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METABOLIC ACTIVATION AND CYTOTOXICITY OF CYCLOPHOSPHAMIDE IN PRIMARY CULTURES OF POSTNATAL RAT HEPATOCYTES*

DANIEL ACOSTA† and DAVID B. MITCHELL
Department of Pharmacology, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712, U.S.A.

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Abstract—We have developed a model of primary cultures of postnatal rat hepatocytes to characterize the metabolic activation of xenobiotics to toxic intermediates and to study the mechanism(s) by which these chemicals produce cellular injury. This model was employed to investigate the cytochrome P-450 mediated biotransformation of cyclophosphamide (CP) to cytotoxic metabolites that nonspecifically alkylate DNA and cellular proteins. The parenchymal cells were isolated by an *in situ* collagenase perfusion technique and cultured for 24 hr prior to drug treatment. The cultures were then exposed to CP concentrations ranging from 1×10^{-4} M to 1×10^{-3} M for 24 hr. Initial studies indicated minimal toxicity to non-replicating parenchymal hepatocytes maintained in ornithine-supplemented, arginine-deficient medium. The addition of arginine permitted the overgrowth of fibroblasts in the same culture system. These fibroblasts then became the target of alkylating CP metabolites produced by the parenchymal cells. By day 3 after CP administration, cell number and total protein per dish decreased by over 40 percent. The morphology of the cultures changed dramatically because of fibroblast destruction. The cytotoxicity to dividing fibroblasts was eliminated by administering 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525-A), an inhibitor of the cytochrome P-450 monooxygenase system, to the co-cultures treated with CP. The alkylating metabolites of CP produced by the parenchymal cells and released into the culture medium were quantitated by reacting aliquots of medium from CP-treated cells with 4-(*p*-nitrobenzyl)pyridine. These results provide both direct and indirect evidence of drug metabolism in cultured cells and suggest that this co-culture system can be utilized to evaluate the metabolic activation of xenobiotics.

Mammalian cells in culture have been proposed as experimental models to help elucidate the mechanism(s) by which xenobiotics produce cytotoxicity [1, 2]. In recent years, a significant body of information has accumulated on the role of metabolism in the activation of foreign compounds to toxic metabolites that may be carcinogenic, mutagenic, or teratogenic, or that may be responsible for tissue injury and necrosis [3–6].

The value of an *in vitro* system as a probe for evaluating potentially hazardous chemicals is enhanced significantly if it has a functional cytochrome P-450 monooxygenase system [7, 8]. Cellular systems that are developed for use in biochemical toxicology ideally should have the internal capacity to metabolize the compounds which are under investigation [8, 9]. Establishment of hepatic culture systems with metabolic activity has been one of the more elusive problems of tissue culture. However, recent reports on isolated adult hepatocyctes [10, 11] and primary adult hepatocyte cultures [12–16] have indicated the capability of these systems to metabolize various classes of xenobiotics and, therefore, may be useful as experimental tools in biochemical toxicology. Studies in our laboratory with primary cultures of postnatal rat hepatocytes have demonstrated their ability to take up and metabolize xenobiotics [17, 18]. Although postnatal rats are generally thought to have lower drug-metabolizing activities and cytochrome P-450 concentrations than adult rats, we have demonstrated recently that cultures of postnatal hepatocytes had concentrations of cytochrome P-450 similar to those of cultured adult hepatocytes [19–22]. More recently we have reported that postnatal rat hepatocytes in culture may serve as experimental models to evaluate the direct cytotoxicity of hepatotoxic agents [23–25] and have suggested that these cultures may be of value in examining agents that require metabolic activation to express their toxicity [24].

The purpose of the present study was to provide more direct evidence that our culture sytem of postnatal hepatocytes could metabolically activate an agent before its toxicity might be expressed and that the toxicity of the formed metabolite(s) could be evaluated by our system. Because of the reported ambiguity over the drug-metabolizing capability of postnatal rat hepatocytes in comparison to adult hepatocytes [26–29], we conducted a study similar to one that had been reported previously with a co-culture system of adult hepatocytes and fibroblasts [14]. The parenchymal hepatocytes served as the metabolizing units and the fibroblasts as the

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† Author to whom all correspondence should be addressed.

targets of the toxic metabolite(s). Cyclophosphamide was chosen as the test agent because its metabolic conversion by the cytochrome P-450 microsomal system is well known [30–32] and because the formation of metabolites which alkylate nonspecifically to DNA and other macromolecules may be the possible mechanism of its cytotoxicity [33–35]. The results demonstrate that non-replicating hepatocytes maintained in culture are effective cell models to study metabolism-mediated cytotoxicity of xenobiotics.

MATERIALS AND METHODS

Reagents. Mechlorethamine HCl, 4(*p*-nitrobenzyl)pyridine, and other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO. Culture medium and serum were purchased from the Grand Island Biological Co., Grand Island, NY.

Culture procedure. The isolation and primary culture of parenchymal hepatocytes from 10- to 11-day-old Sprague–Dawley rats has been described in detail elsewhere [17]. In brief, the procedure utilized an *in situ* perfusion technique of the liver with 0.05% (w/v) collagenase in Ca^{2+}-free Hanks' balanced salt solution to dissociate the tissue into individual cells in suspension. About 5×10^6 cells were plated into individual 60 × 15 mm plastic culture dishes (Falcon, Cockeysville, MD). The cells were cultured in Dulbecco–Vogt's modification of Eagle's minimum essential medium containing 0.1% (w/v) bovine serum albumin and 10% (v/v) newborn bovine serum and this medium was changed every 24 hr. The cultures were placed in a humidified environment of 5% CO_2–95% air and 37° (model CO-20 CO_2 incubator, New Brunswick Scientific, New Brunswick, NJ). Culture medium deficient in arginine but supplemented with 1×10^{-4} M ornithine prevented the overgrowth of the parenchymal hepatocytes with fibroblast and other nonparenchymal cells [36], and thus relatively pure cultures of parenchymal hepatocytes were obtained. The addition of 200 mg/liter arginine permitted the overgrowth of fibroblasts in the same culture system, and these cultures were designated as "co-cultures". Pure cultures of fibroblasts were obtained by taking advantage of the sequential attachment of the various liver cell types to the plastic culture dishes [37]. Fibroblasts attached to the culture dishes in the first 40 min after plating of the cell suspension, after which the culture medium was poured off to eliminate the parenchymal hepatocytes which had not become attached to the culture dish [38].

Drug treatments. Cyclophosphamide (CP*, Mead Johnson & Company, Evansville, IN) was dissolved directly into the culture medium to prepare concentrations ranging from 1×10^{-4} M to 1×10^{-3} M. Three-ml portions of each solution were added to individual dishes of 1-day-old cultures described above. The cells were exposed to CP-containing medium for 24 hr, and fresh drug-free medium was changed thereafter every 24 hr.

* Abbreviations: CP, cyclophosphamide; LDH, lactate dehydrogenase; NBP, 4-(*p*-nitrobenzyl)pyridine; 4-OHCP, 4-hydroxycyclophosphamide; and SKF 525-A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride.

Assays. The total amount of protein per culture dish was determined by the Folin phenol method described by Lowry *et al.* [39]. Cell cultures were stained with hematoxylin to enhance the clarity of nuclei [40] for cell counts. Leakage of the intracellular enzyme, lactate dehydrogenase (LDH), was measured in the medium as an index of cytotoxicity according to procedures previously described [23].

Colorimetric determination of alkylating activity. A method useful for the colorimetric assay of alkylating substances in biological fluids first described by Epstein *et al.* [41] and modified by Friedman and Boger [42] was utilized to estimate the quantity of alkylating CP metabolites produced by the parenchymal hepatocytes. In this reaction, NBP is alkylated on the nitrogen of the pyridine ring by the substance to be assayed. A 2-ml aliquot of culture medium from CP-treated parenchymal hepatocytes was deproteinized with 1 ml of 5.5% (w/v) aqueous solution of $ZnSO_4 \cdot 7H_2O$, followed by 1 ml of 4.0% (w/v) aqueous $Ba(OH)_2 \cdot 8H_2O$. The resulting solution was centrifuged at 500 *g* for 10 min and 2 ml of the supernatant fraction were used for the assay. Each 2-ml aliquot was placed in a culture tube with 1 ml of 0.2 M acetate buffer (pH = 4.0). After agitation of the sample, 0.4 ml of NBP in acetone (50 mg/ml) was added to each tube. The tubes were placed in a 100° bath for 20 min to complete the reaction between the metabolites and NBP. The samples were allowed to cool and 7 ml of a 5:2 (v/v) mixture of ethyl acetate and acetone was added to each tube. The color was developed by the addition of 1.5 ml of 5 M NaOH and each tube was shaken vigorously. The tubes were centrifuged briefly to facilitate separation of the organic and aqueous phases, and the absorbance of the organic phase was read at 540 nm in a Beckman model 25 spectrophotometer. Standard curves were determined using medium containing 1×10^{-3} M CP spiked with known amounts of mechorethamine HCl as the alkylating agent.

Statistical analysis. Comparisions of mean values of the experimental data were made using the analysis of variance with $P < 0.05$ as the limit of significance [43].

RESULTS

Figure 1 illustrates the accepted route of biotransformation of cyclophosphamide by the cytochrome P-450 mixed-function oxygenase system to reactive metabolites that result in cytotoxicity. The production of these alkylating metabolites, phosphoramide mustard and nor-HN_2, was quantified by the 4-(*p*-nitrobenzyl)pyridine assay, as shown in Fig. 2. One-day-old primary cultures of parenchymal hepatocytes in arginine-free medium were incubated with 1×10^{-3} M CP, and aliquots of the medium were then withdrawn at the time intervals indicated. There were detectable levels of alkylating metabolites as early as 2 hr after exposure. The production of alkylating metabolites gradually reached a plateau after 16 hr but continued throughout the entire 24-hr incubation period. The metabolism of CP was not demonstrated in cultures containing only fibroblasts.

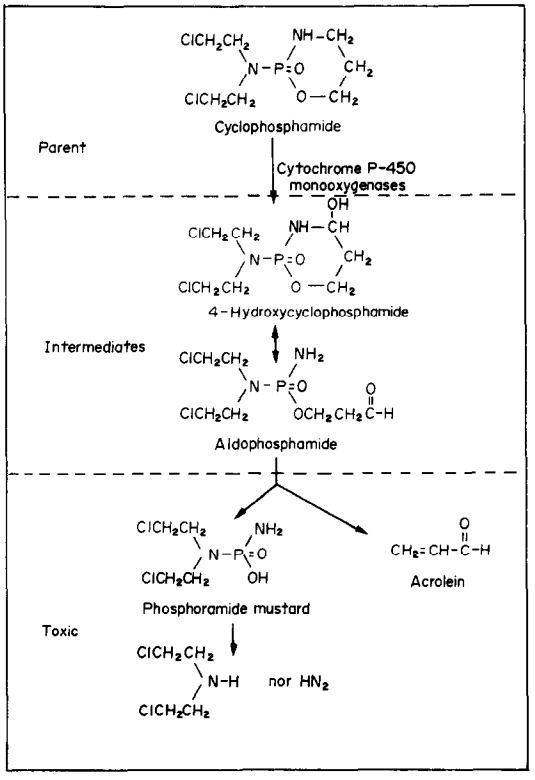

Fig. 1. Scheme for the metabolic activation of cyclophosphamide to toxic metabolites.

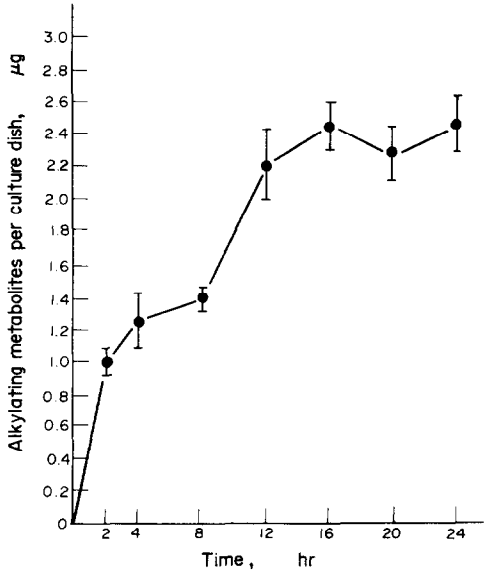

Fig. 2. Estimation by the NBP assay of alkylating metabolites of CP produced by 1-day-old primary cultures of postnatal rat hepatocytes as a function of time of exposure to 1×10^{-3} M cyclophosphamide. Each point is the mean ± S.E. $N = 4$.

Initial experiments were designed to evaluate CP toxicity to the parenchymal hepatocytes maintained in ornithine-enriched, arginine-deficient culture medium which inhibited fibroblast overgrowth. There was no toxicity to parenchymal hepatocytes produced by CP as measured by trypan blue dye exclusion tests for viability, LDH leakage, measurement of total protein per culture dish, or nuclei count data (data not shown).

Such results were expected because CP and its metabolites act upon cells which are undergoing DNA replication [33–35] and thus parenchymal cells which do not divide in culture were not affected. To demonstrate the toxicity of CP, we needed cells which were able to replicate to serve as targets for the alkylating metabolites of CP. By growing the liver cultures in the presence of arginine, an essential amino acid for the growth of fibroblasts, we were able to establish a co-culture system of parenchymal hepatocytes with rapidly dividing fibroblasts which after several days in culture may overgrow the parenchymal cells [14, 17].

In previous studies, we have employed the measurement of leakage of the intracellular enzyme, LDH, into the culture medium as an index of cellular injury [23–25]. In these experiments, co-cultures of parenchymal and fibroblast cells were treated with 1×10^{-3} M CP for 24 hr and LDH leakage was subsequently measured daily for 7 days. The data (not shown) indicated a gradual increase over time in LDH leakage that reached a peak on day 4 after CP treatment. The apparent lag time for LDH leakage to occur and the changing fibroblastic morphology of the primary monolayer as viewed by phase contrast microscopy prompted us to further characterize the CP-induced alterations in the co-cultures. Fibroblastic proliferation was evaluated by measuring the total protein content per culture dish [44] and by counting cell nuclei [40].

Figure 3 compares the amount of total protein per culture dish of control and CP-treated co-cultures grown in arginine-supplemented medium. The protein content of control cultures remained constant or increased slightly as the parenchymal cell monolayers became overgrown with fibroblastic cells. The amount of protein decreased significantly after day 3 for the three doses of CP employed, which indicated a lag time before the toxicity of the metabolites was expressed in proliferating fibroblasts. The values for total protein levelled off between days 5 and 7 because the number of parenchymal cells remained fairly constant.

The number of cells per culture dish was counted after staining the monolayer with hematoxylin to improve the visualization of the cell nucleus [40]. Figure 4 relates the manner in which actual cell number decreased due to CP treatment. The results of diminished cell count closely paralleled the protein loss reported in Fig. 3. Nuclei counts decreased very rapidly after day 1 and reached a plateau between days 3 and 7; microscopic observation revealed a preferential destruction of fibroblast cells with the maintenance of relatively constant numbers of parenchymal hepatocytes.

The cytotoxcity of CP to dividing fibroblasts was mitigated significantly by administering SKF 525-A,

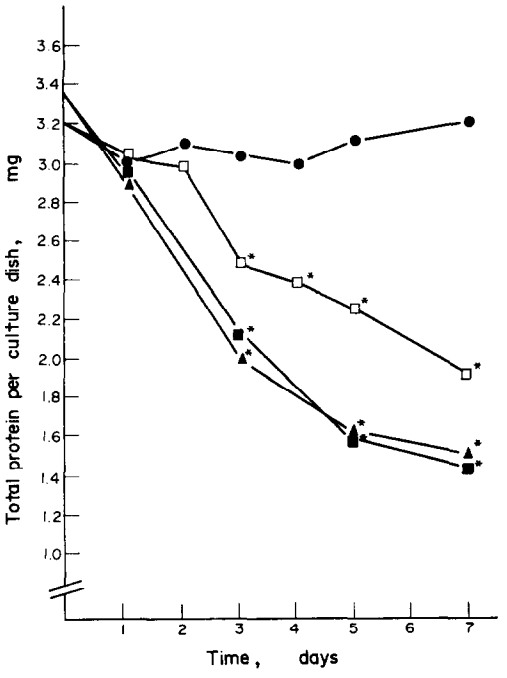

Fig. 3. Dose-dependent effects of CP on the total amount of protein per culture dish of co-cultures of parenchymal hepatocytes and fibroblasts versus time after 24 hr exposure to CP. Four CP concentrations were employed: (●) none (controls); (■) 1×10^{-4} M; (▲) 5×10^{-4} M; and (□) 1×10^{-3} M. Each point is the mean of four separate experiments differing by less than 10 percent from each other. Key: (*) statistically different from controls, $P < 0.05$.

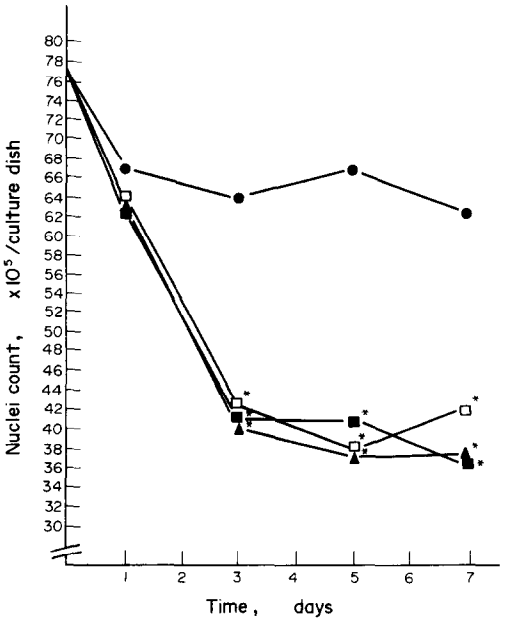

Fig. 4. Dose-dependent effects of CP on cell number per culture dish of co-cultures of parenchymal hepatocytes and fibroblasts versus time after 24 hr exposure to CP. Four CP concentrations were employed: (●) none (control); (■) 1×10^{-4} M; (▲) 5×10^{-4} M; and (□) 1×10^{-3} M. Each point is the mean of four separate experiments differing by less than 10 percent from each other. Key: (*) statistically different from controls, $P < 0.05$.

an inhibitor of the cytochrome P-450 monooxygenase system, to the co-cultures at the same time the cells were exposed to CP. Data for total protein and nuclei counts presented in Table 1 illustrated no significant differences between control co-cultures and CP + SKF 525-A treated co-cultures.

DISCUSSION

Increased studies in the area of toxicology have demonstrated that the widely held assumption that drug metabolism is always synonymous with detoxification is no longer acceptable [45]. Our laboratory has developed a model of primary cultures of postnatal rat hepatocytes to characterize the metabolic activation of xenobiotics to toxic intermediates and to study the mechanism(s) by which these chemicals produce cellular injury. This study has provided evidence that postnatal hepatocytes in primary culture are metabolically competent to activate protoxic compounds via a functional cytochrome P-450 monooxygenase system. We utilized a co-culture system of parenchymal hepatocytes as metabolizing units and proliferating fibroblasts as targets for the toxic metabolites of CP. These metabolites were produced in sufficient quantities and were stable enough to allow passage out of the hepatocytes and entry into the fibroblasts. Our results complement and confirm the work of Fry and Bridges who utilized adult hepatocytes in a co-culture system with fibroblasts [14] to investigate the metabolism-mediated cytotoxicity of carcinogens and non-carcinogens [15]. The evidence presented in this paper suggests that

Table 1. Effect of SKF 525-A on cyclophosphamide-induced inhibition of cell growth

Days in culture	Controls*		Treated cultures†	
	Protein (mg/dish)	Cell count ($\times 10^5$ cells/dish)	Protein (mg/dish)	Cell count ($\times 10^5$ cells/dish)
0	2.97 ± 0.19	60.5 ± 3.51	—	—
1	2.80 ± 0.09	53.3 ± 4.43	2.78 ± 0.06	53 ± 1.41
3	2.83 ± 0.17	54.8 ± 5.12	2.60 ± 0.09	51 ± 4.40
5	2.75 ± 0.18	55.5 ± 4.12	2.71 ± 0.17	50 ± 0.63
7	2.73 ± 0.15	50.5 ± 5.80	2.63 ± 0.21	47 ± 1.60

* Control co-cultures contained neither cyclophosphamide nor SKF 525-A.

† Co-cultures were treated for 24 hr with SKF 525-A (1×10^{-5}M) and cyclophosphamide (1×10^{-4} M).

a co-culture system of postnatal parenchymal hepatocytes and fibroblasts can be employed in evaluations of the metabolic activation of xenobiotics and in studies designed to determine the mechanisms by which chemicals produce cellular injury.

The NBP assay for alkylating metabolites presented in Fig. 2 provides direct evidence of CP metabolism in postnatal hepatocyte cultures. The assay procedure did not quantify the total production of alkylating metabolites because only free alkylating species in the culture medium were measured. The metabolites remaining in the hepatocytes and taken up into the fibroblasts were not measured.

A definite delay existed before significant toxicity became evident for the three parameters utilized to indicate cytotoxicity: LDH leakage, total protein per culture dish, and nuclei counts. This consistent lag time probably indicated the time required for the fibroblasts with alkylated DNA to begin active proliferation that led to cell destruction.

The activation of CP to cytotoxic metabolites was greatly diminished by treating the co-culture simultaneously for 24 hr with cyclophosphamide and SKF 525-A, an inhibitor of the cytochrome P-450 monooxygenase system [46]. SKF 525-A presumably prevented the cytochrome P-450 mediated biotransformation of the parent compound CP to 4-OHCP, which is the "activated" metabolite that leaves the parenchymal hepatocyte and is further converted to alkylating metabolites.

A somewhat unexpected result was obtained during the completion of the dose–response studies. The data for total protein and nuclei counts with CP-treated cultures revealed a trend toward decreasing cytotoxicity as the dose increased. The diminished toxicity may be due to an interaction of CP metabolites and the cytochrome P-450 monooxygenase system that results in denaturation of the heme moiety. The CP metabolite, acrolein, has been reported to react with the sulfhydryl groups in cytochrome P-450 [47–49] causing decreased drug-metabolizing capability. Thus, the highest dose of CP tested (1×10^{-3} M) would presumably result in higher levels of acrolein and would lead to a subsequent reduction of metabolism of the remaining CP in the cells, leading to less cytotoxicity in the cultures.

REFERENCES

1. J. W. Grisham, *Int. Rev. expl Path.* **20**, 123 (1979).
2. J. R. Fry and J. W. Bridges, in *Reviews in Biochemical Toxicology* (Eds. E. Hodgson, J. R. Bend and R. M. Philpot), pp. 201–47. Elsevier/North Holland, New York (1979).
3. E. C. Miller and J. A. Miller, *Pharmac. Rev.* **18**, 805 (1966).
4. A. S. Wright, *Mutation Res.* **75**, 215 (1980).
5. J. R. Mitchell, D. J. Jollow, J. R. Gillette and B. B. Brodie, *Drug Metab. Dispos.* **1**, 418 (1973).
6. J. R. Mitchell and D. J. Jollow, *Gastroenterology* **68**, 392 (1975).
7. F. J. Wiebel, S. Brown, H. L. Waters and J. K. Selkirk, *Arch. Tox.* **39**, 133 (1977).
8. C. M. King, in *Short Term* In Vitro *Testing for Carcinogenesis, Mutagenesis, and Toxicity* (Eds. J. Berky and P. C. Sherrod), pp. 311–23. The Franklin Institute Press, Philadelphia (1978).
9. R. E. Kouri and L. M. Schechtman, in *Short Term* In Vitro *Testing for Carcinogenesis, Mutagenesis, and Toxicity* (Eds. J. Berky and P. C. Sherrod), pp. 423–38. The Franklin Institute Press, Philadelphia (1978).
10. P. Moldeus, R. Grundin, H. Vadi and S. Orrenius, *Eur. J. Biochem.* **46**, 351 (1974).
11. J. R. Fry and J. W. Bridges, in *Progress in Drug Metabolism* (Eds. J. W. Bridges and L. F. Chasseaud), pp. 71–85. John Wiley, Chichester (1977).
12. P. S. Guzelian, D. M. Bissel and U. A. Meyer, *Gastroenterology* **72**, 1232 (1977).
13. G. M. Williams, *Cancer Res.* **37**, 1845 (1977).
14. J. R. Fry and J. W. Bridges, *Biochem. Pharmac.* **26**, 969 (1977).
15. P. Wiebkin, J. R. Fry and J. W. Bridges, *Biochem. Pharmac.* **27**, 1849 (1978).
16. K. K. Dougherty, S. D. Spilman, C. E. Green, A. R. Steward and J. L. Byard, *Biochem. Pharmac.* **29**, 2117 (1980).
17. D. Acosta, D. D. Anuforo and R. V. Smith, *In Vitro* **14**, 428 (1978).
18. D. Acosta, D. C. Anuforo, R. McMillin, W. H. Soine and R. V. Smith, *Fedn Proc.* **38**, 366 (1979).
19. D. C. Anuforo, D. Acosta and R. V. Smith, *In Vitro* **15**, 189 (1979).
20. D. Acosta, D. C. Anuforo, R. McMillin, W. H. Soine and R. V. Smith *Life Sci.* **25**, 1413 (1979).
21. M. Dickins and R. E. Peterson, *Biochem. Pharmac.* **29**, 1231 (1980).
22. D. M. Bissell and P. S. Guzelian, in *Gene Expression and Carcinogenesis in Cultured Liver* (Eds. L. E. Gerschenson and E. B. Thompson), pp. 119–36. Academic Press, New York (1975).
23. D. C. Anuforo, D. Acosta and R. V. Smith, *In Vitro* **14**, 981 (1978).
24. D. Acosta, D. C. Anuforo and R. V. Smith, *Toxic. appl. Pharmac.* **53**, 306 (1980).
25. D. B. Mitchell and D. Acosta, *J. Toxic. environ. Hth.* **7**, 83 (1981).
26. G. Dallner, P. Siekevitz and G. E. Palade, *J. Cell Biol.* **30**, 73 (1966).
27. P. T. Henderson, *Biochem. Pharmac.* **20**, 1225 (1971).
28. T. K. Basu, J. W. Dickerson and D. V. Parke, *Biochem. J.* **124**, 19 (1971).
29. A. L. Jones and E. S. Mills, *Am. J. Drug Alcohol Abuse* **1**, 111 (1974).
30. N. E. Sladek, *Cancer Res.* **31**, 901 (1971).
31. T. A. Connors, P. J. Cox, P. B. Farmer, A. B. Foster and M. Jarman, *Biochem. Pharmac.* **23**, 115 (1974).
32. B. E. Domeyer and N. E. Sladek, *Cancer Res.* **40**, 174 (1980).
33. M. Colvin, R. B. Brundrett, M. N. Kan, I. Jardine and C. Fenselau *Cancer Res.* **36**, 1121 (1976).
34. R. F. Struck, M. C. Lirk, M. H. Witt and W. R. Laster, Jr., *Biomed. Mass Spectrom.* **2**, 46 (1975).
35. S. Waxman, in *Clinical Cancer Chemotherapy* (Ed. E. M. Greenspan), pp. 17–32. Raven Press, New York (1975).
36. H. L. Leffert and D. Paul, *J. cell. Physiol.* **81**, 113 (1973).
37. G. M. Williams, E. K. Weisburger and J. H. Weisburger, *Expl Cell Res.* **69**, 106 (1971).
38. B. J. Dean and G. Hodson-Walker, *Mutation Res.* **64**, 329 (1979).
39. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
40. D. R. Roberts, *J. med. Lab. Technol.* **23**, 119 (1966).
41. J. Epstein, R. W. Rosenthal and R. J. Ess, *Analyt. Chem.* **27**, 1435 (1955).
42. O. M. Friedman and E. Boger, *Analyt. Chem.* **33**, 906 (1961).
43. G. Keppel, *Design and Analysis*, pp. 85–105. Prentice-Hall, Englewood Cliffs, NJ (1973).

44. V. I. Oyama and H. Eagle, *Proc. Soc. exp Biol. Med.* **91**, 305 (1956).
45. P. Mazel and D. Pessayre, in *Advances in Modern Toxicology* (Eds. M. A. Mehlman, R. E. Shapiro and H. Blumenthal), pp. 307–43. Hemisphere Publishing Corp., Washington (1976).
46. J. R. Gillett, D. C. Davis and H. A. Sasame, *A. Rev. Pharmac.* **12**, 57 (1972).
47. K. M. Ivanetich, S. Lucas and J. Marsh, *Drug Metab. Dispos.* **6**, 218 (1978).
48. A. J. Marinello, H. L. Gurtoo, R. F. Struck and B. Paul, *Biochem. biophys. Res. Commun.* **83**, 1347 (1978).
49. M. J. Berrigan, H. L. Gurtoo, S. D. Sharma, R. F. Struck and A. J. Marinello, *Biochem. biophys. Res. Commun.* **93**, 797 (1980).