THE BINDING OF CARCINOGENIC HYDROCARBONS TO EPIDERMAL PROTEINS*

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The binding of polycyclic carcinogenic hydrocarbons to the epidermal proteins of mice was first demonstrated by Miller (7). These observations have been confirmed by other investigators and extensive studies along these lines have been carried out by Heidelberger and his associates (3, 6, 12, 13). The experiments of Woodhouse indicated to him that there was no correlation between carcinogenic activity and protein binding (14, 15). This is in contrast to the interpretations of the results obtained by Miller (7), Heidelberger *et al.* (3, 6, 12, 13), and Moodie *et al.* (9). As a part of a comprehensive study on the partial characterization of the structural and other proteins of human and mouse epidermis (4, 5), and of the malignant states of this tissue, the binding of several carcinogenic hydrocarbons particularly to these proteins and to the crude alcohol-trichloracetic acid insoluble protein (7) was determined.

METHODS

The hair from the back of male Bagg albino mice was removed with an electric clipper. The carcinogens (dissolved in benzene) were applied topically (about 0.3 mg. per mouse) with a Camel's hair brush No. 4 to an area about 2.5 cm. wide and 4.5 cm. long. In some experiments the initial applications were made in ordinary light, but all final applications were made in a dark room under a red light to minimize photo-decomposition of the carcinogens (7). For most of the work the carcinogens (3:4 benzpyrene, BP, methylcholanthrene (MC) and 9:10-dimethyl-1:2-benzanthracene (DMBA)) were purified chromatographic ally by the procedure of Miller and Baumann (8).

For analysis the epidermis, after dry shaving to remove all the hair, was separated from the dermis at 50° C. by the procedure of Baumberger *et al.* (1). In some experiments the crude epidermal protein (proteins of epidermis which were insoluble in a solution containing 85% ethanol and 10% trichloroacetic acid) was analyzed in duplicate or triplicate from several different samples (5 to 15 mice) for fluorescence whereas in others the structural and other proteins were isolated (4, 5) for this purpose. The epidermis was extracted with ethanol for 48 hours (Soxhlet) prior to the isolation of the structural and other proteins. Then the neutral and acid fractions of the proteins were extracted with benzene (7) and their fluorescence intensities determined in Farrand fluorimeter with Farrand instrument blue and yellow filters. The former transmits fluorescent radiation between wave lengths 350 and 450 mμ (maximum transmission at 400–425 mμ).

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Aided in part by an Institutional Grant from the American Cancer Society to the University of Buffalo.

Received for publication December 8, 1956.

Epidermis (MC-treated, 80 mice); Extracted for 48 hrs. with ethanol (Soxhlet); Epidermis air dried (2,298 mg.); Homogenized in 50 ml. 6 M urea; Extracted for 1 week at 2°C.

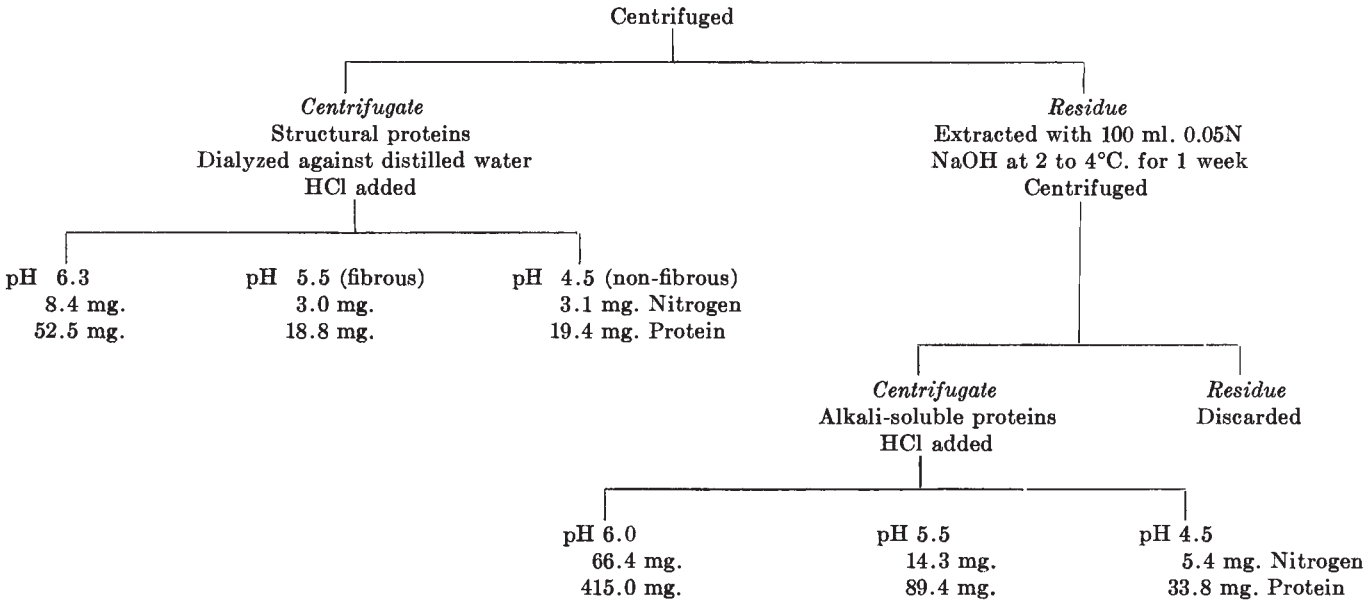

CHART 1. Flow diagram showing the extraction procedure for the isolation of the urea soluble and dilute alkali soluble proteins of mouse epidermis.

The yellow filter cuts out any primary radiation transmitted by Corning filter No. 5874 (maximum transmission at 360–365 mμ). The results are expressed in μg. hydrocarbon, but it is realized that the bound carcinogens probably have fluorescent intensities different from those of the parent hydrocarbons.

Although the all-glass redistilled solvents employed were relatively free of fluorescence, reagent blanks including 2 ml. ethanol, 2 to 5 ml. 4N KOH, 5 ml. toluene and 1.6 gm. zinc dust were refluxed at the same time with 2 samples containing protein. The fluorescence of the neutral and acid fractions of these blanks was subtracted from that obtained from the protein digests.

RESULTS

The procedure employed for the isolation of the structural and certain alkali soluble proteins of MC-treated mouse epidermis is given in Chart 1. This method for the preparation of the structural proteins is essentially that of Rudall (10). In this procedure the epidermis was extracted for one week at 2° C. with a 6 M urea solution. The unextracted part of the epidermis was separated by centrifugation and saved. The supernatant fraction was dialyzed against distilled water of about pH 7.4 with frequent changes during an 8 hour period to remove the urea. The contents of the dialysis sac were removed and the pH (glass electrode) was lowered gradually by the addition of dilute HCl. An insoluble protein flocculated maximally at about pH 6 and it was removed by centrifugation at 1800–2000 rpm for 10 minutes. In a similar fashion the fibrous and non-fibrous proteins were flocculated respectively at pH's of 5.5 and 4.5 and then removed by centrifugation. The epidermal residue from the urea extracted epidermis was treated with dilute alkali at 0° C to remove the alkali soluble proteins. These proteins were separated by centrifugation following their flocculation by the careful addition of dilute HCl at pH values of 6.0, 5.5 and 4.5. The yield of the urea-extractable proteins was considerably less than that obtained from the alkaline

TABLE I

Binding of 3:4-benzpyrene and 9:10-dimethyl-1:2-benzanthracene to epidermal proteins

Treatment of Mice	BP and DMBA Bound	
	Neutral fraction	Acid fraction
	μg. per/100 mg. protein	
Untreated	0.06	0.07
Do.	0.06	0.03
One application BP[1]	0.70	0.90
Three applications BP[1]	0.70	0.90
Three applications BP[1]	0.70	0.90
Six applications BP[1]	0.90	1.0
Three applications DMBA[2]	0.84	0.43
Six applications DMBA[2]	0.82	0.42

[1] 0.2% solution; applications on alternate days.

[2] 0.05% solution; applications on alternate days. In all cases the mice were sacrificed one day following the last application of the carcinogens.

TABLE II

Influence of repeated applications of benzpyrene on the binding of this carcinogen to epidermal proteins

Treatment of Mice	BP Bound	
	Neutral fraction	Acid fraction
	μg./100 mg. protein	
Seven applications BP[1], wait 2 days, then 2 applications BP[2]	1.5	2.0
Do	1.4	1.1
Ten applications BP[1], wait 5 days, then 2 applications BP[2]	0.9	0.9
Do	0.8	1.0
Twelve applications BP[1], wait 5 days, then 2 applications BP[2]	0.8	0.5
Do	0.8	0.7
Six applications BP[1], mice sacrificed 6 days after last application[3] BP	0.05	0.04

[1] BP, 0.6%, applied to mice in animal room.

[2] BP, 0.2% applied to mice in dark room.

[3] Mice were kept in the dark room for 6 days after last application BP. Mice in the other groups were killed one day after last application BP.

TABLE III

Influence of repeated applications of methylcholanthrene on the binding of this carcinogen to epidermal proteins

Treatment of Mice	MC Bound	
	Neutral fraction	Acid fraction
	μg./100 mg. protein	
Three applications MC[1], wait 5 days, then 3 applications MC[2]	0.70	0.37
Do	0.88	0.57
Six applications MC[1], wait 5 days, then 3 applications MC[2]	0.61	0.35
Do	0.52	0.36
Nine applications MC[1], wait 5 days then 3 applications MC[2]	0.49	0.34
Do	0.48	0.45
Six applications MC[1], mice sacrificed 5 days after last application MC[3]	0.41	0.23

[1] MC, 0.3%, applied to mice in animal room.

[2] MC, 0.3%, applied to mice in dark room.

[3] Mice were kept in the dark room for 5 days after last application MC. Mice in other groups were killed one day after last application MC.

digest of the urea-treated epidermis. Although the proteins of the urea and alkali extracts precipitated maximally at nearly the same pH, the solubility and sedimentation properties were different for these proteins (11).

The amount of BP and DMBA bound to the crude epidermal protein was about the same (Table I). There appeared to be no significant change in the amount of binding of both BP (Table II) and MC (Table III) to the proteins of an epidermis made more hyperplastic by multiple applications of these carcinogens. However, MC is retained for a longer period of time than is BP following the last of six applications of these carcinogens.

TABLE IV

Binding of benzpyrene to urea extracted proteins of epidermis

Group and No. Mice	Nature of Protein	Treatment of Mice	Amount BP Bound	
			Neutral fraction	Acid fraction
	pH		*μg./100 mg. protein*	
A, 63	6.2	5 applications 0.6% BP[1], wait 2 days then 3 applications 0.6% BP[2] in the dark; mice killed 3 days after last application BP	0.4	0.2
	5.5		1.8	0.7
B, 60	6.2	6 applications 0.6% BP[1], wait 5 days then 3 applications 0.2% BP[2] in the dark; mice killed 1 day after last application BP	0.9	0.6
	5.5		2.2	1.2
	4.5		1.7	0.7
C, 57	6.2	8 applications 0.6% MC[1], wait 5 days then 3 applications 0.2% BP[2] in the dark; mice killed 1 day after last application BP	0.6	0.4
	5.5		2.3	0.9
	4.5		2.0	0.9

[1] Not purified.
[2] Purified.

TABLE V

Binding of methylcholanthrene to urea extracted proteins of epidermis

Group and No. Mice	Nature of Protein	Treatment of Mice	Amount of MC Bound	
			Neutral fraction	Acid fraction
	pH		*μg./100 mg. protein*	
A, 71	6.2	5 applications 0.3% MC[1], wait 5 days then 3 applications 0.3% MC[2] in the dark; mice killed 1 day after last application MC	0.23	0.11
	5.5		0.22	0.24
	4.5		0.18	0.05
B, 80	6.2	8 applications 0.3% MC[1], wait 5 days then 3 applications 0.3% MC[2] in the dark; mice killed 1 day after last application MC	0.57	0.17
	5.5		0.90	0.58
	4.5		0.27	0.14
C, 90	6.2	11 applications BP[1], wait 5 days then 3 applications 0.3% MC[2] in the dark; mice killed 5 days after last application MC	0.12	0.27
	5.5		0.49	0.14
	4.5		0.67	0.55
D, 50	6.2	12 applications 0.3% MC[1], wait 5 days then 3 applications 0.3% MC[2] in the dark; mice killed 1 day after last application MC	0.40	0.16
	5.5		2.24	0.77
	4.5		2.03	0.83

[1] Not purified.
[2] Purified.

The binding of BP by the proteins isolated from urea treated epidermis is shown in Table IV. The fibrous (isoelectric point of pH 5.5) and the non-fibrous (isoelectric point of pH 4.5) proteins bound more BP (neutral fraction) than did the protein having its least solubility at pH 6.3. The acid fractions of these proteins showed less uptake of BP than the neutral fraction and the aklali soluble proteins (Chart 1) showed much less binding of BP than did the urea extractable proteins. The urea-extracted proteins of MC-treated epidermis also showed considerable binding of this carcinogen, particularly to the fibrous and non-fibrous proteins (Table V). The amount of MC bound increased with the number

of applications of this carcinogen. The fibrous and the non-fibrous protein of group D contained the largest amounts of MC following 12 applications of this carcinogen. The alkali soluble proteins of MC treated epidermis bound much smaller amounts of this carcinogen than did the urea-extractable proteins.

DISCUSSION

The carcinogens BP, MC and DMBA are bound to about the same extent to the crude proteins of epidermis. Heidelberger and Moldenhauer found with radio-labelled carcinogens that BP, MC and DMBA were bound to about the same extent to denatured skin protein (6). BP and MC are bound in larger amounts to the urea-extractable proteins than to the crude proteins of epidermis, and BP accumulated more rapidly into these proteins than did MC. The greater incorporation into the urea extractable proteins is based upon the assumption that the fluorescence of the metabolites in the proteins is not greater than that of the carcinogens. A greater binding of BP and MC into these proteins than into the crude epidermal proteins may imply a correlation between binding and carcinogenesis, but proof of this relationship is not yet available. In fact, Woodhouse (14, 15) has shown that the non-carcinogenic hydrocarbons 2,6-dimethyl-1,2-benzanthracene, 3:4 benztetraphene and perylene and Heidelberger *et al.* (6) that 1,2,3,4-dibenzanthracene are bound to about the same extent as carcinogens to epidermal proteins. There may be differences in the proteins involved in the metabolism of carcinogenic vs. non-carcinogenic hydrocarbons. Such compounds generally may be metabolized via a protein pathway in epidermis since this tissue is (1) avascular and alymphatic and thus may employ channels for metabolism other than those used by vascular organs, and is (2) continually undergoing cell division and keratinization in which processes metabolites may be desquamated with keratin. Actually other non-carcinogenic hydrocarbons than those mentioned above are bound to some extent to epidermal proteins (6).

Practically all of the work in this report has dealt with multiple applications of carcinogens to maintain a thick hyperplasia for a good source of epidermis, and since the single painting technique deals primarily with papillomatosis, or at best with a low incidence of carcinomas (2) whereas multiple applications result in a high incidence of skin cancers.

SUMMARY

1. The crude epidermal protein fraction of mouse epidermis bound BP (3:4 benzpyrene), MC (methylcholanthrene), and DMBA (9:10-dimethyl-1:2-benzanthrene) in about equal amounts.

2. The structural proteins of mouse epidermis bound more MC (methylcholanthrene) and BP (3:4 benzpyrene) than did the crude epidermal proteins.

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