

Decrease of Epidermal Histidase Activity by Tumor-promoting Phorbol Esters¹

Nancy H. Colburn,² Shigeko Lau, and Rebecca Head

Departments of Dermatology and Biological Chemistry, The University of Michigan Medical School, Ann Arbor, Michigan 48104

SUMMARY

The potent skin tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) stimulates epidermal macromolecular synthesis as well as proliferation, but little is known of specific functional aberrations produced by TPA. This report presents results of a study on the effects of TPA on epidermal histidase (L-histidine ammonia lyase), an enzyme found in normal epidermis but not in dermis or in mouse squamous cell carcinomas. Histidase activity was assayed on postmitochondrial supernatants obtained from hairless mouse epidermis after removal by keratome. Topical TPA treatment at doses active in tumor promotion (1.7 to 17.0 nmoles/application) produced dose-dependent decreases in epidermal histidase specific activity at 19 hr posttreatment. The onset of the decrease occurred at 12 hr with recovery to control level specific activity by 5 days, showing kinetics similar to those obtained for stimulation of DNA synthesis. This decrease in histidase could not be attributed to a general inhibition of soluble protein synthesis or to the appearance of an inhibitor of histidase activity. The strong promoter TPA produced a greater histidase decrease than did the moderate promoter and mitogen 12,13-didecanoyl phorbol at equimolar dose, while phorbol, a nonpromoter and nonmitogen, produced no effects on histidase. The relationship of this histidase depression to tumor promotion and not initiation is further indicated by the finding that (a) Tween 60, a structurally unrelated tumor promoter, also produced a decrease in histidase; and (b) the tumor initiator urethan and an initiating dose of 9,10-dimethylbenz(a)anthracene showed no effects on histidase activity.

INTRODUCTION

The enzyme histidase (L-histidine ammonia lyase) occurs in normal adult epidermis of a number of species (3) but is absent from dermis (8) and from early fetal epidermis (4).

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² Recipient of NIH Special Research Fellowship 5 FO3-AM54259-02. To whom requests for reprints should be addressed, at Department of Environmental and Industrial Health, 1620 School of Public Health I, The University of Michigan, Ann Arbor, Mich. 48104.

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Possible involvement of the enzyme in the neoplastic process has been suggested by the finding that histidase activity is very low or absent from mouse and human squamous cell carcinomas (2, 4). In addition, rat liver histidase decreases in response to continuous feeding of hepatic carcinogens, diethylnitrosamine, acetylaminofluorene, and dimethylaminoazobenzene (20-22), as well as in several hepatomas (16).

Tumor-promoting phorbol esters have been shown to stimulate epidermal DNA, RNA, protein and phospholipid synthesis, and histone phosphorylation and synthesis, as well as mitosis³ (7, 13, 19, 23, 26, 28, 29, 33). Recently, phorbol esters have been shown to produce changes in certain cell membrane proteins (18, 33) as well as enhancement of certain soluble protein bands (30, 31).

Although ultrastructure studies suggest changes in the differentiated state of the epidermis following phorbol ester treatment (23, 24), it is not clear what biochemical changes are directly associated with the observed morphological changes. Since histidase may be considered a marker enzyme of differentiated epidermis and since the enzyme has been implicated in the neoplastic process, we asked whether epidermal histidase might be subject to regulation during skin carcinogenesis. The present report deals with the effect of tumor-promoting phorbol esters on epidermal histidase and its relationship to tumor-promoting action.

MATERIALS AND METHODS

TPA⁴ was obtained from Schuchardt via Consolidated Midland Corp., Brewster, N. Y. DDP and phorbol were the gifts of Dr. Tucker Helmes and Dr. R. K. Boutwell of the McArdle Laboratory. Reagent grade acetone was used for solution preparation. Concentrations of phorbol and phorbol ester solutions were determined by using the molar absorbance values of $\epsilon_{234} = 4370$ in 95% ethanol, $\epsilon_{232} = 5400$ in 95% ethanol, $\epsilon_{233} = 5170$ in methanol, for phorbol, DDP, and TPA, respectively. Urethan and Tween 60 were obtained from Sigma Chemical Co., St. Louis, Mo., and DMBA was obtained from K and K Laboratories, Plainview, N. Y. Male hairless mice were obtained from

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⁴ The abbreviations used are: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; DDP, 12,13-didecanoyl phorbol; DMBA, 9,10-dimethylbenz(a)-anthracene.

The Jackson Laboratory, Bar Harbor, Maine, and used at 3 to 4 months of age. Male Swiss haired mice (CD-1) were obtained from Charles River Breeding Laboratories and used at 2 to 4 months of age.

Treatments with Tumor Promoters. Hairless mice were treated both on the dorsal and ventral surfaces with 0.1 ml of an acetone solution containing the specified concentration of tumor promoter or initiator. At times from 4 hr to 5 days posttreatment the mice were killed by cervical dislocation, and the treated epidermis was removed (about 16 sq cm and 50 mg/mouse) by a Castroviejo keratome using a 0.2-mm setting. The epidermis so obtained was greater than 90% pure as judged by light microscopy. This purity was obtained whether or not the mice had been previously treated with tumor promoters. The epidermis was immediately dropped into liquid nitrogen; then epidermis pooled from 2 to 3 mice/sample was pulverized in liquid nitrogen using a mortar and pestle. Three- to 4-month-old CD-1 haired male mice were shaved on the back 1 day prior to treatment (0.2 ml of acetone solution). Immediately after being killed, the CD-1 mice were placed on ice and treated with Nair (Carter Products, Cranbury, N.J.) for 8 min; then the skins were immediately washed thoroughly with cold water and blotted dry, and epidermis was removed by keratome. In some experiments, Nair treatment (3 min) was carried out 3 days prior to tumor promoter treatment.

Assay of Histidase-specific Activity. Pulverized epidermis was homogenized in 3 ml of a buffer containing 0.006 M 2-mercaptoethanol; 0.25 M sucrose; 0.01 M Tris, pH 7.5; 0.06 M KCl; and 0.005 M $MgCl_2$ using a Polytron homogenizer (Brinkmann) 15 sec at setting 6 and adjusted to a volume:weight ratio of 3 ml/0.1 g. The homogenate was centrifuged at $23,000 \times g$ for 20 min, and the resulting supernatant was dialyzed 18 hr against 0.01 M sodium pyrophosphate, pH 9.2, at 4° to give the fraction designated as S-23, which was used as enzyme source in the histidase assay⁵ (200 to 500 μg protein per reaction tube). Histidase was assayed essentially according to the procedure of Barnhisel *et al.* (8), in which the formation of urocanic acid from histidine was determined spectrophotometrically at 277 nm. The only modifications in the procedure consisted of the addition of $ZnSO_4$, 2×10^{-3} M, to the reaction mixture and the omission of glutathione. (Under these assay conditions, the $ZnSO_4$ addition was found to ensure optimal and consistent reaction rates, whereas the glutathione showed no effect.) The histidase reaction was linear for at least 2 hr. Aliquots of the reaction mixture were routinely taken at 0, 15, and 30 min, and the reaction rate was determined from the resulting plot of the kinetics of urocanic acid formation. One unit of histidase is defined as 1 nmole urocanic acid formed per min. The molar extinction coefficient of 18,100 at pH 1 was used.

Protein concentration in the S-23 fraction was determined according to the procedure of Lowry *et al.* (17), and histidase-specific activity was routinely expressed as units/mg soluble protein. In addition, aliquots of the

⁵ Initially, it was found that histidase-specific activity in the $150,000 \times g$ supernatant was not significantly different from that in the $23,000 \times g$ supernatant. The $23,000 \times g$ supernatant was subsequently used routinely.

original homogenates were assayed for DNA content by the diphenylamine assay for deoxyribose (11). Histidase-specific activity was frequently expressed as units/mg DNA in addition to units/mg protein.

RESULTS

Effects of TPA on Epidermal Histidase. Hairless mice were treated once with 17 nmoles TPA per application, a standard dose used frequently by others to elicit maximal tumor yield and stimulation of DNA synthesis (5, 7, 25). Chart 1 shows the kinetics of TPA action on epidermal histidase. Histidase-specific activity decreased to about 45% of control level at 19 to 24 hr. The onset of the decrease occurred at 12 hr posttreatment, with a return to nearly control level specific activity by 5 days.

Chart 2 shows the variation with TPA dose of this decrease in histidase. The effective range used in tumor promotion, namely 1.7 to 17 nmoles/application, gave a dose-dependent histidase decrease with little additional effect and considerable skin damage above the standard 17-nmole level.

Since the bulk of tumor promotion data with phorbol esters has been obtained using Swiss haired mice, we assayed Charles River CD-1 mice for histidase-specific activity following TPA treatment, with the results shown in Table 1. Despite the relatively tedious procedure and the relatively high standard error of results, TPA was found to produce a decrease in epidermal histidase-specific activity in CD-1 mice. This depression occurred whether the mice were treated once with TPA or repeatedly following initiation by DMBA as in a tumor experiment.

Mechanism of Histidase Decrease. Regarding the mechanism of the depression in histidase, we considered 5

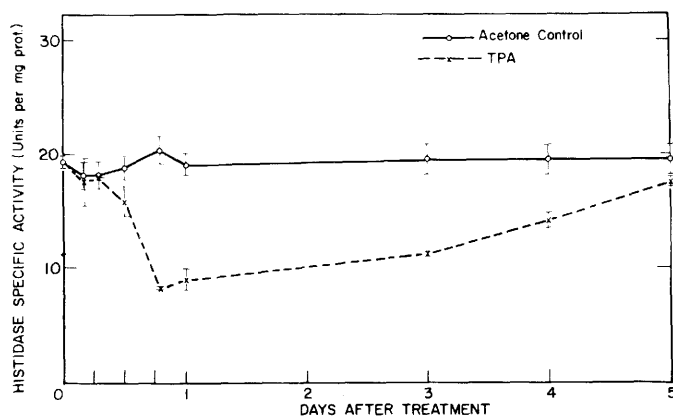


Chart 1. Kinetics of TPA effect on epidermal histidase. Three- to 4-month-old male hairless mice were treated once with TPA in acetone (17 nmoles/0.1 ml). Each mouse received 0.1 ml on the dorsal surface and 0.1 ml on the ventral surface. At 0, 4, 7, 12, 19, and 24 hr and 3, 4, and 5 days after treatment, mice were killed, and the treated epidermis was immediately frozen in liquid nitrogen and the S-23 supernatant was isolated and assayed for histidase activity and protein concentration as described in "Materials and Methods." Histidase-specific activity is expressed as units (nmoles urocanic acid formed per min) per mg soluble protein (*prot.*). Each *point* represents the mean of 3 to 9 separate groups of 2 mice each; *vertical lines*, S.E.

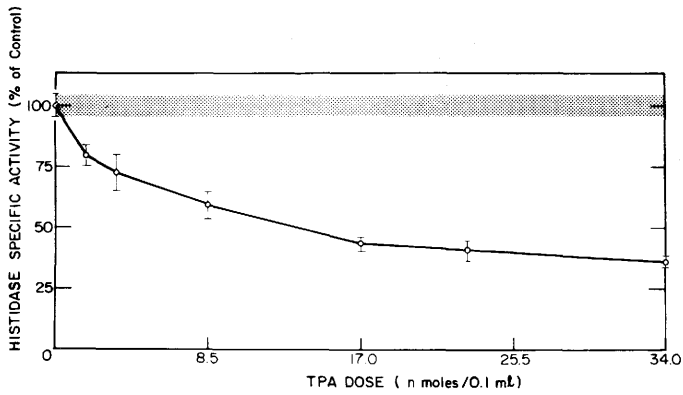


Chart 2. Effect of TPA dose on histidase-specific activity. The procedures for TPA treatment, isolation of epidermal S-23 supernatant, and assay of histidase-specific activity are described in the legend to Chart 1. TPA concentration was varied from 1.7 to 34.0 nmoles/0.1 ml acetone solution, and epidermal S-23 was assayed 19 hr following treatment. Histidase-specific activity is expressed in units/mg soluble protein, as percentage of the value for acetone control. Each point represents the mean \pm S.E. of 3 to 6 separate groups of 3 mice each.

Table 1

Histidase-specific activity in CD-1 haired mouse epidermis after tumor promoter treatment

Hair was removed from CD-1 mice by Nair treatment and epidermis was obtained as described in "Materials and Methods."

Treatment	Histidase-specific activity (units/mg protein)	
	CD-1	Hairless
Acetone control	12.5 \pm 1.8 ^{a, b}	14.0 \pm 1.6 ^c
TPA once (17 nmoles/application)	8.3 \pm 0.9 ^b	
Initiated, then TPA 2 times per wk ^{d, e}	8.9 \pm 2.0 ^f	9.4 \pm 0.6 ^c
DMBA 2 times per week (25 μ g/application ^d)	6.3 \pm 0.9 ^f	

^a Mean \pm S.E.

^b Assayed 20 hr after treatment.

^c Assayed 24 hr after the last treatment.

^d Assayed after 25 to 35 weeks of treatment and after appearance of carcinomas in some mice.

^e Hairless mice were initiated with β -propiolactone, 240 μ moles 2 times per week for 2 weeks; CD-1 mice were initiated with DMBA (50 μ g). TPA dose for hairless mice, 17 nmoles/application; for CD-1 mice, 3.4 nmoles/application.

^f Assayed 1 week after the last treatment.

possibilities: (a) that the decreased specific activity is an artifact due to choice of an inappropriate denominator; (b) that in response to TPA there is a decrease in histidase synthesis as part of an overall decrease in the rate of soluble protein synthesis; (c) that an inhibitor of histidase activity appears following TPA treatment; (d) that histidase activity decreases due to a structural change that gives rise to a higher K_m ; and (e) that TPA produces a real decrease in the amount of histidase enzyme present due to an increased rate of degradation or a decreased rate of synthesis.

Since soluble protein concentration might be an inappropriate denominator for expressing histidase-specific activity, we also calculated the results using a different data base,

namely DNA concentration. Typical results shown in Table 2 indicate that TPA treatment produced a substantial decrease in histidase-specific activity expressed as units/mg DNA. The magnitude of the decrease was similar although usually about 5 to 10% less than the decrease in specific activity expressed as units/mg protein.

Since the decreased specific activity might arise completely from an increase in both DNA and soluble protein following TPA treatment, we tested the possibility that the amount of soluble protein or DNA per unit wet weight of epidermis might increase significantly following TPA treatment. Table 3 shows that TPA treatment produced a small but insignificant increase in soluble protein content and a small but insignificant decrease in DNA content of hairless mouse epidermis at 19 hr posttreatment. In contrast, histidase activity/mg wet weight showed a significant decrease following TPA treatment.

Having established that the histidase depression was real, we next tested the possibility of a general decrease in the rate of soluble protein synthesis as shown in Chart 3. The rate of soluble protein synthesis was not decreased during the 1st 48 hr after TPA treatment but was substantially stimulated from 7 to 24 hr posttreatment, similar to the reported effect on total protein synthesis (7).

A histidase inhibitor, if it appeared in response to TPA treatment, would be expected to inhibit the control enzyme. The presence of a dialyzable inhibitor would be ruled out, since supernatant fractions were routinely dialyzed prior to histidase assay. The possibility of a nondialyzable inhibitor was tested by mixing supernatants from *in vivo* TPA-treated mice with control supernatants as indicated in Table 4. The specific activity of the mixed fractions was almost identical to the theoretical value for no inhibitor.

Determination of the K_m for histidase following TPA treatment (20 hr) yielded a K_m value for histidine of $7.5 \times 10^{-4} \pm 2.4 \times 10^{-5}$ M compared with $7.5 \times 10^{-4} \pm 8.8 \times 10^{-5}$ M for the control enzyme.

Relationship of Histidase Levels to Tumor Promotion. We next investigated the possible relationship of the enzyme decrease to tumor-promoting activity of phorbol esters. Comparison of the effects of TPA, a strong promoter, with

Table 2

Effect of data base on expression of TPA effects on histidase-specific activity

Experiment	TPA concentration (nmoles/0.1 ml)	Assay time (hr)	Histidase-specific activity ^a	
			% of control/mg protein ^b	% of control/mg DNA ^c
1	17.0	19	41.8	46.0
2	17.0	24	43.1	54.5
3	17.0	19	44.4	50.6
4	8.5	19	62.0	58.5

^a Mean for 2 separate groups of 3 mice each, expressed as percentage of specific activity in acetone control groups.

^b Determined on dialyzed soluble S-23 fraction.

^c DNA content was assayed using an aliquot of the same homogenate from which the S-23 fraction was subsequently obtained.

Table 3

Effect of TPA on the soluble protein and DNA content of hairless mouse epidermis

After removal from control or TPA-treated hairless mice (17 nmoles/application), epidermis was frozen in liquid nitrogen and weighed. The homogenates were assayed for DNA content and the dialyzed 23,000 × g supernatants were assayed for protein content as described in "Materials and Methods."

	μg soluble protein/ mg wet wt	μg DNA/ mg wet wt	10 ³ × units histidase/ mg wet wt
Acetone control	14.1 ± 1.3 (10) ^a	2.9 ± 0.4 (6)	251.5 ± 36.7 (7)
TPA-treated (19 hr)	15.8 ± 0.8 (9)	2.4 ± 0.4 (6)	136.3 ± 15.0 (7)
Probability of <i>t</i> ^b	0.2 > <i>p</i> > 0.1	0.4 > <i>p</i> > 0.2	0.025 > <i>p</i> > 0.01

^a Mean ratio ± S.E. for the number of experimental groups of mice indicated in parentheses.

^b Student's *t* test using the 2-sided hypothesis.

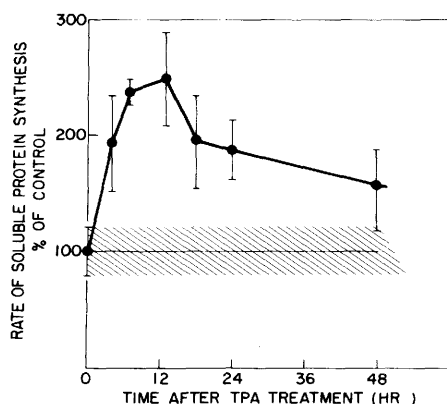


Chart 3. The effect of TPA treatment on the rate of soluble protein synthesis. Hairless mice were treated once topically with 17 nmoles TPA per application as described for Chart 1. At 3, 6, 12, 18, 23, and 47 hr after treatment the mice were given injections of tritiated leucine, and 1 hr later the epidermis was removed and the dialyzed supernatant (S-23) was obtained as described in "Materials and Methods." Rate of protein synthesis was determined by measurement of protein specific radioactivity using liquid scintillation spectrometry and the Lowry protein assay. Each point represents the mean for 2 mice; vertical lines, range. Two additional experiments verified the results of the 1 shown.

Table 4

Effect of S-23 from TPA-treated mice on activity of the control enzyme

Hairless mice were treated with TPA, 17 nmoles/0.1 ml acetone solution, and the epidermis was removed 19 hr later. The dialyzed S-23 fraction was assayed for histidase-specific activity as described for Chart 1. The mixed enzyme fraction was composed of equal volumes (not equal protein concentrations) of the control and *in vivo* TPA-treated S-23 fractions. Results are the means of duplicate determinations.

Source of S-23	Histidase-specific activity (units/mg protein)		
	Experi- ment 1	Experi- ment 2	Experi- ment 3
Acetone control	17.4	18.2	18.2
TPA-treated	6.2	6.4	7.3
Control + TPA-treated (1/1, v/v)	11.8	13.3	12.5
Theoretical for no inhibitor	11.8	12.4	12.8
Mixed S-23 as % of theoretical	100	107	98

DDP, a moderate promoter (6), and with phorbol, the parent compound and nonpromoter at equimolar doses,

indicated that phorbol produced no effect while didecanoyl phorbol produced an intermediate effect on histidase-specific activity (Chart 4). An assay of enzyme activity at 44 hr following application of phorbol indicated no effect, just as at 19 hr (data not shown).

The structurally unrelated moderately active tumor promoter Tween 60 (32) was tested for its effect on histidase, with the result that a substantial decrease occurred at both 20 hr and 44 hr following treatment (Chart 5). In contrast, neither the pure initiator urethan, administered topically or i.p., nor an initiating dose of DMBA produced any significant effect on epidermal histidase (Chart 5). However, DMBA used as a complete carcinogen and applied repeatedly on CD-1 mice as shown in Table 1 produced a substantial decrease in histidase, further demonstrating the correlation between tumor promotion and decreased histidase levels.

DISCUSSION

Application of TPA decreases epidermal histidase in a dose-dependent manner over a dose range that is active in tumor promotion (5, 25, 35). The kinetics are similar to those for stimulation of DNA synthesis in haired (7) and hairless (N. H. Colburn, unpublished observation) mice, with maximal histidase effect at 19 to 24 hr posttreatment.

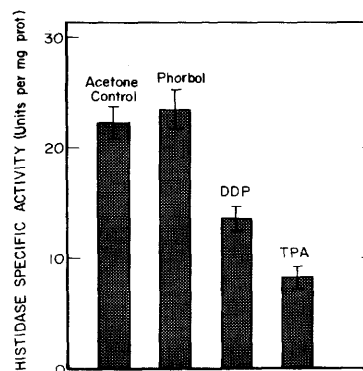


Chart 4. Effects on histidase-specific activity of phorbol and 2 phorbol diesters. Hairless mice received 17 nmoles/0.1 ml acetone solution of phorbol, DDP, or TPA. After 19 hr, epidermal histidase-specific activity was assayed as described in "Materials and Methods." Each bar represents the mean of 2 groups of 3 mice each; vertical lines, range; prot, protein.

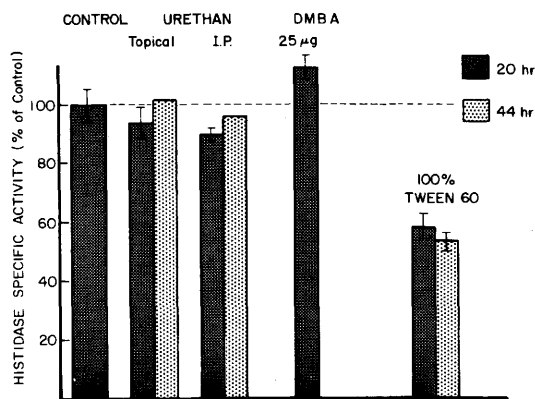


Chart 5. Effects of 2 tumor initiators and a nonphorbol promoter on epidermal histidase-specific activity. Hairless mice were treated topically with 680 μ moles urethan per 0.1 ml of acetone solution, or by i.p. injection of urethan (224 μ moles in 0.1 ml aqueous solution), or topically with 25 μ g of DMBA per 0.1 ml acetone solution, or topically with 100% Tween 60, 0.1 ml each to the dorsal and ventral skin. Histidase-specific activity was determined at 20 and 44 hr posttreatment, by methods indicated in Charts 1 and 2. Vertical lines, ranges of histidase-specific activity for 2 groups of 3 mice each.

The onset of the enzyme decrease occurs at 12 hr, or after the beginning of stimulated phospholipid (29), protein (7), and RNA (7) synthesis in haired mice. These observations are compatible with the concept that both DNA stimulation and histidase decrease may be coordinately regulated by an early response to phorbol ester treatment such as stimulated synthesis of a certain class of phospholipids or RNA species.

Since tumor promotion is reversible (10), and since, for optimal tumor yield, promoter application must be repeated at intervals of 3 to 4 days, this suggests that the duration of the pertinent components of promoting action is 4 days or less (at least for TPA). The histidase decrease is 1 event that shows a duration compatible with what is expected for such a relevant component of tumor promotion.

The histidase decrease could not be attributed to a general decrease in the rate of soluble protein synthesis or to the appearance of an inhibitor of histidase activity. Furthermore, since enzyme fractions are dialyzed prior to assay, it is unlikely that TPA could be acting as an *in vitro* inhibitor. The possibility that a tightly bound form of TPA or another small molecule acts to inhibit histidase is not ruled out by these experiments. However, since major modes of depressing activity independently of any effects on synthesis have been ruled out, it appears probable that TPA treatment results in an *in vivo* decrease in the amount of histidase enzyme present. Additional experiments will be needed to test this possibility directly.

The data suggest that the decrease in epidermal histidase is related to tumor promotion, since there is a positive correlation between the magnitude of histidase decrease and tumor-promoting activity for phorbol and 2 phorbol esters. This correlation is indicated in Chart 6. Using data of Baird *et al.* (6), we calculated an index of tumor-promoting activity that was plotted against the magnitude of histidase decrease. It will be of interest to determine whether other phorbol esters fit this relationship.

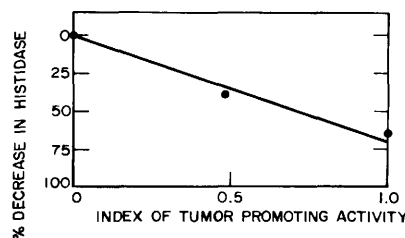


Chart 6. Relationship of the decrease in histidase activity to tumor-promoting activity of phorbol esters. An index of tumor-promoting activity calculated from Baird *et al.* (6) gives equal weight to the average number of papillomas (P_a) per mouse at 14 weeks of promotion and to the percentage of incidence of papillomas as measures of tumor-promoting potency.

$$\text{Index} = \frac{\left(\frac{Pa/\text{mouse}_x}{Pa/\text{mouse}_{TPA}} \right) + \left(\frac{\% Pa_x}{\% Pa_{TPA}} \right)}{2}$$

The index for TPA is 1.0 by definition. Histidase-specific activity data are taken from Chart 4.

The data further suggest that the histidase decrease is specifically related to tumor promotion and not initiation, since Tween 60 produces the decrease but urethan and an initiating dose of DMBA do not.

Although there has been no clear demonstration of 2-stage carcinogenesis in liver, it seems possible that the histidase decreases that occur during continuous feeding of hepatocarcinogens (20–22) may reflect promoting actions of these complete carcinogens. Furthermore, in view of the observations regarding the absence of histidase from human and mouse squamous cell carcinomas (2, 4), as well as hepatomas (16), it could be that decrease or loss of this enzyme is specifically related to neoplasia.

Alternatively, this decrease may be related to stimulated proliferation in general⁶ or to differentiation or dedifferentiation. Regardless of the mechanism, it appears that the ability to decrease epidermal histidase may be 1 marker of tumor promoters.

We suggest the following hypothesis relating this effect to epidermal differentiation. In view of the reports that agents that stimulate epidermal proliferation also stimulate a wave of keratinization about 1 to 2 days following the peak of DNA synthesis (1), and in view of the histochemical findings showing a high concentration of bound histidine in the region of epidermis where keratinization occurs (12, 27), we suggest that the decrease in histidase that occurs during the peak of DNA synthesis may be involved in priming the cells for the subsequent wave of keratinization. A decrease in histidase may permit a rise in the cellular concentration of histidine that can then be utilized for stimulated synthesis of histidine-rich protein (9, 14) in keratohyalin granules of granular cells. This histidine-rich protein may then play a role in stimulated production of “keratin” in stratum corneum at the end of the keratinization process. We suggest that tumor promoters act on initiated or uninitiated basal cells to stimulate their traverse into the keratinization process. But, while uninitiated cells are stimulated to

⁶ N. H. Colburn, R. A. Head, and S. O. Lau, manuscript in preparation.

keratinize normally as in wound healing, initiated cells are, by virtue of having acquired an altered genetic program, stimulated to differentiate abnormally, giving rise to squamous cell carcinomas.

Recently, Krieg *et al.* (15) have reported that TPA (20 nmoles) treatment produced peaks of histidine incorporation into histidine-rich protein of epidermis at intervals of 18 hr after the DNA synthesis maxima. This kinetics of stimulated synthesis of histidine-rich protein is consistent with the above hypothesis regarding epidermal histidase.

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