Biochemical Pharmacology, Vol. 17, pp. 2421–2434. Pergamon Press. 1968. Printed in Great Britain

STUDIES ON LYSOSOMES—X

EFFECTS OF TUMOR-PROMOTING AGENTS UPON BIOLOGICAL AND ARTIFICIAL MEMBRANE SYSTEMS*

GERALD WEISSMANN,† WALTER TROLL, BENJAMIN L. VAN DUUREN and GRAZIA SESSA

Departments of Medicine and Institute of Environmental Medicine, New York University School of Medicine, New York, N.Y., U.S.A.

(*Received* 2 *April* 1968; *accepted* 14 *June* 1968)

Abstract—Six tumor-promoting compounds (crude croton oil, croton oil resin, phorbol esters, Tween 80, Tween 60 and an extract of tobacco leaves) were tested for their activity on biological and artificial membranes. Each of the agents was capable of releasing aryl sulfatase, β-glucuronidase and acid phosphatase from large-granule fractions of rabbit liver (0·25 M sucrose). Release of enzymes was dependent upon time, temperature and concentration of the tumor-promoting agent. The order of activity upon lysosomes closely followed the order of activity of a given agent as a tumor-promoter (co-carcinogen). Although most of the agents were hemolytic and released malate dehydrogenase from mitochondria, their order of activity on red cells and mitochondria was not directly related to tumor-promoting action. All tumor-promoting agents did not release marker anions from artificial lipid spherules, differing in this property from other membrane-active substances such as steroids or polyene antibiotics. Lysosomes from liver were not disrupted by tumor-initiating agents nor by complete, systemic carcinogens, although "proximate carcinogens" other than *N*-hydroxy-2-acetylaminofluorene were not tested. The results suggest that tumor-promoting agents disrupt biological membranes *in vitro* and that their order of action *in vivo* is paralleled by their effects upon lysosomes.

WHEN A subcarcinogenic dose of a tumor-initiating agent such as 7,12-dimethylbenz-(a)anthracene (DMBA) is applied once to the skin of mice, no tumors arise. Multiple tumors are induced, however, in almost all animals if one application of DMBA is followed by repeated applications of tumor-promoting substances at intervals ranging from 3 days to 1 yr after DMBA.[1] Such tumor-promoting agents, also called co-carcinogens, include croton oil (from the seed of *Croton tiglium L.*), phorbol esters derived from the oil or an extract from the seeds,[2] the non-ionic "Tween" group of detergents,[3] extracts prepared from tobacco leaves[4] and hyperoxia.[5] In the absence of a tumor-initiator, these agents usually do not produce skin tumors; however, applications of pure phorbol esters result in low tumor yield.[6] The active principle of crude croton oil has been identified as fatty acid esters of phorbol, a tetracyclic, diterpene alcohol.[2, 7, 8] The fraction containing these esters, termed fraction A, is 500 times as effective as crude croton oil (in terms of equivalent dosage required to give similar tumor yields) and 30,000 times more effective than Tween 60.[1, 3] Thus, there are

* Aided by grants from the USPHS (AM 08363, GM 11949, CA 08580, ES 00260 and Contract PH 43-64-938).

† Career Investigator of the Health Research Council of the City of New York (1-467).

available for study a number of tumor-promoting substances, the biological activities of which vary by many orders of magnitude. Nevertheless, no satisfactory hypothesis has been suggested to account for their biological activity. Nor have these diverse agents been shown to possess other biological properties in common.

Because phorbol esters and the Tweens possess hydrophilic and hydrophobic groupings (amphipaths), it was considered likely that these agents might interact with lipids such as those which bound the membranes of cells or their organelles. Therefore the effects were studied of a series of tumor-promoting agents upon lysosomes, mitochondria and erythrocytes, as well as upon artificial lipid spherules which serve as models for biomembranes. Other amphipathic molecules (polyene antibiotics, steroids, lysolecithin, detergents) act upon biological and artificial membrane-bounded structures in distinctve way.[9–12]

The studies reported below suggest that tumor-promoting compounds share the capacity to disrupt the membranes of erythrocytes, mitochondria and lysosomes, but have no clear-cut effects on artificial lipid spherules. Indeed their order of activity as tumor-promoters was similar to their order of activity upon lysosomes, but bore no direct relation to hemolytic action. This spectrum of membrane activity differed considerably from those displayed by other amphipaths and may therefore suggest a general mode of action for tumor-promoting agents.

MATERIALS AND METHODS

Tumor-promoting agents and carcinogens

Croton oil was commercially available material (Magnus, Mabee and Reynard, Ltd., London, England). Croton resin was prepared by solvent extraction of the seeds of *Croton tiglium L.* followed by solvent partitioning, as described previously.[2]

Phorbol esters (fraction A) were also prepared as previously described[2] by counter-current distribution from croton resin followed by TLC. This material is a mixture of two phorbol esters (mol. wt., approx. 600).

Tobacco leaf extract was an ether extract of flue-cured tobacco leaf prepared as previously described.[4]

N-hydroxy-2 acetylaminofluorene (*N*-Hydroxy-AAF) was prepared according to Miller *et al.*[13] and recrystallized from 50% ethanol. 2-Acetylaminofluorene (AAF), 7,12-dimethylbenz(a)anthracene (DMBA), 3-methylcholanthrene, β-propiolactone and the Tweens were obtained commercially from Eastman Distillation Product Industries (Rochester, N.Y.).

Preparation of a large-granule fraction from rabbit liver

Livers were removed at 4° from young, albino, male rabbits. Homogenates (1 : 10, w/v) were prepared in unbuffered, chilled, 0·25 M sucrose with a Teflon-glass motor-driven homogenizer (Tri-R-Instruments, Jamaica, N.Y.). After unbroken cells were removed by centrifugation for 1 min at 500 *g* in a Sorvall SS-3 centrifuge, nuclei and further debris were separated by sedimentation at 800 *g* for 10 min. The resultant supernatant was next sedimented at 20,000 *g* for 20 min, and this large-granule fraction was twice washed and resuspended 1 : 5 (v/v) in 0·25 M sucrose. Ultrastructural studies and further density gradient analyses of such fractions have shown that these are composed of 85–90 per cent mitochondria with other organelles (lysosomes, dense bodies, fragments of endoplasmic reticulum) comprising the remainder.[14, 15]

Release of acid hydrolases and mitochondrial enzymes

Aliquots (5 ml) of large-granule fractions were incubated with test substances (0·5 ml) in dimethylsulfoxide (DMSO) or solvent alone for periods and at temperatures indicated below. After incubation, the suspensions were again centrifuged at 20,000 *g* for 20 min, and the clear supernatants were assayed. To determine maximum enzyme activities which could be released, 0·1% (v/v) of Triton X-100 was added to two samples of each homogenate. Previous studies have indicated that measurements of hydrolases or of malate dehydrogenase which have been released into the 20,000 *g* supernatants indicate irreversible damage to the membranes of lysosomes and mitochondria respectively.[16]

Enzyme assays

Acid β-glycerophospatase was measured by a modification of the method of Valentine and Beck[17] as previously described.[18] With β-glycerophosphate (Sigma) as substrate, samples of enzyme-rich supernatants (0·2 ml) were added to 1·8 ml of buffer-substrate mixture. Incubation was for 30 min; P_i was determined by the method of Chen *et al.*[19] β-Glucuronidase was measured by the method of Talalay *et al.*[20] with phenolphthalein glucuronidate (0·01 M, Sigma) as substrate. Incubation was for 60 min with 0·5 ml of enzyme-rich supernatants, 1·5 ml acetate buffer (pH 4·5, 0·1 M) and 0·1 ml substrate. The reaction was stopped with 5 ml glycine buffer, 0·2 M in 0·2 M NaCl, pH 10·4. Aryl sulfatase was measured by a modification[18] of the procedure of Roy[21] with nitrocatechol sulfate (Sigma) as substrate. Enzyme-rich supernatants (0·2 ml) were incubated for 60 min at 37° with 0·2 ml acetate buffer (pH 5, 0·5 M) and 0·4 ml of 0·01 M substrate. Malate dehydrogenase, a soluble mitochondrial enzyme, was determined at 23° by the method of Mehler *et al.*[22] as previously described.[16] Croton oil, croton oil resin and phorbol ester fraction A each caused a concentration-dependent inhibition of malate dehydrogenase activity, assayed on samples in which the enzyme had been completely solubilized by 0·1% (v/v) Triton X-100. Therefore these activities have been corrected for the degree of inhibition caused by a given agent at each concentration tested, e.g. croton oil resin (500 μg/ml) inhibited malate dehydrogenase to 72·5 per cent of control values. Results are expressed as μmoles of $NADH_2$ oxidized/milligram of protein/min.

Monoamine oxidase, a marker enzyme for the outer mitochondrial membrane,[23] was determined by the method of Tabor *et al.*,[24] measuring the oxidative deamination of benzylamine at 30° by increments of absorbance at 250 mμ in a Gilford recording spectrophotometer. To 1 ml phosphate buffer (0·2 M, pH 7·2) were added 0·1 ml benzylamine sulfate (Fisher) and 0·01 to 0·05 ml of enzyme-rich supernatant; the final volume was adjusted to 3 ml with water.

Each of the enzymes assayed showed linear reaction kinetics over the period of assay and with appropriately varied amounts of enzyme preparations. With the exception noted above, the agents did not inhibit soluble enzyme activity. Protein was determined by the method of Lowry *et al.*[25] with standards of crystalline egg-white lysozyme.

Hemolysis

Lysis of rabbit erythrocytes was determined as previously described.[26] Solutions of test materials in DMSO (0·05 ml) were added to 0·5 ml of 4 × washed erythrocytes

(10%, v/v) and 2·0 ml of phosphate-buffered (0·01 M, pH 7·2) saline (0·145 M). In other tubes, similar numbers of red cells were suspended in distilled water and these were used to assess 100 per cent hemolysis. After incubation for 3 hr at 37°, the suspensions were centrifuged and the cell-free supernatants removed. The hemoglobin released was determined at 540 mμ in a Beckman DB spectrophotometer after adding 1·0 ml of supernatant to 4·0 ml of 0·6% ammonium hydroxide.

Preparation of phospholipid spherules and membrane-active agents

The preparation and properties of these artificial structures, which serve as models for biological membranes, have been extensively described.[11, 12, 27] Spherules were prepared to sequester chromate ions as a marker for their internal aqueous environment. Release of this divalent anion from its sequestration within the spherules into the suspending medium, NaCl–KCl (0·145 M), is considered to represent exchange diffusion across the individual lipid lamellae which compose the spherules. These were with molar ratios of ovolecithin, 70: cholesterol, 10: dicetylphosphate, 20. Release of anions was measured 30 min after addition of the test agent (in 0·05 ml DMSO) or solvent alone. Results are expressed as a percentage of anion released from solvent-treated control samples. Etiocholanolone was obtained from Steraloids Inc. (Pawling, N.Y.), filipin from the Upjohn Company, (Kalamazoo, Mich.) and Triton X-100 from Rohm & Haas (Philadelphia, Pa.).

RESULTS

Relationship of tumor-promoting to hemolytic activity. When five tumor-promoting agents were incubated with rabbit erythrocytes (Fig. 1), it was found that each agent

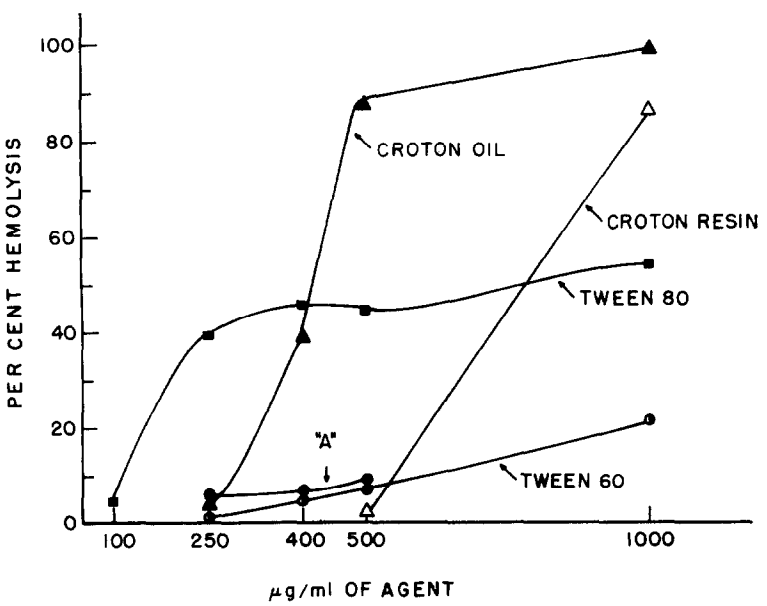

FIG. 1. Release of hemoglobin by tumor-promoting agents from washed rabbit erythrocytes incubated in buffered saline (pH 7·2) for 3 hr at 37°. Results expressed as percent of hemolysis in distilled water. Agents suspended in dimethylsulfoxide. "A" = phorbol esters (fraction A), see text. Means of 3 experiments.

was hemolytic. Yet the most active hemolysin (at 500 μg/ml) was crude croton oil, followed by Tween 80, phorbol esters (fraction A), Tween 60 and croton resin. If hemolytic activity was expressed as a function of the lowest quantity necessary to induce hemolysis, Tween 80 was most active, followed by phorbol esters, croton oil, Tween 60 and croton resin. This order bore no direct relationship to the order of tumor-promoting activity[2–4, 6] (Table 1). Indeed fraction A was 30,000 times more

TABLE 1. COMPARISON OF PROMOTING AGENTS*

Promoting agent†	Weekly dose (mg)	No. of applications per week	No. of mice with tumors/ total number of animals	References
Tobacco leaf extract	25·0	3	4/20	4
Tween 60	522·0	6	37/49	3
Croton oil	1·0	3	6/20	6
Croton resin	0·0075	3	15/20 (3)‡	6
Phorbol esters (fraction A)	0·0015	2	8/20 (1)‡	2

* Results at 1 yr.

† Following a single application of DMBA or benzo(a)pyrene.

‡ Number of mice with squamous carcinomas is given in parentheses.

effective than Tween 60 as a tumor-promoter but was not markedly hemolytic, while Tween 80 caused nearly 50 per cent hemolysis. Nevertheless, this group of compounds induced hemolysis of approximately the same order of magnitude as that induced by neutral steroids or bile acids.[26]

Relationship of tumor-promoting activity to concentration-dependent enzyme release from organelles. Six tumor-promoting agents were tested for their capacity to release lysosomal hydrolases from large-granule fractions of rabbit liver. Dose-response curves were plotted for each of the agents (Fig. 2). The data indicate that the order of activity of these compounds upon lysosomes paralleled their order of activity in tumor promotion, i.e. phorbol esters > croton oil resin > croton oil ⩾ Tween 80 > Tween 60 > tobacco leaf extract. Activity upon liver granules was examined in some detail by using the three promoting agents from *Croton tiglium L.* Release of two lysosomal hydrolases (β-glucuronidase, aryl sulfatase) was compared with release of malate dehydrogenase from mitochondria (Table 2). Both lysosomal hydrolases and malate dehydrogenase were released by the croton fractions. Indeed at 400 μg/ml, the most active tumor-promoting agent (phorbol esters or fraction A) released 71·6 and 68·8 per cent of β-glucuronidase and aryl sulfatase, respectively, while releasing 46·1 per cent of malate dehydrogenase. Furthermore, with progressively purer materials (from crude croton oil to croton oil resin to fraction A), the effect of a given concentration of agent upon lysosomes was progressively increased. Thus at 400 μg/ml, croton oil released 28·1 per cent, the resin 44·0 per cent and phorbol esters 71·6 per cent of β-glucuronidase. In contrast, at the same concentration, release of mitochondrial enzyme did not vary with degree of purity of the fractions (e.g. croton oil released 36·3 per cent, the resin 45·5 per cent and phorbol esters 46·6 per cent). Therefore both types of organelle were affected by tumor-promoting agents. Their

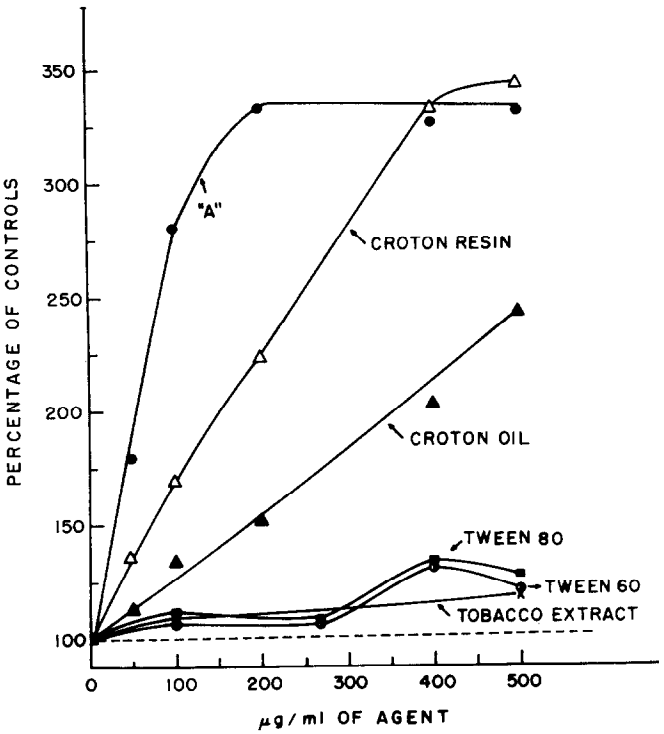

FIG. 2. Release of β-glucuronidase by tumor-promoting agents from large-granule fractions of rabbit liver (in 0·25 M sucrose) into 20,000 $g \times 20$ min supernatants. Results expressed as percent of enzyme released from control samples incubated with solvent (dimethylsulfoxide) alone at 37° for 60 min. Means of 3 experiments. "A" = phorbol esters (fraction A).

TABLE 2. RELEASE OF ENZYMES FROM LARGE-GRANULE FRACTION OF RABBIT LIVER

Agent	Concn (μg/ml)	Percentage of total enzyme activity in fraction* β-Gluc.	S.E.M.	Aryl sulf.	S.E.M.	Mal. dehy'ase	S.E.M.
Croton oil	1000	(4) 44·8	±12·2	(4) 49·0	±18·0	(2) 52·1	
	500	(6) 27·3	± 5·4	(4) 19·4	± 5·3	(4) 55·5	± 8·6
	400	(5) 28·1	± 3·4	(4) 22·1	± 7·3	(4) 36·3	± 7·2
	100	(3) 20·3	± 8·1	(4) 13·4	± 4·3	(4) 17·9	± 4·8
	50	(2) 16·3				(2) 15·6	
Croton oil resin	1000	(3) 52·0	±15·2	(4) 61·5	±14·3	(4) 61·0	±16·0
	500	(4) 49·0	±12·1	(4) 50·0	±11·7	(4) 65·0	±17·4
	400	(4) 44·0	±10·0	(4) 57·7	± 9·4	(4) 45·5	±12·1
	100	(4) 27·4	± 6·3	(2) 19·2	± 3·4	(3) 33·0	± 7·3
	50	(3) 20·8	± 5·2	(4) 18·1	± 2·5	(3) 18·6	± 4·1
Phorbol esters (fraction A)	400	(4) 71·6	±11·4	(4) 68·8	±12·8	(4) 46·1	± 8·4
	100	(3) 34·8	± 9·5	(4) 31·7	± 7·4	(2) 25·0	
	50	(3) 21·2	± 4·3	(4) 18·7	± 2·9	(2) 19·7	
Controls	—	(22) 15·9	± 2·8	(8) 9·7	± 2·1	(8) 15·8	± 3·1

* Results (±S.E.M.) represent enzyme activities released into supernatants (20,000 g, 20 min) of large-granule fractions from rabbit liver and are expressed as percentage of total enzyme present in such supernatants after treatment with Triton X-100, 0·1% (v/v). Absolute total values are: β-glucuronidase (β-Gluc.), 142 μg phenolphthalein released/mg protein/hr; aryl sulfatase (aryl sulf.), 104 μg nitocatechol released/mg protein/hr.; and malate dehydrogenase (Mal. dehy'ase), 30 μm $NADH_2$ oxidized/mg protein/min. Numbers in parentheses represent number of experiments.

order of activity upon lysosomes, however, appeared to be more directly related to tumor-promoting action than did their effect upon mitochondria.

Another possible action of croton fractions upon mitochondria was tested. Monoamine oxidase has been shown to be associated with the outer membrane of the mitochondria, disruption of which by agents such as digitonin releases monoamine oxidase activity from particulate form.[23] Therefore the release of monoamine oxidase was determined simultaneously with release of aryl sulfatase (Fig. 3). Whereas croton

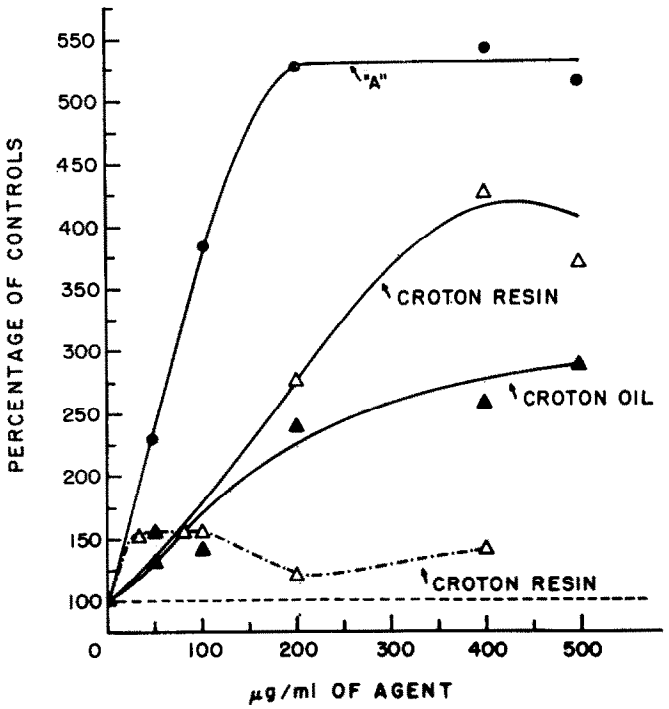

FIG. 3. Release of aryl sulfatase (———) and monoamine oxidase (-·-·-·-) by tumor-promoting agents from large-granule fractions of rabbit liver (in 0·25 M sucrose) into 20,000 $g \times 20$ min supernatants. Results expressed as percent of enzyme released from control samples incubated with solvent (dimethylsulfoxide) alone at 37° for 60 min. Means of 3 experiments. "A" = phorbol esters (fraction A).

oil resin liberated significant quantities of the lysosomal hydrolase, it had no effect upon monoamine oxidase (see below).

Time and temperature dependence of enzyme release. Release of lysosomal hydrolases was also studied as a function of time and temperature. The three preparations from *Croton tiglium L.* maintained their order of activity in promoting release of aryl sulfatase (Fig. 4), β-glucuronidase (Fig. 5) and acid phosphatase (Fig. 6). Indeed the kinetics of enzyme release were related to the purity of tumor-promoting preparations; phorbol esters (fraction A) were more rapid in onset of action than the resin and this preparation exceeded crude croton oil in onset. Although there was some variation in the pattern of release among the three hydrolases, the initial slopes of release (0–30 min) clearly indicated the order of activity. Such variations have been described

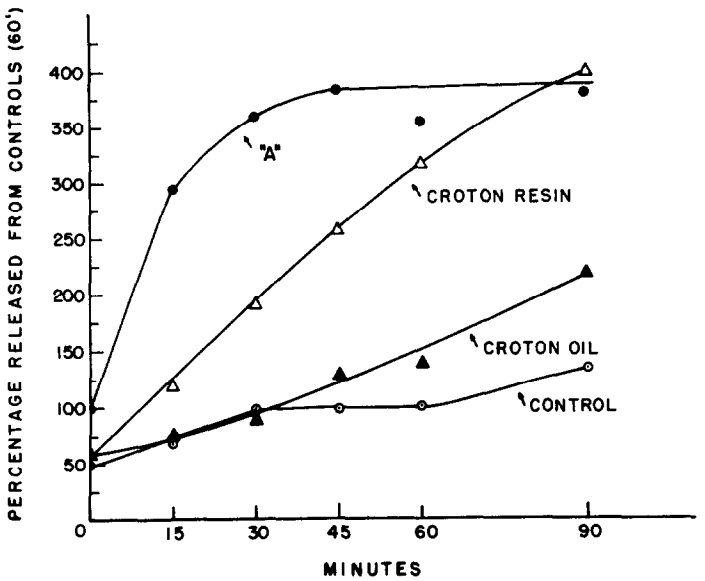

FIG. 4. Release of β-glucuronidase by tumor-promoting agents (500 μg/ml) from large-granule fractions of rabbit liver (in 0·25 M sucrose) into 20,000 *g* × 20 min supernatants. Results expressed as percent release from control samples incubated with solvent (dimethylsulfoxide) alone at 37° for 60 min. Means of 3 experiments. "A" = phorbol esters (fraction A).

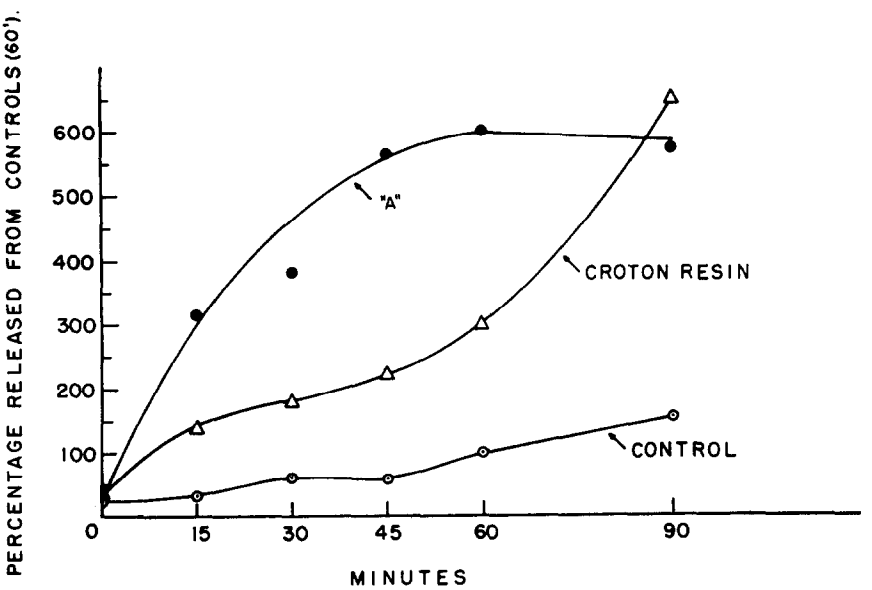

FIG. 5. Release of aryl sulfatase by tumor-promoting agents (500 μg/ml) from large-granule fractions of rabbit liver (in 0·25 M sucrose) into 20,000 *g* × 20 min supernatants. Results expressed as per cent released from control samples incubated with solvent (dimethylsulfoxide) alone at 37° for 60 min. Means of 3 experiments. "A" = phorbol esters (fraction A).

previously and may be due to the heterogeneity of lysosomes or to the differential access of lytic agents to the target organelles, or to both.[10, 15, 16]

The rates of release of β-glucuronidase by croton oil resin were compared at various temperatures (Fig. 7). Release was both markedly accelerated and augmented at higher temperature. This temperature-dependence is entirely analogous to the effects of several other (nondetergent) agents which disrupt lysosomes: vitamin A, steroids, polyenes, etc.[9, 10, 16]

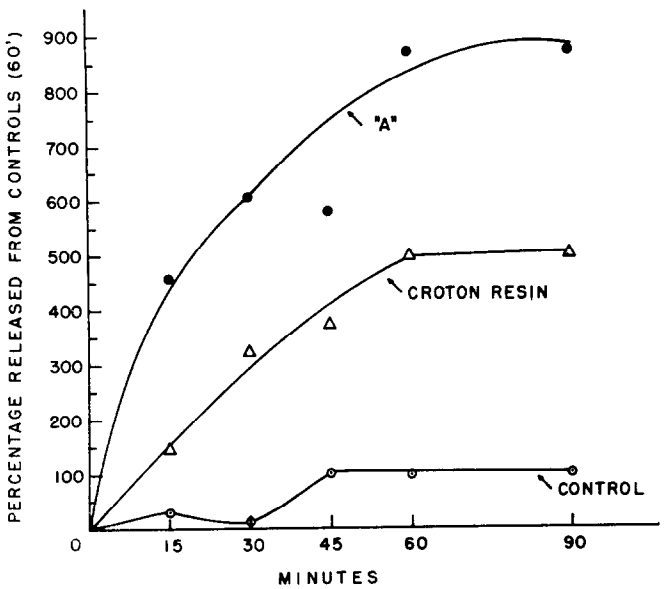

FIG. 6. Release of acid phosphatase by tumor-promoting agents (500 μg/ml) from large granule fractions of rabbit liver (in 0·25 M sucrose) into 20,000 *g* × 20 min supernatants. Results expressed as percent release from control samples incubated with solvent (dimethylsulfoxide) alone at 37° for 60 min. Means of 3 experiments. "A" = phorbol esters (fraction A).

Croton resin did not release comparable amounts of monoamine oxidase (Fig. 7). Complete release of either lysosomal hydrolase or mitochondrial oxidase was achieved at all three temperatures after incubation (60 min) with Triton X-100. Plotted as a function of this value, the data suggest that solubilization of acid hydrolases was not accompanied by significant release of outer membrane-associated enzymes from mitochondria.

Effect of carcinogens and phorbol esters(A) on release of enzymes. Because croton oil is a complex chemical mixture and croton resin is a mixture of phorbol esters with substantial range of molecular weights, it was not possible to express their concentrations in molarity. Therefore only phorbol esters, fraction A (mol. wt., approx. 600) were compared with other carcinogens as to activity upon lysosomes. Three well known tumor-initiating compounds (DMBA, 3-methylcholanthrene and β-propiolactone) did not release significant β-glucuronidase activity (Table 3). The liver carcinogen, AAF, had no effect on the granules, whereas its suggested "proximate carcinogenic metabolite",[28] *N*-hydroxy-AAF, released β-glucuronidase at a concentration of 10^{-8} M, an order of magnitude above that required for the phorbol ester. The

data suggest that these cutaneous and systemic carcinogens, some of which are also tumor-initiating agents, do not share the capacity to disrupt lysosomes from liver. Clearly, further experiments with "proximate carcinogens" are required to exclude effects of other agents in this class.

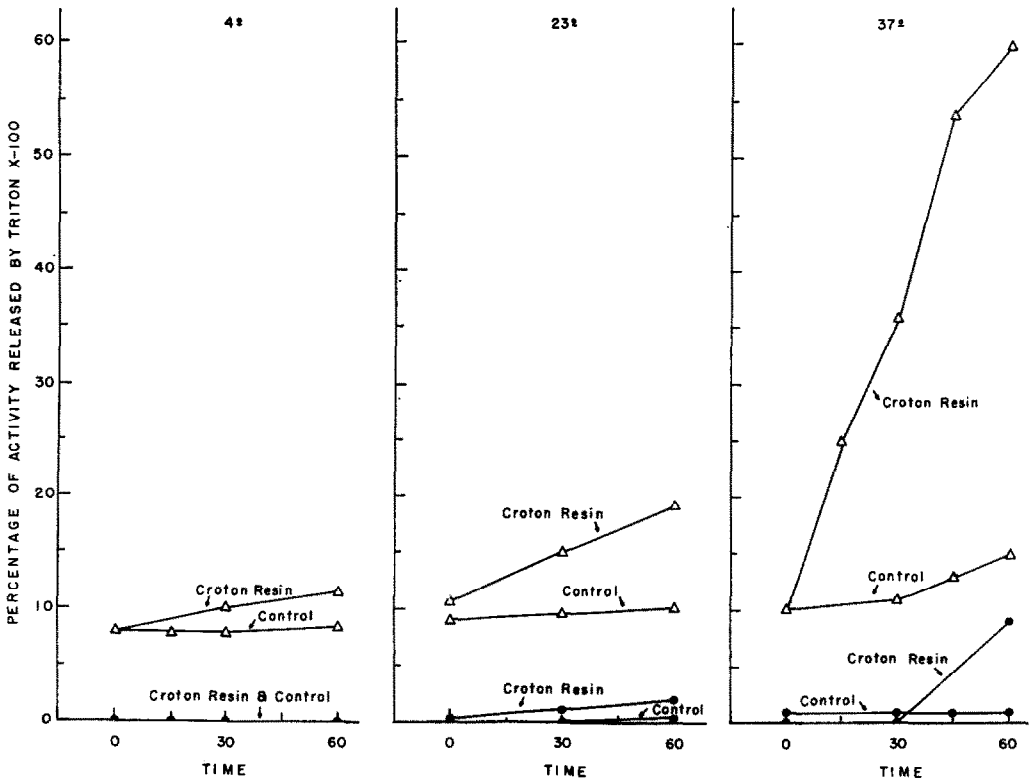

FIG. 7. Release of beta-glucuronidase (△—△) and monoamine oxidase (●—●) by croton resin (500 μg/ml) from large-granule fractions of rabbit liver (in 0·25 M sucrose) into 20,000 $g \times 20$ min supernatants. Results expressed as percent released into supernatants after incubation with 1% Triton X-100 for 60 min at each temperature. Controls (5 ml) incubated with solvent (dimethyl, sulfoxide) alone (0·5 ml).

TABLE 3. RELEASE OF BETA-GLUCURONIDASE FROM RABBIT LIVER GRANULES INDUCED BY CARCINOGENS AND PHORBOL ESTER FRACTION A*

Agent	Concentration (M)		
	10^{-3}	10^{-4}	10^{-5}
Phorbol ester (fraction A)	514	228	101
N-OH acetylaminofluorene	288	106	98·5
3-Methylcholanthrene	128	98·4	95·0
Beta-propiolactone	122	89·5	
DMBA (7,12-dimethybenz(a)-anthracene)	120	105	89·4
Acetylaminofluorene	102	92·4	93·5

* Expressed as per cent of enzyme activity released from controls + solvent (DMSO) at 60 min, 37°; mean of 3 experiments.

Release of marker anions from artificial lipid spherules. Artificial lipid spherules (ovolecithin, 70: cholesterol, 10: dicetylphosphate, 20; molar ratios) were prepared to sequester chromate as marker anion. Release of anion from the spherules was measured by its absorbance at 340mμ after the test agents in dimethylsulfoxide, or the solvent alone, were added to the lipid suspensions (15 μmole lipid/ml). One representative agent from each of three groups of surfactants was chosen as a "positive control". At widely varying concentrations, filipin (a polyene antibiotic) Triton X-100 (a non-ionic detergent) and etiocholanolone (a steroid) induced gross changes in the overall permeability of the lipid structures to cations (Table 4). In contrast, three tumor-

TABLE 4. RELEASE OF $CrO_4^=$ FROM ARTIFICAL LIPID SPHERULES

Agent	Concn (mg/ml)	Per cent anion released by controls at 30 min
Control*		100
Triton X-100	2·0	1080
Filipin	0·057	845
Etiocholanolone	1·45	330
Tween 60	1·0	130
	2·0	160
Tween 80	1·0	180
	2·0	190
Phorbol esters (fraction A)	0·60	91
Croton oil resin	1·0	108
	3·0	95
Croton oil	1·0	110
	3·0	92

* Controls = 7·1 per cent of total trapped anions. Results represent means of 3 experiments.

promoting agents from *Croton tiglium L.* were devoid of action upon the spherules. Tween 80 and Tween 60 disrupted the spherules to a far lesser extent than did Triton, filipin or etiocholanolone. Since each of the latter membrane-disruptive agents was as active upon erythrocytes and lysosomes[11,12,26] as upon artificial structures, the relative lack of effect of tumor-promoting agents upon lipid spherules is unusual.

DISCUSSION

The experiments detailed above demonstrate a new biological property which is shared by several tumor-promoting agents: the capacity to disrupt the membranes of lysosomes, mitochondria and erythrocytes. While this behavior was, in part, predictable on the basis of their amphipathic structure, it is still not clear whether the agents interact with lipids or with other components of biological membranes. Indeed, since the phorbol esters and croton oil failed to interact with artificial lipid spherules (which resemble natural organelles in responding to the lytic effects of steroids, polyene antibiotics, toxins, etc.), it becomes probable that their primary reaction is with polysaccharide or protein. Protein, in fact, may constitute at least some of the sub-units of a mosaic, micellar structure within biomembranes and it has been postulated that globules of proteins may be intercalated as hydrophobic-hydrophilic areas among arrays of lipid globules.[29] The alternate explanation for a relative lack of response by artificial lipid spherules to tumor-promoting agents is that the spherules are inadequate

models for even the lipid "skeleton" of biomembranes. This possibility cannot be excluded, although a remarkable correspondence between the behavior of the model structures and natural membranes has been described in recent reviews.[27, 30]

Therefore the intimate mechanisms whereby tumor-promoting agents disrupt biological membranes are unclear. It is nevertheless noteworthy that their order of activity upon lysosomes so closely resembled their order of activity in tumor promotion. This correspondence did not extend to their order of activity upon mitochondria or upon erythrocytes. Moreover, with progressive purification of fractions from the best known tumor-promoting agent (croton oil), these became progressively more active upon lysosomes. Differential effects of membrane-active agents have been described before. Thus mitochondria are less sensitive than lysosomes to streptolysins, but are more readily disrupted by vitamin A at 23°.[16] Polyene antibiotics disrupt lysosomes, but have no effect on mitochondria,[10] and Allison and Dingle have shown that whereas lysosomes from adrenal are disrupted by 7-hydroxy-methyl-12 methyl-(a)benzanthracene and DMBA, those from liver are unaffected.[31] The latter part of their experiment is confirmed by our findings. Such differences *in vitro* may not, however, necessarily reflect differential susceptibility of cells or organelles *in vivo*. Indeed, the outer membranes of cells other than the erythrocyte (i.e. mouse epidermis) may respond more nearly like rabbit liver lysosomes to the various promoters. More direct evidence to this point could have been obtained if tumor-promoting agents had been added to lysosomes from mouse skin, a tissue particularly susceptible to studies of tumor initiation and promotion.[32] Unfortunately, although mouse epidermis is rich in lysosomes,[33] it is notoriously difficult to homogenize adequately. Furthermore, it remains to be demonstrated that lysosomes in intact mouse skin are affected by the tumor-promoting agents, and such experiments are in progress.[34] Finally, the disparity should be noted between tumor-promoting doses *in vivo* (Table 1) and the larger amounts required to disrupt lysosomes *in vitro*.

Indeed, the correspondence of tumor-promoting activity with capacity to disrupt lysosomes could conceivably be fortuitous. On the other hand, this relationship suggests several approaches to the mode of action of tumor-promoting agents. Allison and Mallucci[35] found that "hydrocarbon carcinogens such as DMBA, methylcholanthrene and benzpyrene", were each localized to lysosomes of macrophages and monkey kidney cells *in vitro*, as judged by fluorescence microscopy. Moreover, they demonstrated that DMBA was concentrated in lysosomes of mouse connective tissue cells and they reported histochemical experiments which indicated that the permeability of lysosomes of macrophages and monkey kidney cells was increased after treatment with croton oil and Tween 60. These experiments were used as evidence for their hypothesis of chemical carcinogenesis, which suggested that tumor-promoting agents release lysosomal enzymes by potentiating the disruptive effects of initiators. Release of lysosomal hydrolases was thought to account for the induction of chromosomal injury and subsequent neoplasia.[36]

This hypothesis seems quite unlikely in view of the long interval that can elapse between initiation and promotion (1 yr), since the initiating agent would have to be sequestered in, and be disruptive to, lysosomes for the entire period. It is known, for example, that DMBA disappears from the site of application between 24 and 48 hr.[37] Such tumor-initiators as β-propiolactone and urethane are diffusible, water-soluble compounds which would not be expected to be localized in lysosomes. In addition,

β-propiolactone has a half-life of less than 1 hr[38] and would not be expected to survive intact in the lysosome. Indeed its activity has been related to its capacity to form an N-7 adduct to the guanine of DNA.[39]

Because of these data, and others which suggest the interaction of tumor-initiating agents with DNA,[28] an alternate hypothesis can be considered. Initiating agents may induce somatic mutations by virtue of their direct interaction with DNA. Under conditions of widespread gene activation (derepression), the chances for expression of such an error would be increased manyfold. Lysosomal hydrolases have been implicated in the derepression of lymphocytes by phytohemagglutinin[40] and the role of proteases in hydrolysing repressor materials of mammalian cells has been studied in detail.[41] Thus a number of nonspecific stimuli (viruses, hyperoxia, etc.) as well as tumor-promoting agents release enzymes from lysosomes[36] which could potentially increase the expression of altered DNA. Indeed phorbol esters are known to induce the expression of neoplastic potential in a cell culture system, and it was suggested that the phorbol esters might interact with biomembranes so as to induce loss of control of cell division.[42]

Whatever the general implications of the data prove to be, the findings that tumor-promoting agents from *Croton tiglium L.* act to disrupt lysosomes and cell membranes may also provide an explanation, at least in part, for the irritative and inflammatory properties of croton oil. Although phorbol esters can clearly promote tumors at doses below which skin necrosis or cell death is evident,[6] crude croton oil has for some time been a model "nonspecific" inflammatory agent.[43] This agent can therefore be added to the list of compounds which possess both the capacity to disrupt biomembranes and to provoke inflammation.

Acknowledgement—The invaluable technical assistance of Miss Kathrin Krakauer is gratefully acknowledged.

REFERENCES

1. B. L. VAN DUUREN, L. ORRIS and E. ARROYO, *Nature, Lond.* **200**, 1115 (1963).
2. B. L. VAN DUUREN and L. ORRIS, *Cancer Res.* **25**, 1871 (1965).
3. H. SETALA, *Acta path. microbiol. scand. suppl.* **115**, 7 (1956).
4. B. L. VAN DUUREN, A. SIVAK, A. SEGAL, L. ORRIS and L. LANGSETH, *J. natn. Cancer Inst.* **37**, 519 (1966).
5. W. E. HESTON and A. W. PRATT, *J. natn. Cancer Inst.* **22**, 707 (1959).
6. B. L. VAN DUUREN, *Prog. exp. Tumor Res.* in press.
7. R. C. PETTERSEN and G. FERGUSON, *Chem. Communications, Lond.* **14**, 716 (1967).
8. W. HOPPE, F. BRANDL, I. STRELL, M. ROHRL, J. GASSMANN, E. HECKER, H. BARTSCH, G. KREIBICH and CH. V. SZCZEPANSKI, *Angew. Chem.* **79**, 824 (1967).
9. G. WEISSMANN, *Biochem. Pharmac.* **14**, 537 (1965).
10. G. WEISSMANN, G. SESSA, M. PRAS, V. A. H. BEVANS and R. HIRSCHHORN, *Biochem. Pharmac.* **16** 1057 (1967).
11. G. WEISSMANN and G. SESSA, *J. biol. Chem.* **242**, 616 (1967).
12. A. D. BANGHAM, M. M. STANDISH and G. WEISSMANN, *J. molec. Biol.* **13**, 253 (1965).
13. E. C. MILLER, J. A. MILLER and H. A. HARTMANN, *Cancer Res.* **21**, 815 (1961).
14. K. SAITO and E. SUTER, *J. exp. Med.* **121**, 739 (1965).
15. G. WEISSMANN and J. W. UHR, *Biochem. Pharm. suppl.* **17**. 5 (1268).
16. H. KEISER, G. WEISSMANN and A. W. BERNHEIMER, *J. Cell Biol.* **22**, 101 (1964).
17. W. N. VALENTINE and W. S. BECK, *J. Lab. clin. Med.* **38**, 39 (1951).
18. R. HIRSCHHORN, K. HIRSCHHORN and G. WEISSMANN, *Blood* **30**, 84 (1967).
19. P. S. CHEN, T. Y. TORIBARA and H. WARNER, *Analyt. Chem.* **28**, 1756 (1956).

20. P. TALALAY, W. H. FISHMAN and C. HUGGINS, *J. biol. Chem.* **166**, 757 (1946).
21. A. B. ROY, *Biochem. J.* **53**, 12 (1953).
22. A. H. MEHLER, A. KORNBERG, S. GRISOLIA and S. OCHOA, *J. biol. Chem.* **174**, 961 (1948).
23. C. SCHNAITMAN, G. U. ERWIN and J. W. GREENAWALT, *J. Cell. Biol.* **32**, 719 (1967).
24. C. W. TABOR, H. TABOR and S. M. ROSENTHAL, *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), vol. 11, p. 390. Academic Press, New York (1955).
25. C. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951)
26. G. WEISSMANN and H. KEISER, *Biochem. Pharmac.* **14**, 537 (1965).
27. G. SESSA and G. WEISSMANN, *J. Lipid Res.*, **9**, 3 (1968).
28. E. C. MILLER and J. A. MILLER, *Pharmac. Rev.* **18**, 805 (1966).
29. J. A. LUCY and A. M. GLAUERT, *J. molec. Biol.* **8**, 727 (1964).
30. A. D. BANGHAM, *Progr. Biophys. biophys. Chem.* in press.
31. A. C. ALLISON and J. T. DINGLE, *Nature, Lond.* **209**, 303 (1966).
32. I. BERENBLUM and P. SHUBIK, *Br. J. Cancer* **1**, 383 (1947).
33. J. SUGAR, *Acta morph. hung.* **15**, 93 (1967).
34. A. SIVAK and B. L. VAN DUUREN, unpublished experiments.
35. A. C. ALLISON and L. MALLUCCI, *Nature, Lond.* **203**, 1024 (1964).
36. A. C. ALLISON, *Scient. Am.* **217**, 62 (1967).
37. F. BOCK, *Prog. exp. Tumor Res.* **4**, 131 (1964).
38. B. L. VAN DUUREN and B. M. GOLDSCHMIDT, *J. med. Chem.* **9**, 77 (1966).
39. N. H. COLBURN and R. K. BOUTWELL, *Cancer Res.* **26**, 1701 (1966).
40. G. WEISSMANN, W. TROLL, G. BRITTINGER and R. HIRSCHHORN, *J. Cell. Biol.* **35**, 140A (1967).
41. V. G. ALLFREY, V. C. LITTAU and A. E. MIRSKY, *Proc. natn. Acad. Sci. U.S.A.* **49**, 414 (1963).
42. A. SIVAK and B. L. VAN DUUREN, *Science* **157**, 1443 (1967).
43. A. ROBERT and J. NEZAMIS, *Acta endocr., Copenh.* **25**, 105 (1957).