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METABOLIC ACTION OF URETHAN ON PROTEIN SYNTHESIS AND DRUG OXIDATION IN NORMAL AND REGENERATING LIVER*

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Abstract—Administration of the carcinogen urethan to sham-operated rats resulted in a dose-dependent increase in the synthesis of protein on membrane-bound polyribosomes and to a lesser degree on free polyribosomes 6 hr after drug. Maximal elevation of the specific radioactivity of nascent peptide synthesized on both classes of polyribosomes of sham-operated animals occurred 6–12 hr after the administration of urethan (1 g/kg). Animals injected with urethan 12 hr after partial hepatectomy showed no increase in protein synthesis on membrane-bound polyribosomes above that occurring in saline-treated rats 2–24 hr after urethan and only a slight elevation in peptide synthesis on free polyribosomes 12 hr after the carcinogen. Aniline hydroxylation was elevated 2-fold 12–24 hr after the administration of urethan (1 g/kg) to sham-operated animals, while little or no change occurred in: (1) rate of demethylation of aminopyrine, (2) NADPH cytochrome *c* reductase activity, and (3) levels of cytochrome b_5 and cytochrome P-450. Mixed-function oxidase activities in partially hepatectomized animals injected with urethan 12 hr after the operation were either unaffected or increased to a lesser degree than in sham-operated animals.

URETHAN (ethyl carbamate) has been reported to be a potent liver carcinogen in 70 per cent hepatectomized mice, producing hepatomas in a large percentage of treated animals.[1–3] This compound is particularly useful for investigating the means by which a carcinogenic agent alters normal functional processes, since it produces only minimal damage to liver parenchyma.[4] A number of investigations have indicated that chemical carcinogens or their reactive metabolites have the ability to interact with macromolecules such as nucleic acids and proteins.[5,6] Several reports have shown that hepatocarcinogens such as 2,7-diaminofluorene, aflatoxin B_1 and azo-dyes interact with the endoplasmic reticulum of the liver and thereby affect polysome–membrane interactions,[7–11] and that metabolites of urethan bind to nucleic acids and proteins of the liver *in vivo*.[12,13] In view of the ability of urethan to decrease the hexobarbital sleeping time of normal animals[14] and of partial hepatectomy to prolong the hypnotic effects of urethan,[15] the present investigation was undertaken to investigate the action of urethan on polyribosome–membrane interactions through an analysis of: (1) the synthesis of nascent protein on free and membrane-bound

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polyribosomes, which appears to be obligatory for the induction of drug-metabolizing enzymes; and (2) mixed-function oxidase activities associated with the endoplasmic reticulum of the liver.

MATERIALS AND METHODS

Materials. Aminopyrine was purchased from Matheson, Coleman & Bell, and urethan, cytochrome *c* (type VI) and NADPH from Sigma Chemical Co. Aniline was obtained from Merck and was distilled under vacuum before use. [4,5-^{3}H]-L-leucine (55 c/m-mole) was purchased from New England Nuclear Corp. All other chemicals were of reagent grade and glass-distilled water was used.

Preparation of animals. Male Sprague–Dawley rats (Charles River Laboratories, Inc.) weighing 175–225 g were employed in all experiments. Animals were housed over corn cob bedding and alternating periods of 12 hr dark and 12 hr light were maintained. Purina rat chow and water were available *ad lib.*

Partial hepatectomies and sham operations were performed under ether anesthesia between 6.00 and 8.00 p.m. according to the method of Higgins and Anderson.[16] No diurnal variations were noted in any of the biochemical parameters measured in these experiments.

Urethan was dissolved in 0·9% NaCl and injected intraperitoneally 12 hr after partial hepatectomy or sham operation; control animals received an equivalent volume (approximately 1·0 ml) of 0·9% NaCl.

[4,5-^{3}H]-L-leucine was diluted in 0·9% NaCl and injected into the portal vein after laporotomy at a dose of 100 μc/kg.

Preparation of hepatic polyribosomes. After [^{3}H]-leucine was allowed to incorporate for 3 min, the liver was rapidly excised and placed into a beaker of ice-cold 0·25 M sucrose containing 50 mM Tris-HCl (pH 7·8), 50 mM KCl and 5 mM $MgCl_2$ (TKM). Liver homogenates (4 g liver in 8 ml of 0·25 M sucrose–TKM) were prepared using ten strokes of a motor driven glass-Teflon tissue grinder operating at 2000 rev/min. Homogenates were centrifuged at 12,000 *g* for 20 min at 4° and the overlying layer of fat was removed. Free and membrane-bound polyribosomes were prepared on a discontinuous sucrose gradient without the use of deoxycholate by the method of Blobel and Potter.[17] Five ml of the 12,000 *g* supernatant was layered over a discontinuous gradient of 4 ml of 0·5 M sucrose–TKM over 4 ml of 2·0 M sucrose–TKM. Centrifugation was carried out in a Spinco SW 40 Ti rotor for 2 hr at 284,000 *g*. Free polyribosomes were obtained as a pellet and bound polyribosomes as a band at the 0·5–2·0 M sucrose–TKM interface. Aliquots of free and bound polyribosomes were heated at 90° for 30 min with 1 ml of 15% trichloroacetic acid. Precipitated protein was collected by filtration on paper discs, washed twice with 10 ml of cold 15% trichloroacetic acid, twice with 10 ml of absolute alcohol and air dried. Protein was solubilized by heating the discs at 60° for 20 min with 1 ml of Protosol (New England Nuclear Corp.) and radioactivity was determined after addition of 10 ml of Liquifluor scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer with a counting efficiency of 30 per cent.

Protein was determined by the method of Lowry *et al.*[18]

Assays. Hepatic microsomes were prepared as previously described.[19] NADPH cytochrome *c* reductase (EC 1.6.2.3) was determined by the method indicated previously,[19] and aminopyrine demethylation and aniline hydroxylation were determined

by the procedure of Schenkman *et al.*[20] Cytochrome b_5 and cytochrome P-450 were assayed spectrophotometrically as reported by Omura and Sato[21] and Schenkman *et al.*,[20] respectively.

RESULTS

Partial hepatectomy caused a pronounced increase in the rate of synthesis of protein with a 3·5-fold elevation occurring on membrane-bound polyribosomes and a 2·5-fold increase in the formation of nascent peptide taking place on free polyribosomes of liver 12–18 hr after the operation (Fig. 1). The increase in specific radioactivity of nascent protein on both populations of polyribosomes remained elevated by approximately 200 per cent for up to 36 hr after partial hepatectomy.

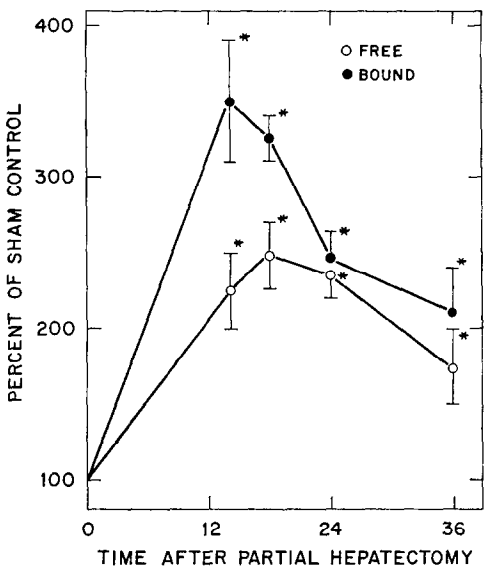

FIG. 1. Incorporation of [^{3}H]-leucine into nascent protein synthesized on free and membrane-bound polyribosomes of regenerating liver. The specific radioactivities of free and membrane-bound polyribosomes were determined as described in Materials and Methods. Each point represents the mean ±S.E. of results obtained from the separate analyses of preparations from four animals. Control activities (Counts/min/mg protein) were 1100 ± 200 for free polyribosomes and 1240 ± 150 for bound polyribosomes. Asterisk (*) indicates statistical significance ($P < 0·05$) when compared with sham-operated controls.

A pronounced increase occurred in the labeling of nascent protein synthesized on free and membrane-bound polyribosomes 6–12 hr after the administration of urethan to sham-operated animals (Fig. 2). In contrast, injection of the carcinogen 12 hr after partial hepatectomy resulted in no significant alteration of the operation-induced increased incorporation of [^{3}H]-leucine into peptide on membrane-bound polyribosomes and only a 50 per cent increase on free polyribosomes 12 hr after urethan.

A dose-dependent increase in the degree of pulse-labeling of nascent protein on membrane-bound polyribosomes occurred following urethan administration to sham-operated animals (Fig. 3); however, no demonstrable effect of urethan upon peptide synthesis on free polyribosomes was produced at doses lower than 1 g/kg, in contrast

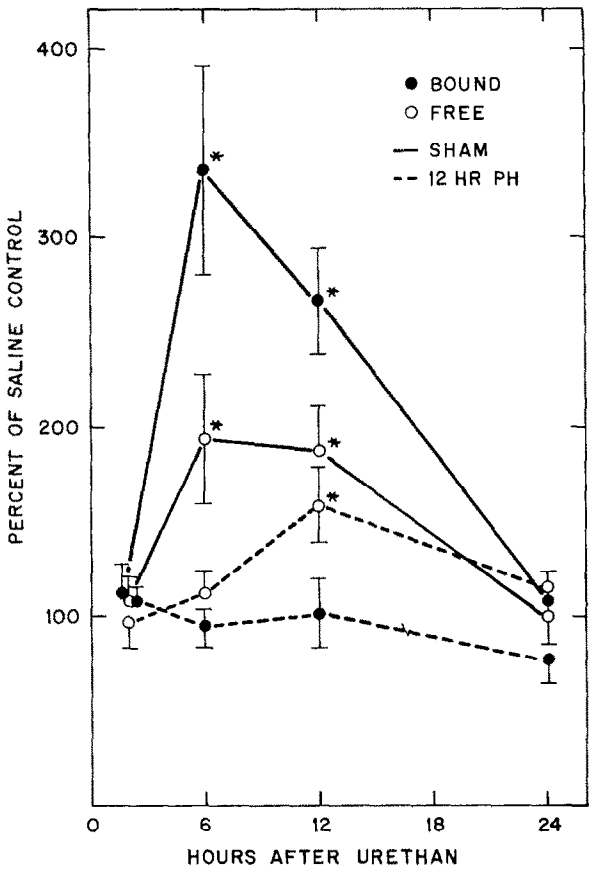

FIG. 2. Effect of urethan on the incorporation of [^{3}H]-leucine into nascent protein on free and membrane-bound polyribosomes of normal and 12-hr regenerating liver. See text for experimental details. Each point represents the mean ±S.E. of results obtained from the separate analyses of preparations from five to eight animals. Asterisk (*) indicates statistical significance ($P < 0.05$) when compared with saline-treated controls. PH = partial hepatectomy.

to the 2- to 2·5-fold increase in labeling observed with membrane-bound polyribosomes at doses of 0·5–0·75 g/kg of urethan.

Analysis of the effects of the carcinogen upon drug oxidation in sham-operated animals revealed that aniline hydroxylation increased significantly as early as 6 hr after injection of urethan and reached maximal induction by 12 hr (Table 1). In contrast, no significant increase in the rate of either aminopyrine demethylation or NADPH cytochrome *c* reductase occurred until 24 hr after administration of urethan.

Treatment of partially hepatectomized animals with urethan 12 hr after surgery resulted in an approximately 60–70 per cent lower induction of aniline hydroxylation than took place in sham-operated rats (Table 2). Neither NADPH cytochrome *c* reductase nor aminopyrine demethylation was significantly elevated by urethan in partially hepatectomized animals.

The response of aniline hydroxylation to different doses of inducer was investigated (Fig. 4); the results indicated that approximately a 200 per cent increase in enzyme

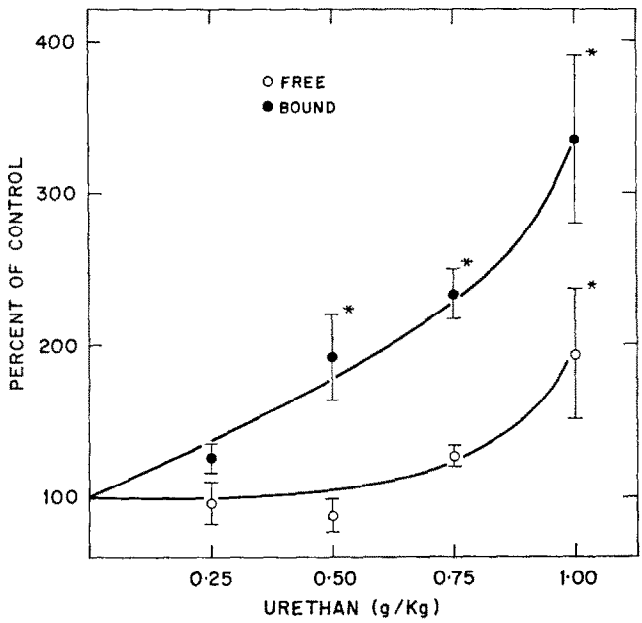

FIG. 3. Effect of various doses of urethan on incorporation of [^{3}H]-leucine into nascent protein on free and membrane-bound polyribosomes of normal liver. See text for experimental details. Each point represents the mean ±S.E. of results obtained from the separate analyses of preparations from four animals. Control activities (Counts/min/mg protein) were 900 ± 120 for free polyribosomes and 1500 ± 250 for bound polyribosomes. Asterisk (*) indicates statistical significance ($P < 0{\cdot}05$) when compared with saline-treated controls. Measurement of the specific radioactivities of protein on free and membrane-bound polyribosomes was made 6 hr after treatment with urethan.

activity occurred at 24 hr after the administration of 0·6–1·5 g/kg of urethan. It should be noted that drug toxicity was evident at a dose of 1·5 g urethan/kg, resulting in approximately 50 per cent mortality; however, no deaths occurred at the lower doses used. NADPH cytochrome *c* reductase was affected to a lesser degree than aniline hydroxylation, showing a 25–64 per cent increase in activity at 0·6–1·5 g/kg of urethan.

DISCUSSION

A number of carcinogens stimulate specific microsomal mixed-function oxidase activities. Polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, 3,4-benzpyrene and azo-dyes initiate the induction of azodye demethylation,[22–25] azo-dye reductase,[22] zoxazolamine hydroxylation[23] and aniline hydroxylation,[26] while having little effect on ethylmorphine demethylation,[25] aminopyrine demethylation[27] and NADPH cytochrome *c* reductase.[27,28]

The action of urethan as an inducer of the mixed-function oxidase system in sham-operated animals appears to be unique in several important respects: (1) aniline hydroxylation is stimulated at an earlier time after administration of urethan than is NADPH cytochrome *c* reductase; (2) the induction of aniline hydroxylation and NADPH cytochrome *c* reductase activities is accompanied by a marginal change in aminopyrine demethylation and no significant alteration in the level of cytochrome P-450. These results pose several interesting questions concerning the number and

TABLE 1. NADPH CYTOCHROME *c* REDUCTASE, AMINOPYRINE *N*-DEMETHYLATION AND ANILINE HYDROXYLATION ACTIVITIES AFTER URETHAN TREATMENT OF SHAM-OPERATED RATS*

Activity	Urethan treatment	Specific activity (nmoles/min/mg microsomal protein) 6 hr†	12 hr	24 hr	48 hr
NADPH cytochrome *c* reductase	−	81·6 ± 6·0	69·5 ± 2·6	74·3 ± 3·6	70·0 ± 2·9
	+	87·9 ± 1·7 (108)	69·2 ± 4·7 (100)	120·1 ± 8·5 (162)‡	103·0 ± 7·6 (148)‡
Aminopyrine demethylation	−	8·9 ± 0·5	10·8 ± 0·5	9·8 ± 0·6	8·1 ± 0·1
	+	10·0 ± 0·3 (112)	8·7 ± 0·2 (81)‡	12·6 ± 0·2 (129)‡	10·0 ± 1·2 (124)
Aniline hydroxylation	−	0·60 ± 0·07	0·55 ± 0·06	0·71 ± 0·03	0·78 ± 0·09
	+	0·84 ± 0·06 (140)‡	1·15 ± 0·10 (209)‡	1·40 ± 0·09 (196)‡	1·36 ± 0·05 (174)‡

* Rats received a single intraperitoneal injection of urethan (1 g/kg) or an equivalent volume of 0·9% sodium chloride 12 hr after sham operation, and NADPH cytochrome *c* reductase, aminopyrine *N*-demethylation and aniline hydroxylation activities were measured at the indicated times thereafter. NADPH cytochrome *c* reductase activity is expressed as nanomoles of reduced cytochrome *c* per minute per milligram of protein, aminopyrine *N*-demethylation as nanomoles formaldehyde per minute per milligram of protein and aniline hydroxylation as nanomoles *p*-aminophenol per minute per milligram of protein. Each value represents the mean ±S.E. of results obtained from the separate analyses of microsomal preparations from three (saline-injected) or five (urethan-injected) rats.

† Time after urethan or 0·9% sodium chloride.

‡ Statistically significant ($P < 0·05$) from saline-injected sham-operated rats. Numbers in parentheses represent percentages relative to appropriate saline-injected rats taken as 100 per cent.

TABLE 2. NADPH CYTOCHROME *c* REDUCTASE, AMINOPYRINE *N*-DEMETHYLATION AND ANILINE HYDROXYLATION ACTIVITIES AFTER URETHAN TREATMENT OF PARTIALLY HEPATECTOMIZED RATS*

Activity	Urethan treatment	Specific activity (nmoles/min/mg microsomal protein) 12 hr†	24 hr	48 hr
NADPH cytochrome *c* reductase	−	68·2 ± 6·8	64·9 ± 9·5	59·7 ± 4·3
	+	79·8 ± 6·5 (117)	66·7 ± 6·6 (103)	70·1 ± 2·0 (117)
Aminopyrine demethylation	−	6·3 ± 0·8	4·6 ± 0·9	4·7 ± 0·1
	+	6·8 ± 0·6 (108)	5·3 ± 0·3 (115)	4·0 ± 0·4 (85)
Aniline hydroxylation	−	0·59 ± 0·04	0·61 ± 0·05	0·61 ± 0·04
	+	0·80 ± 0·10 (136)‡	0·84 ± 0·08 (138)‡	0·70 ± 0·05 (115)

* Rats received a single intraperitoneal injection of urethan (1 g/kg) or an equivalent volume of 0·9% sodium chloride 12 hr after partial hepatectomy, and NADPH cytochrome *c* reductase, aminopyrine *N*-demethylation and aniline hydroxylation activities were measured at the indicated times thereafter. Each value represents the mean ±S.E. of results obtained from the separate analyses of microsomal preparations from three (saline-injected) or five (urethan-injected) rats.

† Time after urethan or 0·9% sodium chloride.

‡ Statistically significant ($P < 0·05$) from saline-injected partially hepatectomized rats. Numbers in parentheses represent percentages relative to appropriate saline-injected rats taken as 100 per cent.

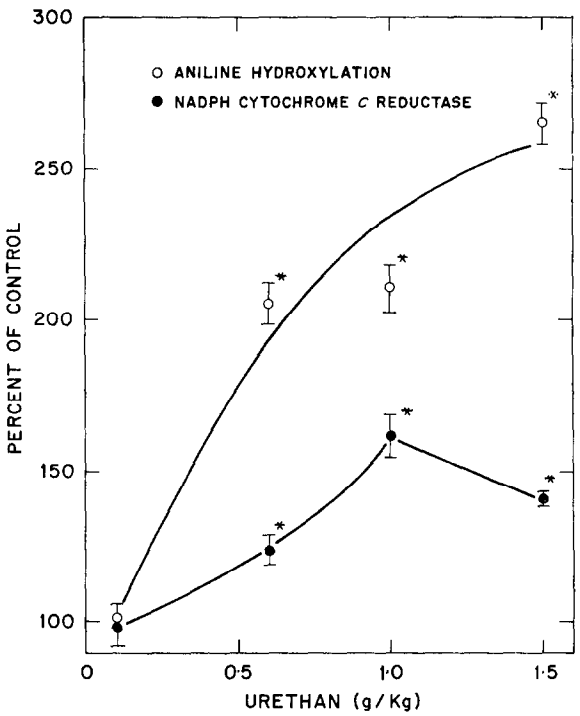

FIG. 4. Activities of aniline hydroxylation and NADPH cytochrome *c* reductase of normal liver 24 h after the administration of different doses of urethan. See text for experimental details. Each poin represents the mean ±S.E. of results obtained from the separate analyses of three animals. Contro activities (nmoles/min/mg microsomal protein) were 100 ± 4 for NADPH cytochrome *c* reductase and 0·83 ± 0·03 for aniline hydroxylation. Asterisk (*) indicates statistical significance ($P < 0·05$) when compared with saline-treated controls.

function of the oxidative pathways involved in drug metabolism in the liver. Our data suggest that urethan may uncouple aniline hydroxylation from aminopyrine demethylation or possibly stimulate a separate pathway involved in the ring oxidation of primary aromatic amines. Since the latter activity was temporarily dissociated from changes in NADPH cytochrome *c* reductase activity, aminopyrine demethylation and the level of cytochrome P-450 (Table 1), the second possibility is favored. In agreement with the concept of a separate pathway for aniline hydroxylation are: (1) the greatly different activation energies observed for aniline hydroxylation and aminopyrine demethylation,[29] and (2) the different effects that polycyclic aromatic hydrocarbons and starvation have on these oxidative pathways.[26,27,30]

In addition, we have found that urethan does not alter the levels of cytochromes b_5 or P-450 or exhibit any type of binding spectrum (unpublished results), which is a characteristic of many drugs oxidized via the mixed-function oxidase system.[20] Urethan also does not influence the binding of aniline or aminopyrine to microsomes or the rate of reduction by NADPH of cytochrome P-450 in the presence or absence of aniline or aminopyrine (unpublished results). Thus, it appears that urethan has the unique ability to stimulate the hydroxylation of aniline and, to a lesser extent, the NADPH-mediated reduction of cytochrome *c* without influencing the spectral

characteristics and concentration of the terminal oxidase of the microsomal electron transport system(s).

The mechanism by which urethan stimulates nascent protein synthesis on free and membrane-bound polyribosomes is uncertain. The ability of several carcinogens to cause deaggregation of membrane-bound polyribosomes[8] indicates that they have the potential to interfere with normal polyribosome function. However, the increase in synthesis of nascent peptide on both populations of polyribosomes after urethan treatment of sham-operated animals indicates that interference with the integrity of the polyribosome–membrane complex is probably not involved in the action of this carcinogen. On the other hand, the temporal relationship between the elevation of protein synthesis and the induction of enzyme activities indicates that these processes may be interrelated. The greater response in the synthesis of protein on membrane-bound polyribosomes following administration of urethan (Figs. 2 and 3) also suggests that bound polyribosomes may be preferentially involved in the synthesis of specific mixed-function oxidase activities. These findings are similar to results obtained with phenobarbital* and suggest that protein synthesis is obligatory for induction of drug-metabolizing enzymes.

Imposition of the stress of partial hepatectomy upon animals subsequently treated with urethan resulted in a diminished capacity of the carcinogen to elevate the rate of synthesis of protein on free and membrane-bound polyribosomes and to increase mixed-function oxidase activities. The findings are in agreement with the concepts of previous reports from this laboratory showing the competitive effects of hepatic cellular proliferation upon the ability of the normal liver cell to respond to the functional demands of an inducer such as phenobarbital or 3-methylcholanthrene.[19,31,32]

Urethan is rapidly decarboxylated in normal and partially hepatectomized animals where complete elimination of drug is achieved within 24 hr.[33–36] However, the 5- to 6-fold greater retention of [ethyl-1-^{14}C]-urethan compared to [carboxy-1-^{14}C]-urethan,[37] as well as the sustained labeling for 5 days by [ethyl-2-^{3}H]-urethan of both nuclear and cytoplasmic proteins of rat liver,[12] suggests that metabolites of urethan are retained by the liver for a considerable time. Although urethan had a marked ability to interact with the mixed-function oxidase system, our data do not rule out the possibility that a metabolite of urethan mediates the effects noticed in our experiments.

Since the regenerating liver is in a state of hyperplasia, the increased requirement for proteins involved in replication results, as shown in the present report, in an elevated rate of protein synthesis on both free and bound polyribosomes. The apparent competitive process of growth and enzyme induction, a functional process, may reside, at least in part, at the level of translation.

REFERENCES

1. C. F. HOLLANDER and P. BENTVELZEN, *J. natn. Cancer Inst.* **41,** 1303 (1968).
2. I. N. CHERNOZEMSKI and G. P. WARWICK, *Cancer Res.* **30,** 2685 (1970).
3. M. LANE, A. LIEBELT, J. CALVERT and R. A. LIEBELT, *Cancer Res.* **30,** 1812 (1970).
4. I. N. CHERNOZEMSKI and G. P. WARWICK, *Chem. Biol. Interact.* **2,** 29 (1970).
5. E. C. MILLER and J. A. MILLER, *Pharmac. Rev.* **18,** 805 (1966).
6. E. FARBER, *Cancer Res.* **28,** 1859 (1968).
7. D. J. WILLIAMS and B. R. RABIN, *Fedn Eur. Biochem. Soc. Lett.* **4,** 103 (1969).
8. D. J. WILLIAMS and B. R. RABIN, *Nature, Lond.* **232,** 102 (1971).

* R. I. Glazer and A. C. Sartorelli, unpublished observations.

9. A. E. M. McLean and E. K. McLean, *Br. med. Bull.* **25,** 278 (1969).
10. T. Hultin, *Expl. Cell Res.* **10,** 71 (1956).
11. S. Fiala and A. E. Fiala, *Br. J. Cancer* **13,** 236 (1959).
12. G. Prodi, P. Rocchi and S. Grilli, *Cancer Res.* **30,** 2887 (1970).
13. E. Boyland and K. Williams, *Biochem. J.* **111,** 121 (1969).
14. J. M. Fujimoto, D. E. Blickenstaff and F. W. Schueler, *Proc. Soc. exp. Biol. Med.* **103,** 463 (1959).
15. F. Antal, T. Lajos and V. Istvan, *Kiscérlo Orvostud.* **15,** 555 (1963).
16. G. M. Higgins and R. M. Anderson, *Arch. Path.* **12,** 186 (1931).
17. G. Blobel and V. R. Potter, *J. molec. Biol.* **26,** 279 (1967).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
19. R. I. Glazer and A. C. Sartorelli, *Biochem. Pharmac.* **20,** 3521 (1971).
20. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3,** 113 (1967).
21. T. Omura and R. Sato, *J. biol. Chem.* **239,** 2370 (1964).
22. E. C. Miller, J. A. Miller, R. R. Brown and J. C. MacDonald, *Cancer Res.* **18,** 469 (1958).
23. J. C. Arcos, A. H. Conney and N. P. Buu-Hoi, *J. biol. Chem.* **236,** 1291 (1961).
24. C. J. Parli and G. J. Mannering, *Molec. Pharmac.* **6,** 178 (1970).
25. N. E. Sladek and G. J. Mannering, *Molec. Pharmac.* **5,** 174 (1969).
26. D. W. Shoeman, M. D. Chaplin and G. J. Mannering, *Molec. Pharmac.* **5,** 412 (1969).
27. H. Glaumann, *Chem. Biol. Interact.* **2,** 369 (1970).
28. R. I. Glazer, J. B. Schenkman and A. C. Sartorelli, *Molec. Pharmac.* **7,** 683 (1971).
29. J. B. Schenkman, *Molec. Pharmac.* **8,** 178 (1972).
30. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **150,** 279 (1965).
31. J. Hilton and A. C. Sartorelli, *J. biol. Chem.* **245,** 4187 (1970).
32. J. Hilton and A. C. Sartorelli, *Adv. Enzyme Regulat.* **8,** 153 (1970).
33. A. M. Kaye, *Cancer Res.* **20,** 237 (1960).
34. S. Mirvish, G. Cividalli and I. Berenblum, *Proc. Soc. exp. Biol. Med.* **116,** 265 (1964).
35. D. E. Grogan, M. Lane, R. A. Liebelt and F. E. Smith, *Cancer Res.* **30,** 1806 (1970).
36. E. Boyland and E. Rhoden, *Biochem. J.* **44,** 528 (1949).
37. H. E. Skipper, L. L. Bennett, Jr., C. E. Bryan, L. White, Jr., M. A. Newton and L. Simpson, *Cancer Res.* **11,** 46 (1951).