Chemical Studies on the Mode of Action of Methylcholanthrene on Mouse Epidermis*

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Previous studies (2, 6) have shown that methylcholanthrene produced a notable decrease in the iron and calcium content of mouse epidermis while the sodium and ascorbic acid content was not significantly altered. For a more complete understanding of the events occurring in the epidermis under the influence of this carcinogen, the effect of the latter on epidermal magnesium and potassium was investigated.

The Mineral Content of Mouse Epidermis

The mineral content of mouse epidermis is detailed in Table I. The potassium content is more than twice that of the sodium, and these metals account for 88 per cent of the total cations. Calcium and magnesium occur in the ratio of about 2 to 1, and make up 11 per cent of the metals. On the other hand iron is low, and preliminary experiments with large amounts (400 to 800 mgm. of epidermis from 15 to 20 mice) indicate a copper content of 0.2 to 0.4 microgram per 100 mgm. Since the epidermis is avascular inorganic analysis is facilitated owing to the noninterference of blood. This fact is well illustrated in Table I by the excellent agreement in the normal content of each of the metals in different samples.

The methods of separating the epidermis from the dermis were devised by Baumberger, Suntzeff, and Cowdry (1), and the adaptation of microchemical methods to the small amounts of tissue thus obtained should be of great value in skin physiology and in dermatology. Microchemical methods are essential because only limited amounts of epidermis can be obtained unless many mice are sacrificed or large pieces of human or other skin are removed. For example, only 150 to 200 mgm. of epidermis can be secured from the entire backs of 10 to 12 mice at the age of 3 to 4 months. After incineration there is but 1 to 2 mgm. of ash left for inorganic analysis.

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Experimental Procedure

New Buffalo mice of both sexes and 3 to 4 months old were used, as there was no apparent sex difference. The methods of shaving, applying the carcinogen, and of separating the epidermis from the dermis have been described (2). In this investigation, as in those already completed, nucleoprotein phosphorus was used as a basis of reference for the amount of tissue in-

Table I: The Mineral Composition of Mouse Epidermis

Metal	Number of mice represented	Mgm. metal per 100 mgm. epidermis	Total cations, per cent
Potassium	10	0.3480	
	10	0.3475	
	8	0.3400	
	10	0.3507	
	38 (total)		
	Average...	0.3465	59.4
Sodium	8	0.1707	
	7	0.1697	
	8	0.1490	
	8	0.1829	
	8	0.1677	
	39 (total)		
	Average...	0.1680	28.7
Calcium	7	0.0358	
	8	0.0420	
	11	0.0505	
	11	0.0400	
	10	0.0405	
	12	0.0520	
	59 (total)		
	Average...	0.0435	7.5
Magnesium	12	0.0195	
	12	0.0176	
	11	0.0210	
	10	0.0164	
	45 (total)		
	Average...	0.0186	3.3
Iron	108 (total)		
	Average...	0.0064	1.0

volved. The necessity of an adequate base in these studies has been given (2).

The magnesium content of the epidermis was determined by a micro-polarographic method (3) devised in this laboratory. The potassium content was determined as follows: The sample was ashed at 450° C. in a silica crucible in a muffle furnace until all the organic matter had been destroyed. The ash remaining was dissolved with 1 cc. of 0.1 *N* hydrochloric acid and the crucible contents were rinsed with several 2 cc. portions of distilled water into a pyrex test tube. The solution in the test tube was evaporated to dryness in a beaker of boiling water, and after cooling to room temperature, sufficient platinic chloride (5 per cent solution in 0.1 *N* hydrochloric acid) was added to combine with all the potassium and sodium present. The resulting mixed solution of platinates was evaporated to dryness as before and the residue remaining was extracted with 2 to 4 cc. of 80 per cent alcohol at room temperature. The test tube was stoppered and allowed to stand overnight to insure complete precipitation of the potassium chloroplatinate. Then the latter compound was separated from the mother liquor by aspiration with a pyrex filter stick (10 mm. disk, medium porosity) covered with a layer of asbestos. The precipitate of potassium chloroplatinate was washed with two 2 cc. portions of freshly prepared alcohol wash solution. The wash solution of 95 per cent alcohol was saturated with potassium chloroplatinate and filtered just before use through a sintered glass filter covered with a layer of asbestos. The rest of the procedure was that of Consolazio and Talbott (4) except that 0.005 *N* sodium thiosulfate was used to titrate (from a microburette) the potassium iodoplatinate.

RESULTS

Magnesium

A comparison of the analysis for magnesium of normal controls, benzene-treated controls, and methylcholanthrene-treated mice is shown in Table II. The results are expressed in terms of milligrams of magnesium (Mg) per 100 mgm. of tissue, of milligrams of nucleoprotein phosphorus (denoted by N.P.P.) per 100 mgm. of tissue, and by the significant ratio Mg/N.P.P. × 10 (hereinafter denoted by Mg/N.P.P.). The epidermis of normal mice contained an average of 0.0186 mgm. Mg per 100 mgm. and had an Mg/N.P.P. ratio of 1.44. The benzene-treated epidermis had an average of 0.0189 mgm. Mg per 100 mgm. and an Mg/N.P.P. ratio of 1.53. The effect of benzene alone was therefore not significant. After 1 and 2 applications of methylcholanthrene the magnesium content and the ratios, Mg/N.P.P., were slightly greater than those of the normal. However,

TABLE II: MAGNESIUM/NUCLEOPROTEIN PHOSPHORUS RATIO OF MOUSE EPIDERMIS

Number of mice	Number of paintings	Time after first treatment to killing of mice, days	Mg per 100 mgm. tissue, mgm.	N.P.P. per 100 mgm. tissue, mgm.	Mg/N.P.P × 10
		NORMAL, UNTREATED MICE			
12			0.0195	0.127	1.53
12			0.0176	0.139	1.26
11			0.0210	0.121	1.73
10			0.0164	0.133	1.23
45	(total)				
	Average		0.0186	0.130	1.44
		BENZENE-TREATED MICE			
12	3	10	0.0193	0.116	1.66
11	3	10	0.0178	0.139	1.28
11	3	10	0.0184	0.127	1.45
12	3	10	0.0204	0.113	1.80
46	(total)				
	Average		0.0189	0.124	1.55
		METHYLCHOLANTHRENE-TREATED MICE			
8	1	10	0.0209	0.139	1.50
7	1	10	0.0215	0.134	1.60
8	1	10	0.0202	0.119	1.70
23	(total)				
	Average		0.0208	0.131	1.60
6	2	10	0.0228	0.140	1.63
8	3	10	0.0271	0.134	2.02
8	3	10	0.0311	0.151	2.06
6	3	10	0.0220	0.148	1.48
8	4	10	0.0207	0.121	1.71
30	(total)				
	Average		0.0252	0.138	1.82
6	4	15	0.0229	0.149	1.53
6	4	15	0.0207	0.142	1.52
12	(total)				
	Average		0.0218	0.145	1.53
7	6	20	0.0235	0.142	1.65
5	6	20	0.0266	0.149	1.80
9	6	20	0.0208	0.138	1.50
21	(total)				
	Average		0.0236	0.143	1.65
8	12	30	0.0219	0.124	1.76
8	12	30	0.0193	0.116	1.66
5	12	30	0.0216	0.119	1.81
21	(total)				
	Average		0.0209	0.119	1.74
7	24	60	0.0196	0.112	1.75
4	24	60	0.0214	0.120	1.78
4	24	60	0.0195	0.120	1.62
4	24	60	0.0248	0.117	2.12
4	24	60	0.0189	0.117	1.61
23	(total)				
	Average		0.0208	0.117	1.77

after 3 treatments the magnesium had increased to 0.0252 mgm. (average) per 100 mgm. and the ratio Mg/N.P.P. to 1.82. Likewise the epidermis treated 6 times with the carcinogen showed an elevated Mg content and Mg/N.P.P. ratio.

In order to determine whether the increase in magnesium was significant, samples were removed at 15 days after 4 applications of the carcinogen. However, both the Mg content and Mg/N.P.P. ratios were only slightly above normal.

After 12 and 24 treatments of mice that were killed at 30 and 60 days respectively, there was excellent agreement in the magnesium content and in the Mg/N.P.P. ratios, the latter being about 20 per cent higher than those of the normal controls. The importance of the increase in the Mg/N.P.P. ratios of most of the methylcholanthrene-treated mice may be of questionable significance since mice treated 4 times had a nearly normal ratio. Moreover, the epidermises having the greatest rise (after 3 and 6 applications and killed at 10 and 20 days respectively) in magnesium and in Mg/N.P.P. ratios showed considerable variation.

Potassium

The results for potassium are given in Table III, and are expressed in the same manner as those for magnesium except that the ratio K/N.P.P. was not multiplied by 10. Normal epidermis contained an average of 0.3465 mgm. potassium per 100 mgm. and had a K/N.P.P. ratio of 2.68, and the benzene-treated had an average of 0.3511 mgm. potassium per 100 mgm. and a K/N.P.P. ratio of 2.64. It was evident that benzene alone had no effect on epidermal potassium. Mice killed at 10 days after 1 and 3 applications of methylcholanthrene did not show any appreciable change in the K content or in the K/N.P.P. ratios. Likewise the epidermises removed at 20, 30, and 60 days after 6, 12, and 24 treatments respectively had nearly normal K range and K/N.P.P. ratios. Therefore epidermal potassium was not altered under the influence of the carcinogen.

DISCUSSION

The effect of methylcholanthrene upon epidermal iron, sodium, calcium, magnesium, potassium, and ascorbic acid is shown graphically in Fig. 1. The time in days is plotted against the percentage of change in the metal/nucleoprotein phosphorus ratios. The number of applications of the carcinogen corresponding to various times in days is expressed at the top of the graph. As early as 10 days after 1 application of methylcholanthrene the Fe and Ca/N.P.P. ratios had dropped to about 50 per cent of the normal. There was a further reduction in the Fe/N.P.P. ratio,

Table III: Potassium/Nucleoprotein Phosphorus Ratio of Mouse Epidermis

Number of mice	Number of paintings	Time after first treatment to killing of mice, days	K per 100 mgm. tissue, mgm.	N.P.P. per 100 mgm. tissue, mgm.	K/N.P.P.
			NORMAL, UNTREATED MICE		
10			0.3480	0.128	2.72
10			0.3475	0.124	2.80
8			0.3400	0.129	2.64
10			0.3507	0.136	2.58
38	(total) Average		0.3465	0.129	2.68
			BENZENE-TREATED MICE		
10	3	10	0.3547	0.125	2.84
9	3	10	0.3503	0.121	2.89
10	3	10	0.3313	0.153	2.16
9	3	10	0.3681	0.136	2.70
38	(total) Average		0.3511	0.134	2.65
			METHYLCHOLANTHRENE-TREATED MICE		
6	1	10	0.3242	0.109	2.97
6	1	10	0.3220	0.136	2.37
6	1	10	0.3642	0.156	2.34
18	(total) Average		0.3368	0.134	2.56
5	3	10	0.3615	0.126	2.87
6	3	10	0.3430	0.106	3.23
6	3	10	0.3609	0.149	2.42
17	(total) Average		0.3551	0.127	2.84
6	6	20	0.3870	0.132	2.93
5	6	20	0.3180	0.131	2.43
6	6	20	0.3815	0.148	2.58
6	6	16	0.3553	0.135	2.63
23	(total) Average		0.3604	0.136	2.64
8	12	30	0.3456	0.140	2.47
3	12	30	0.3504	0.119	2.94
4	12	30	0.3526	0.119	2.96
3	12	30	0.3310	0.123	2.69
4	12	30	0.3280	0.123	2.66
22	(total) Average		0.3415	0.125	2.73
4	24	60	0.3329	0.125	2.66
4	24	60	0.3401	0.125	2.72
4	24	60	0.3284	0.130	2.52
6	24	50	0.3546	0.131	2.70
18	(total) Average		0.3390	0.128	2.65

while the Ca/N.P.P. ratio diminution remained fairly constant. The decrease in iron may be associated with a lowering of the cytochrome *c* content, since the latter was found to be less in neoplastic growths (5) than in the normal tissues from which the malignant growths arose. The chemical changes, if any, associated with the lowering of calcium must await further investigation.

ments the ratio was essentially normal. Although the late hyperplasias (30 and 60 days) showed about a 20 per cent augmentation in the ratios, it was difficult to evaluate the rise owing to the variation in the Mg content of different samples. The K/N.P.P. ratio was notably constant throughout the period of carcinogenesis.

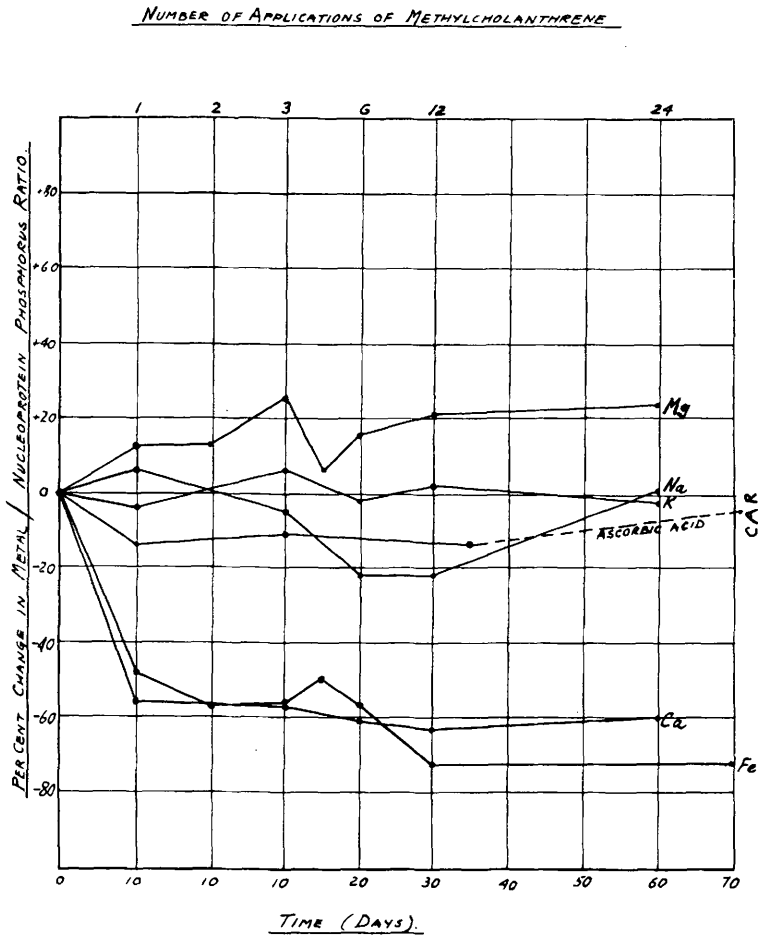

FIG. 1.—The influence of methylcholanthrene on epidermal iron (Fe), sodium (Na), calcium (Ca), magnesium (Mg), potassium (K), and ascorbic acid.

On the other hand the ascorbic acid/N.P.P. ratio was not appreciably affected. This constituent was also determined in epidermal carcinomas (denoted on the graph by CAR) and the ratio was found to be nearly the same as that of normal epidermis. At 20 and 30 days after 6 and 12 treatments respectively the decrease in the Na/N.P.P. ratio was about 20 per cent, but at 60 days the ratio was normal. It was therefore concluded that epidermal sodium was not significantly altered. The Mg/N.P.P. ratio was increased by nearly 25 per cent at 10 and 20 days after 3 and 6 applications respectively, but at 15 days after 4 treat-

$$\frac{\text{K/N.P.P.}}{\text{Na/N.P.P.}} \quad \text{and} \quad \frac{\text{Ca/N.P.P.}}{\text{Mg/N.P.P.}}$$

ratios of normal and methylcholanthrene-treated epidermis are shown in Table IV. These data further demonstrate that potassium and sodium were not significantly changed in the process of carcinogenesis although the ratio was increased somewhat in the epidermis treated 6 and 12 times. On the other hand, the Ca/Mg ratio decreased by more than 60 per cent after the application of methylcholanthrene, the diminution remaining remarkably constant from 10 to 60 days. It is obvious, therefore, that the alkaline earths, especially

calcium, play an important role in the transformation of normal to "precancerous" tissue.

The data expressed graphically in Fig. 1 also demonstrate that the response of mouse epidermis to methylcholanthrene is rapid and chemically complex even in 10 day samples following a single treatment with the carcinogen. Wicks and Suntzeff (7) have also shown that the total lipid/protein nitrogen ratio of mouse epidermis was much lowered as early as 5 days after one application of methylcholanthrene. Therefore it is apparent that the process of epidermal carcinogenesis in mice is initiated early, and that there

TABLE IV: POTASSIUM-SODIUM/NUCLEOPROTEIN PHOSPHORUS RATIO AND CALCIUM-MAGNESIUM/NUCLEOPROTEIN PHOSPHORUS RATIO OF MOUSE EPIDERMIS

Treatment	Number of paintings	Time after first treatment to killing of mice, days	$\frac{K}{Na}$/N.P.P.	$\frac{Ca}{Mg}$/N.P.P.
Normal		10	2.29	2.41
Benzene-treated	3	10	2.18	2.19
Methylcholanthrene-treated	1	10	2.05	0.93
" "	3	10	2.55	0.85
" "	6	20	2.90	0.85
" "	12	30	3.04	0.72
" "	24	60	2.24	0.82

are at least several chemical changes occurring simultaneously.

These investigations also reveal from a chemical standpoint the existence of "precancerous" tissue. This is apparent from Fig. 1, which shows that the methylcholanthrene-treated cells have lost 50 per cent of their calcium and iron, and about the same amount of total lipid (7), and also may be richer in magnesium. Nevertheless these cells are capable of continued existence and proliferation to form extensive hyperplasias and carcinomas. It is not yet known whether the mineral changes are maintained in epidermal squamous cell carcinomas.

SUMMARY

The effect of methylcholanthrene upon epidermal iron, calcium, sodium, magnesium, potassium, and ascorbic acid is discussed. One application of the carcinogen reduced within 10 days the iron and calcium content to approximately 50 per cent of the normal. On the other hand sodium, potassium, and ascorbic acid were not appreciably altered, while the increase in magnesium may be important. The significance of the chemical changes is briefly outlined. Nucleoprotein phosphorus was used as a basis of reference for the amount of tissue involved.

The existence of "precancerous" tissue from a chemical standpoint is postulated.

The mineral analysis of mouse epidermis is also given, and the use of the technics employed in these studies for other biological problems is briefly mentioned.

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