The data in Table 1 indicate that compounds with a variety of aromatic moieties are able to compete with [^{3}H]-TAM for binding to rat liver microsomal AEBS. While the data in Table 2 reaffirm AEBS to bind triarylethylenes such as TAM with highest affinity, our results indicate that these sites will bind a relatively diverse collection of structural types, including SKF 525-A and metyrapone.

Since the last compounds are classical inhibitors of drug metabolism [11, 12], it may be that AEBS are a component of the liver microsomal drug-metabolizing enzyme system, which also is known to have relatively low ligand (substrate) specificity [13]. Cytochrome P-450, a component of this system, has been suggested to interact with SKF 525-A [14], metyrapone [12], and TAM [15, 16] in part on the basis of difference spectra generated by addition of each of these drugs to liver microsomal suspensions. Our results indicated an AEBS level of 1.34 pmoles/mg microsomal protein (Table 1), whereas rat liver contains about 1000 pmoles of cytochrome P-450/mg microsomal protein [17]. Thus, if one assumes that the AEBS are associated with cytochrome P-450, the amount involved must be a small percentage of the total amount present. Studies with purified components of the drug-metabolizing enzyme system, including isoenzymatic forms of cytochrome P-450, will be necessary to determine whether any of these constitute the AEBS.

The apparent distribution volume, and thus the extent and duration of action of triarylethylene antiestrogens, has been suggested to be influenced by the affinity of these compounds for AEBS [4, 18]. Our results implicate AEBS in modulation not only of the pharmacokinetics of TAM and related antiestrogens, but also of those of a wide variety of drugs bearing aminoether side chains and aromatic systems like those of compounds in this study.

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REFEERENCES

1. R. L. Sutherland and L. C. Murphy, *Molec. cell. Endocr.* **25**, 5 (1982).
2. R. L. Sutherland and V. C. Jordan (Eds.), *Nonsteroidal Antiestrogens. Molecular Pharmacology and Antitumor Activity.* Academic Press, Sydney (1981).
3. R. L. Sutherland, L. C. Murphy, M. S. Foo, M. D. Green, A. M. Whybourne and Z. S. Krozowski, *Nature, Lond.* **288**, 273 (1980).
4. K. Sudo, F. J. Monsma, Jr. and B. S. Katzenellenbogen, *Endocrinology* **112**, 425 (1983).
5. C. K. W. Watts, L. C. Murphy and R. L. Sutherland, *J. biol. Chem.* **259**, 4223 (1984).
6. L. J. Brandes and M. W. Hermonat, *Biochem. biophys. Res. Commun.* **123**, 724 (1984).
7. P. C. Ruenitz, J. R. Bagley and C. M. Mokler, *J. med. Chem.* **25**, 1056 (1982).
8. R. E. Allen, F. P. Palopoli, E. L. Schumann and M. G. Van Campen, Jr., U.S. Pat. 2,914,561-4 (1959); *Chem. Abstr.* **54**, 5581e (1960).
9. M. J. K. Harper, D. N. Richardson and A. L. Walpole, Br. Patent 1,064,629 (1967); *Chem. Abstr.* **67**, 90515g (1967).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. J. R. Cooper, J. Axelrod and B. B. Brodie, *J. Pharmac. exp. Ther.* **112**, 55 (1954).
12. H. G. Jonen, B. Hüthwohl, R. Kahl and G. F. Kahl, *Biochem. Pharmac.* **23**, 1319 (1974).
13. B. Testa and P. Jenner, *Drug Metabolism: Chemical and Biochemical Aspects* pp. 3–135. Marcel Dekker, New York (1976).
14. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
15. P. C. Ruenitz and M. M. Toledo, *Biochem. Pharmac.* **29**, 1583 (1980).
16. N. M. Meltzer, P. Stang and L. A. Sternson, *Biochem. Pharmac.* **33**, 115 (1984).
17. E. J. Flynn, M. Lynch and V. G. Zannoni, *Biochem. Pharmac.* **21** 2577 (1972).
18. V. C. Jordan and R. A. Bowser-Finn, *Endocrinology* **110**, 1281 (1982).

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Differential inhibition by T-2 toxin of total protein, DNA and isoprenoid synthesis in the culture macrophage cell line J774

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T-2 toxin (4β,15diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene-3α-ol.) is part of a major family of mycotoxins known as trichothecenes, which have world-wide distribution and which are implicated in a diverse variety of disease processes in animals and man [1–4]. The clinical picture in both man and animals is determined principally by arrest of normally rapidly proliferating tissues followed by their necrosis, as in bone-marrow, skin and gastrointestinal tract [5–6]. Of major interest is the effect of T-2 toxin on immune function, as leukopenia and agranulocytosis are life threatening complications of toxicity states.

In vitro studies have shown a number of the trichothecene toxins to be potent inhibitors of protein synthesis. These compounds, including T-2 toxin, would bind to ribosome thereby inhibiting peptidyl transferase and blocking initiation of synthesized polypeptides [7]. Although the mechanism of protein synthesis inhibition has been studied *in vitro* there have been very few detailed studies on whole cells aimed at accurately elucidating the effects of T-2 toxin on integrated cell function [8–9].

This study was undertaken to more precisely define the differential stability of J774 macrophage cell enzymes important in normal growth behaviour to the toxicity of T-2, by following the inhibition of protein, DNA and isoprenoid synthesis.

Materials and methods

The cells used in this study were derived from the J774 macrophage cell line originally isolated by Ralph and Nakoinz [11] and cloned in the laboratory of Dr. B. Bloom, Albert Einstein College of Medicine, NY. Cells were cultivated in Eagle's Minimal Essential Medium (MEM) supplemented with glutamine, glucose, 20% horse serum (inac-

tivated at 60° for 30 min), gentamycin and ampicillin. Experiments were conducted in either full medium or with omission of horse serum as indicated.

Drugs. T-2 toxin was isolated from *Fusarium sporotrichioides* [10], cycloheximide (CHM) and puromycin were obtained from Sigma Chemical Co., St. Louis, MO. These drugs were added in 3–10 μl of dimethylsulfoxide (DMSO). DMSO content never exceeded 1% of the incubation mixture, a concentration known in these cells to affect neither growth behaviour nor protein synthesis.

Isotopes. [^{14}C]-Acetic acid, sodium salt (45–60 mCi/mmole), L-4.5-^{3}H(*N*)-leucine (40–60 mCi/mmole), [6-^{3}H]-thymidine 15 Ci/mmole were purchased from New England Nuclear, Boston, MA. [^{3}H]Cholesterol was obtained from Amersham, U.K.

Incorporation studies. The J774 macrophage cells were grown for 3 days in Falcon 1008 plates. The medium was exchanged and the incubation continued for an additional 24 hr. The cells were harvested by centrifugation and resuspended in fresh medium with or without serum to a final concentration of 1–2.5 × 10^{6} cells/ml. Radioactive precursors 0.3 μCi/ml [1^{3}H]-thymidine, 0.3 μCi/ml [^{3}H]-leucine or 0.3 μCi/ml [^{14}C]-acetic acid were added and the cell suspension was divided between miniwells. The T-2 toxin, cycloheximide or puromycin in 3–10 μl DMSO were added to each miniwell and incubated at 37° for various time periods (30 min–6 hr). The corresponding controls consisted of 3–10 μl DMSO without the drug. After the desired time, the incubation was stopped by centrifugation and the pellets were washed three times with 1 ml of cold 10% trichloroacetic acid (TCA). The final pellets, from the leucine and thymidine incubation experiments, were dissolved in 0.5 ml of 1N NaOH, kept for 30 min at 60° and the radioactivity of the whole sample was measured by liquid scintillation counting.

The resultant pellets, from the [^{14}C]-acetic acid incorporation experiments were dissolved in 0.5 ml of 70% ethanolic KOH solution containing, 1 μCi [^{3}H]-cholesterol and 0.25 mg non-labelled cholesterol, and incubated for 2 hr at 60°; then 1 ml of butyl alcohol was added to each well. Non-saponifiable lipids were extracted three times with 1 ml ethylacetate. The combined ethylacetate extracts washed with 1 ml of 10% sodium acetate solution and the radioactivity of the ethylacetate fraction was measured by liquid scintillation counting. The biosynthesized ^{14}C-labelled fatty acids remained as potassium salts in the basic alcohol–water solution. By using this procedure the non-saponifiable lipids have been totally extracted with the ethylacetate.

[^{3}H]-Cholesterol has been used to determine the efficiency of the above used extraction procedure. The method used by us for the isolation of isoprenoids was a modification of the technique of Cavenee *et al.* [12].

Results

T-2 toxin was found to be extremely potent in inhibiting protein synthesis. Some variability in sensitivity was observed from one experiment to the next but inhibition was invariably induced by less than 1 ng/ml of T-2 toxin. Figure 1 shows a representative experiment. It was also observed that the presence or absence of serum affected the cell's sensitivity to the toxin. Total protein synthesis is reduced in cells grown in serum free medium. In all cases the effect was rapid and clearly seen within the first hour.

A study was then undertaken to compare the relative potency of the widely used protein inhibitors CHM and puromycin (Fig. 1). The concentrations required for 50% inhibition of protein synthesis (I_{50}) are shown in Table 1. T-2 toxin is the most potent of the three inhibitors with CHM intermediate and puromycin the weakest in effect. All inhibitors are more effective in serum-free conditions of cell growth (Table 1). In the case of DNA synthesis, 52% inhibition of [^{3}H]-thymidine incorporation was observed in under 1 hr with a concentration of 0.05 ng/ml T-2 (Fig. 2).

We then proceeded to study the effect of T-2 toxin on the incorporation of [^{14}C]-acetate into biosynthesized isoprenoids (Fig. 3).

Table 1. Concentrations of puromycin, cycloheximide and T-2 toxin required for 50% inhibition of protein synthesis at 2 hr by cultured macrophage cell line, J774 (2 × 10^{6} cells)

	Puromycin (μg/ml)	Cycloheximide (ng/ml)	T-2 Toxin (ng/ml)
+Serum	10	40	0.8
−Serum	5	33	0.46

It was observed that [^{14}C]-acetate incorporation into non-saponifiable lipids was linear over 3–4 hr following treatment with T-2 toxin in concentrations sufficient to cause inhibition of protein synthesis. Only after this time was there inhibition of acetate incorporation into isoprenoid fraction. The efficiency of the recovery of nonsaponifiable lipids, by the ethylacetate extraction from the basic water–alcoholic solution in samples spiked with 1 μCi [^{3}H]-cholesterol, was about 91 ± 7.8%.

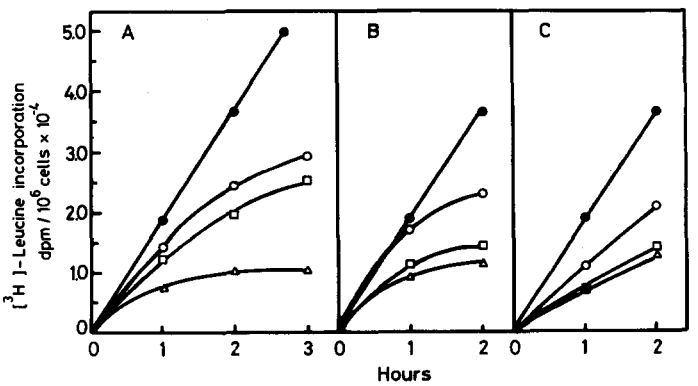

Fig. 1. Effect of T-2 toxin (A), puromycin (B) and cycloheximide (C), on protein synthesis by J774 macrophage cell line *in vitro*. Symbols: controls (●); T-2 toxin—0.3 ng/ml (○), 0.6 ng/ml (□), 1.0 ng/ml (△); puromycin—5 μg/ml (○), 10 μg/ml (□), 15 μg/ml (△); cycloheximide—25 ng/ml (○), 50 ng/ml (□), 100 ng/ml (△).

Discussion

The experiments performed in this study have yielded a number of observations, the most interesting of which is the remarkable sensitivity of the cultured macrophage cell line J774 to the protein inhibitory effects on T-2 toxin. We have shown here that concentrations of less than 1 ng/ml of T-2 toxin substantially suppress [^{3}H]-thymidine incorporation into DNA (Fig. 2) and [^{3}H]-leucine incorporation into protein. These inhibitory effects are comparable to those observed in similar studies on the normal mouse lymphocyte [13] but exceed in sensitivity the response of other mammalian cells tested, including rabbit reticulocytes, Ehrlich ascites tumor cells [8] and human neoplastic cells of intestinal origin [9]. These *in vitro* observations parallel the clinically observed effects of T-2 toxin as an especially powerful inhibitor of the lymphoreticular system.

This sensitivity of the J774 macrophage cell line was also seen in its response to CHM which produced striking (at least 50%) inhibition at ng/ml concentrations (Fig. 1). However this too is a much lower concentration of CHM than is normally used in many mammalian cells to obtain comparable protein inhibition [15]. It is of interest that the sensitivity did not extend to puromycin while in other studies on whole cells, it seems to be about as effective as CHM in causing inhibition of protein synthesis [8].

The greater sensitivity of the protein synthetic mechanism to T-2 toxin in the absence of serum, presumably reflects the normally reduced metabolic activity of cells grown under these conditions [17] (Table 1).

We have observed, as have others [9], that the effects of T-2 on DNA synthesis and cell growth parallel completely its effect as an inhibitor of protein synthesis (Fig. 2).

Our interest in the biosynthesis of isoprenoids in this study was derived from the observation that the growth of cells in culture is dependent on the normal activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), a key regulatory enzyme in the synthesis of cholesterol. Inhibition of this enzyme makes cells dependent on an exogenous source of mevalonate or cholesterol normally obtained from plasma low density lipoprotein for normal growth [14]. In a systematic evaluation of the effect of T-2 toxin, we were therefore interested to determine whether HMG-CoA reductase activity was suppressed. This would have shown as reduced incorporation of [^{14}C]-acetate into the biosynthesized sterols [16]. It is clear from this study that, in J774 macrophage, the effect of T-2 toxin on [^{14}C]-acetate incorporation into isoprenoids was considerably delayed (after 3–4 hr) (Fig. 3). This is presumably due to the greater stability and slower turnover of the rate limiting enzymes mediating the biosynthesis of isoprenoids, as compared to the enzymes more directly associated with DNA synthesis [18].

The findings reported here demonstrate that the early inhibition of DNA synthesis by T-2 toxin is not linked immediately to the inhibition of isoprenoid synthesis which follows much later.

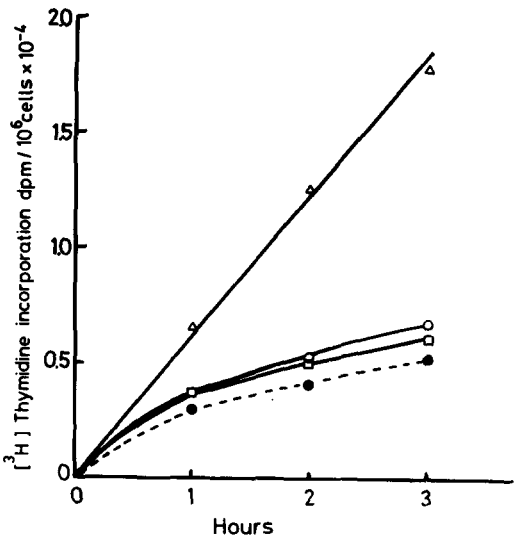

Fig. 3. Effect of T-2 toxin on synthesis of DNA by J774 macrophages *in vitro*. The amount of ^{3}H-Thymidine incorporated into DNA molecules was monitored for 3 hr. Symbols: control (△); T-2 toxin—0.05 ng/ml (○), 0.3 ng/ml (□), 0.6 ng/ml (●).

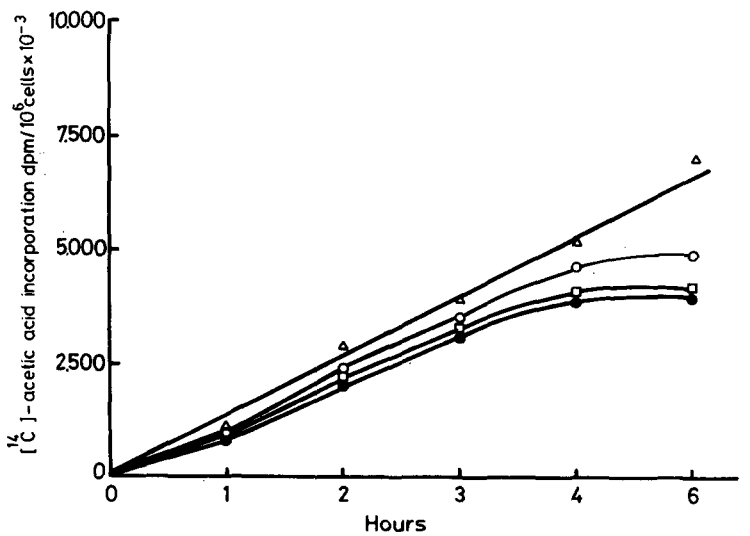

Fig. 2. Effect of T-2 toxin on synthesis of isoprenoids by J774 macrophage cells *in vitro*. The amount of ^{14}C-acetic acid incorporated into macrophage isoprenoid molecules were monitored for 6 hr. Symbols: control (△); T-2 toxin—0.05 ng/ml (○), 0.1 ng/ml (□), 0.3 ng/ml (●).

Summary

The inhibition of total protein, DNA and isoprenoid synthesis by the potent T-2 toxin, was investigated in the murine macrophage cell line, J774. Protein and DNA synthesis were more than 50% inhibited within 1 hr by extremely low doses of the toxin (<1 ng/ml). Isoprenoid synthesis, unlike protein and DNA synthesis, showed delayed inhibition (i.e. after 3–4 hr). This effect of T-2 toxin on protein, DNA and isoprenoid biosynthesis presumably reflects the differential stability of the enzymes involved in these biosynthetic pathways.

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REFERENCES

1. Y. Ueno, in *Advances in Nutritional Research* (Ed. H. H. Draper), Vol. 3, p. 301. Plenum Press, New York (1980).
2. A. Ciegler, *J. Food Protec.* **41**, 399 (1978).
3. B. Yagen and A. Z. Joffe, *Appl. Microbiol.* **32**, 423 (1976).
4. I. Lutsky, N. Mor, B. Yagen and A. Z. Joffe, *Toxic. appl. Pharmac.* **43**, 111 (1978).
5. C. E. Mayer, *Milit. Surg.* **113**, 173 (1953).
6. A. Z. Joffe, in *Mycotoxic Fungi, Mycotoxin, Mycotoxicoses, An Encyclopedic Handbook* (Eds. T. D. Wyllie and L. G. Morehouse), Vol. 3, p. 21. Marcel Dekker, New York (1978).
7. C. S. McLaughlin, M. H. Vaughan, I. M. Cambell, C. M. Wei, M. E. Stafford and B. S. Hansen, in *Mycotoxins in Human and Animal Health* (Eds. J. V. Rodricks, G. W. Hesseltine and M. A. Mehlman), p. 263. Pathotox Publishers, Park Forest South, Illinois (1978).
8. Y. Ueno, M. Nakajima, K. Sakai, K. Ishii, N. Sato and N. Shimada, *J. Biochem. (Tokyo)* **74**, 285 (1973).
9. E. Morel-Chany, P. Burtin, G. Trincal and C. Frayssinet, *Masson (Paris)* **149**, (1980).
10. B. Yagen, A. Z. Joffe, P. Horn, N. Mor and I. I. Lutsky, in *Mycotoxins in Human and Animal Health* (Eds. J. V. Rodricks, G. W. Hesseltine and M. A. Mehlman), p. 329. Pathotox Publishers, Park Forest South, Illinois (1978).
11. P. Ralph and W. Nakoinz, *Nature* **257**, 393 (1975).
12. W. K. Cavenee, D. Johnston and G. Melnykovych, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2103 (1978).
13. C. Lafarge-Frayssinet, G. Lespinats, P. Lafont, F. Loisillier, S. Mousset, Y. Rosenstein and C. Frayssinett, *Proc. Soc. exp. Biol. Med.* **160**, 302 (1979).
14. I. Kaneko, Y. Hazama-Shimada and A. Endo, *Eur. J. Biochem.* **87**, 313 (1978).
15. C. E. Agrelo and R. Schoental, *Toxic. Vet.* **5**, 155 (1980).
16. J. R. Faust, M. S. Brown and J. L. Goldstein, *J. biol. Chem.* **255**, 6546 (1980).
17. D. Barnes and G. Sato, *Cell* **22**, 649 (1980).
18. V. Quesney-Huneeus, M. H. Wiley and M. D. Siperstein, *Proc. natn. Acad. Sci. U.S.A.* **76**, 5056 (1979).

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Adrenergic ligand binding in human serum

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Propranolol is extensively bound in human serum and plasma [1–7]. *Alpha*-1 acid glycoprotein has been shown to be the major determinant for binding variability at therapeutic concentrations [2–8]. The binding of propranolol to this protein is saturable with high affinity [3, 9] and with stereospecificity [10, 11]. Other serum proteins have binding capacity for propranolol as well, but the binding to albumin and to lipoproteins has been reported to be nonsaturable [3, 4, 7]. A number of other adrenergic agents are bound to human serum proteins: alprenolol [5, 12], pindolol [5, 13, 14], timolol [5], oxprenolol [5, 15], prazosin [16], chlorpromazine [2, 7, 9], isoproterenol [1], epinephrine and norepinephrine [17–20].

The purpose of the present study has been to characterize the affinity and stereospecificity of adrenergic ligands in human serum by their ability to displace radiolabelled (−)-propranolol from its binding sites.

Materials and methods

Chemicals. [^{3}H]-(−)-Propranolol hydrochloride (sp. act. 19.6 Ci/mmole) was purchased from New England Nuclear, Dreieich, F.R.G. (−)-/(+)- and (±)-propranolol hydrochloride (Imperial Chemical Industries Ltd., London, UK), (−)- and (+)-alprenolol bitartrate (Haessle, Molndalen, Sweden), prazosin hydrochloride (Pfizer, Sandwich, U.K.) and (±)-hydroxybenzyl-pindolol (Sandoz A. G., Basel, Switzerland) were obtained as gifts. (−)- and (+)-Isoproterenol bitartrate, (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate and ascorbic acid were purchased by Sigma Chemical Company (St. Louis, MO). Chlorpromazine and (±)-isoproterenol sulphate were purchased from the Norwegian Medicinal Depot, Oslo, Norway. All other chemicals were of analytical grade.

Buffers. Modified Krebs Ringer phosphate buffer: NaCl 122 mM, KCl 4.9 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM, Na_2HPO_4 15.9 mM, pH 7.40–7.45.

Modified Krebs Ringer bicarbonate buffer: NaCl 121 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM $NaHCO_3$ 25.3 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.2 mM, pH 7.35–7.40 was achieved by gassing [5% (v/v) carbon dioxide in air].

Serum. Serum was obtained from healthy subjects. Venous blood was sampled after breakfast and left for 1 hr at room temperature and centrifuged at 2000 *g* for 30 min at 22°. Serum was aspirated immediately and stored at −20° until analysis.

Equilibrium dialysis. Serum protein binding of [^{3}H]-(−)-