor weights, but an 8 g tumor produced less of an effect that the larger tumors.

Increased glucuronide production by the hepatoma-bearing rats could signify that the tumor tissue was contributing to the metabolism of *p*-nitrophenol *in vivo*, or that the host liver had a significantly increased glucuronide conjugating capability, or that a combination of both these factors was at play. Lueders *et al.*[19] found glucuronyl transferase activity in a number of transplantable hepatomas, including Reuber

H-35. However, if metabolism in the hepatoma were the only contributing factor, a proportionality between the tumor weights and conjugate levels would have been expected, notwithstanding the possibility that partial necrotization of tumor tissue could have been obscuring the correlation to some extent. Also, a proportional increase in the sulfate conjugate would have been expected, if the hepatoma simply behaved as additional liver tissue for the purpose of conjugations. Sulfotransferase and glucuronyl transferase activities of control and host livers and hepatoma were examined to clarify the basis for the observed changes in conjugation.

TABLE 3. COMPARISON OF *p*-NITROPHENOL SULFOTRANSFERASE AND GLUCURONYL TRANSFERASE ACTIVITIES IN HOMOGENATES OF HEPATOMA, THE CORRESPONDING HOST LIVER AND CONTROL LIVER*

Enzyme	Enzyme activities (nmoles product/g tissue/min) Hepatoma		Host liver		Control liver
Sulfotransferase	5·9 ± 0·3		20·3 ± 5·6		23·7 ± 10·9
		P < 0·005†		NS†	
Glucuronyltransferase	168 ± 16		76 ± 20		60 ± 24
		P < 0·02		NS	

* Incubations were carried out at 37° as described under Methods. Values are means ± S.D.
† P values were computed by Student's *t*-test; NS means not significantly different from each other when P > 0·05.

As can be seen from the experiments summarized in Table 3, sulfotransferase activity in the hepatoma was very much lower than in either host or control liver, whereas glucuronyl transferase activity was about twice as high in the hepatoma than in the livers. Host and control livers did not differ significantly from each other in either conjugation, although a somewhat elevated glucuronyl transferase activity of host liver was consistently observed. When microsomal preparations of host and control liver, and of hepatoma were assayed for glucuronyl transferase activity, the hepatoma preparations always contained more activity than those from either type of liver (Fig. 1). Moreover, the kinetic behavior of the hepatoma glucuronyl transferase was different from that of either liver and conformed to the usual Michaelis-Menten kinetics. The transferase of both the control and the host liver microsomes behaved alike, giving irregular kinetics suggestive of either a mixture of enzymes or a system with regulatory subunits which is subject to activation at higher substrate concentrations. Thus, the results show that the hepatoma glucuronyl transferase is both quantitatively and qualitatively different from the liver enzyme.

If the host and the control liver glucuronyl transferase activity is due to a mixture of enzymes, the behavior depicted in Fig. 1 can be interpreted to indicate the presence of a low affinity component with a K_m value of about 0·5 mM for *p*-nitrophenol, and a high affinity component with a very much lower K_m value. It is interesting that K_m values of 0·55 to 0·62 mM have been reported for microsomal, or partially purified, *p*-nitrophenol glucuronyl transferase of rat liver.[20,21] The K_m value of the hepatoma glucuronyl transferase, as determined from Fig. 1, is 0·22 mM for *p*-nitrophenol, and the V_{max} range is 8·7 to 13·8 nmoles glucuronide formed/mg of microsomal protein/min.

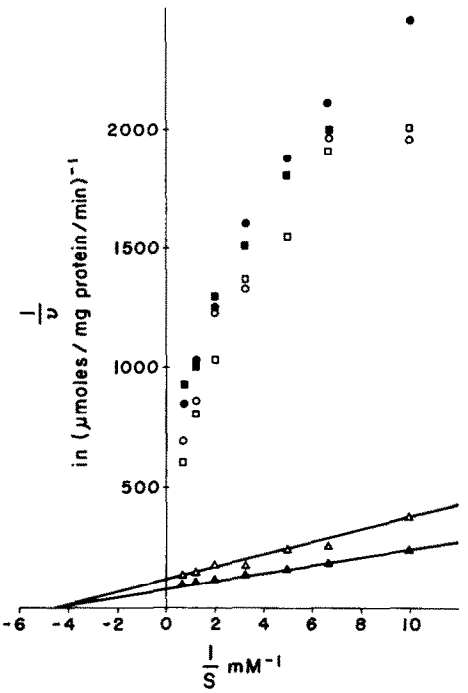

FIG. 1. Double reciprocal plot of v (in μmoles p-nitrophenylglucuronide formed/mg of microsomal protein/min) against S, the mM concentration of p-nitrophenol. ● ○ = Control livers; ■ □ = host livers; ▲ △ = hepatomas, in two experiments represented by open and closed symbols respectively.

In the light of the present results, it seems likely that the major contribution to the increased glucuronide excretion by the hepatoma-bearing hosts comes from the hepatoma tissue itself. The absence of a simultaneous increase in the urinary sulfate can be understood in view of the low sulfotransferase activity found in the hepatoma.

The results reported herein show that host liver glucuronyl and sulfate transferase activity, unlike that of the hepatic mixed-function oxidases, is not markedly affected by the presence of tumor in the host. However, tumors, at least those of liver origin (and possibly others, as indicated by the findings of Dao and Libby[22,23]), can possess varying amounts of conjugating activity and can actively contribute to detoxification by such pathways. The activity may reside in enzymes which may be qualitatively different from those of normal tissues, as our kinetic studies of glucuronyl transferase suggest. It is interesting in this context that Dao and Libby[22,23] have observed that human mammary neoplasms possess a variable pattern of steroid-sulfating activity and that this pattern is different from that in either normal liver of normal breast tissue. In the present study, it was also found that the sulfating activity of the hepatoma is significantly different from that of liver; the decreased activity with respect to p-nitrophenol as substrate could be due to a decreased amount of sulfotransferase in the hepatoma or the presence of an enzyme with a lowered affinity for p-nitrophenol. The above observations raise questions about the possibility that sulfate and glucuronyl transferases of tumors, hepatomas and other neoplasms may be qualitatively different from those of normal tissues.

Acknowledgements—This work was supported by grants CA-14729 and CA-13038 from the National Cancer Institute, United States Public Health Services. Thanks are due to Ms. Diane Bevilaqua for her skilled technical assistance, and to Drs. E. Mihich, F. Rosen and H. Schwartz for their valuable comments.

REFERENCES

1. R. KATO, G. FRONTINO and P. VASSANELLI, *Experientia* **19,** 30 (1963).
2. J. T. WILSON, *Pharmacologist* **9,** 202 (1967).
3. R. KATO, A. TAKANAKA and T. OSHIMA, *Jap. J. Pharmac.* **18,** 245 (1968).
4. R. ROSSO, E. DOLFINI and M. G. DONELLI, *Eur. J. Cancer* **4,** 133 (1968).
5. G. FRANCHI and R. ROSSO, *Biochem. Pharmac.* **18,** 238 (1969).
6. R. KATO, A. TAKANAKA, A. TAKAHASHI and K. ONODA, *Jap. J. Pharmac.* **18,** 224 (1968).
7. J. T. WILSON, *Biochem. Pharmac.* **17,** 1449 (1968).
8. S. K. CHATTOPADHYAY, H. D. BROWN and H. P. MORRIS, *Br. J. Cancer* **26,** 3 (1972).
9. J. T. WILSON, *Endocrinology* **88,** 185 (1971).
10. E. M. BOULOS, M. MACDOUGALL, D. W. SHOEMAN and D. AZARNOFF, *Proc. Soc. exp. Biol. Med.* **139,** 1353 (1972).
11. R. KATO and A. TAKAHASHI, *Cancer Res.* **30,** 2346 (1970).
12. R. KATO, A. TAKANAKA and A. TAKAHASHI, *Gann* **61,** 359 (1970).
13. I. BARTOSEK, A. GUAITANI and M. G. DONELLI, *Biochem. Pharmac.* **21,** 2359 (1972).
14. H. R. HARDING, F. ROSEN and C. A. NICHOL, *Cancer Res.* **24,** 1318 (1964).
15. A. HERTZFELD and D. GREENGARD, *Cancer Res.* **32,** 1826 (1972).
16. J. J. SCHNEIDER and M. L. LEWBART, *J. biol. Chem.* **222,** 787 (1956).
17. T. GESSNER and M. ACARA, *Analyt. Biochem.* **35,** 442 (1970).
18. D. ROBINSON, J. N. SMITH and R. T. WILLIAMS, *Biochem. J.* **50,** 221 (1951).
19. K. K. LUEDERS, H. M. DYER, E. B. THOMPSON and E. L. KUFF, *Cancer Res.* **30,** 274 (1970).
20. E. HALAC, JR. and A. REFF, *Biochim. biophys. Acta* **139,** 328 (1967).
21. G. J. MULDER, *Biochem. J.* **117,** 319 (1970).
22. T. L. DAO and P. R. LIBBY, *J. Clin. Endocr.* **28,** 1431 (1968).
23. T. L. DAO and P. R. LIBBY, *Surgery* **66,** 162 (1969).