Citric Acid Metabolism in Carcinogenesis and Its Relationships to Calcium Metabolism*

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Previous studies on the calcium metabolism of epidermis undergoing carcinogenesis (2, 10, 11) have suggested the possibility that the action of one or more calcium-binding substances may account for the decreased calcium content in hyperplastic epidermis and in a squamous-cell carcinoma. The present investigation has been devoted to a study, in normal and cancerous tissues of the mouse, of the content of citric acid, a substance known to form slightly dissociated complexes with calcium (7).

Several tumor tissues were analyzed by Dickens (5), who concluded that tumor tissue contains more citric acid than most normal tissues, with the exception of skin, bone, hair, and the tissues comprising the seminal vesicle. A high content of citric acid was found in the Walker 256 tumor of the rat by Haven, Randall, and Bloor (8). The latter investigators also reported a markedly higher content of citric acid in the necrotic center of this tumor than in the viable periphery. These findings differ from those of Dickens (5), who found a higher citrate level in the non-necrotic portions of the Crocker sarcoma.

MATERIALS AND METHODS

The following tissues of the mouse were analyzed for their citric acid contents: normal and hyperplastic Swiss strain epidermis, a Swiss strain transplanted squamous-cell carcinoma, C strain liver and transplanted hepatoma,[1] Leaden strain liver and transplanted hepatoma,[2] and C3H strain muscle and transplanted rhabdomyosarcoma.[3]

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[1] Obtained from Dr. H. B. Andervont of the National Cancer Institute.

[2] Obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine.

[3] Otained from Dr. E. U. Green, Institute for Cancer Research and the Lankenau Hospital Research Institute, Philadelphia.

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Technics for shaving the mice, applying methylcholanthrene, and separating the epidermis from the dermis have been previously described (1, 3). Thigh, gluteal, and lower back muscles were used for normal muscle analyses on C3H strain mice. Subcutaneous transplants of the various tumors were removed when the neoplasms attained a diameter of between 0.5 and 1 cm. (with the exception of several C strain hepatomas, which were larger). Fresh tissue was removed, weighed on a Roller-Smith precision balance, and ground in a mortar with about 5 ml. of 10 per cent trichloroacetic acid, with the aid of purified sand when necessary. The contents of the mortar were transferred quantitatively to a 40-ml. centrifuge tube by rinsing both the mortar and pestle several times with 10 per cent trichloroacetic acid and by adding the washings to the centrifuge tube.

The citric acid content of the tissues was determined by the method of Taussky and Shorr (13) and Taussky (12). The color reaction used, however, was that of Natelson, Pincus, and Lugovoy (9). A number of modifications of these procedures were necessitated by the fact that they were originally devised for blood and urine analyses. The entire procedure will, therefore, be outlined, in order to clarify these modifications.

The contents of the centrifuge tube were stirred well, allowed to stand at room temperature for about 10 minutes, and then centrifuged for the same period of time at 1,500 r.p.m. The supernatant was then filtered through a 7-cm. Whatman No. 42 filter paper into a graduated cylinder and the volume of the filtrate recorded. Two ml. of the filtrate was pipetted into a 60-ml. glass-stoppered Pyrex bottle to which 9 ml. of distilled water was added to bring the sample to volume. In order to determine recoveries, a known amount of citric acid was added to the tissue filtrate, and the solution then brought to volume (11 ml.). One ml. of 20 per cent trichloroacetic acid was added to all standard solutions, to maintain a constant concentration of the acid in all samples, before making a similar dilution with distilled water.

The next step was the addition of sulfuric acid. It was found (Table 1) that the addition of 3.7 ml. of 27 N sulfuric acid to epidermal samples gave the best recoveries, and 4.4 ml. of 27 N sulfuric acid also produced better results than any other concentrations of acid with liver and muscle samples. The normalities of sulfuric acid prior to the oxidation stage were 6.36 and 7.24, respectively. It may also be mentioned that, although varying the normality of sulfuric acid had some effect on the results obtained on tissue samples, no changes were noted with standard solutions when the normality of the acid was varied.

TABLE 1

RECOVERIES OF ADDED CITRIC ACID

Tissue	Sample no.	Normality of H_2SO_4	Citric acid content (γ)	Recovery sample* (γ)	Non-recovery sample† (γ)	Added citric acid‡ (γ)	Recovery (per cent)
C strain liver	1	7.24	13.1	6.0	2.8	3.57	95.4
	2	11.6	18.3	5.3	2.8	3.57	83.3
C strain hepatoma	3	7.24	174	11.3	8.2	3.57	96.0
	4	11.6	162	7.8	5.3	3.57	88.0
Leaden strain liver	5	7.24	27.0	8.5	4.3	3.57	108
	6A	1.08	29.7	9.2	4.3	3.57	117
	6B	1.84	39.4	10.2	5.7	3.57	110
	6C	3.86	24.9	8.8	3.6	3.57	123
	6D	11.6	19.4	5.3	2.8	3.57	83.3
Leaden strain hepatoma	7	7.24	69.9	5.7	2.8	3.57	89.6
	8	11.6	52.2		2.5		
C3H strain muscle	9	7.24	24.5	5.3	1.4	3.57	107
	10A	6.10	25.4	5.7	1.1	3.57	122
	10B	6.36	16.2	4.3	0.7	3.57	101
	10C	6.63	32.4	4.3	1.4	3.57	86.6
	10D	7.37	25.4	3.6	1.1	3.57	77.2
	10E	11.6	32.4	4.3	1.4	4.28	77.4
C3H strain rhabdomyosarcoma	11	7.24	47.8	5.3	2.1	3.57	93.5
Swiss strain epidermis	12A	3.86	272	12.0	7.8	3.57	106
	12B	5.40	247	11.6	7.1	3.57	109
	12C	6.75	247	10.2	7.1	3.57	95.8
	12D	11.6	209	6.7	6.0	1.43	90.2
3 paintings, methylcholanthrene	13	6.36	196	11.6	7.8	3.57	102
6 paintings, benzene	14	6.36	529	7.8	3.9	5.00	87.8
12 paintings, benzene	15A	5.96	478	12.4	9.6	3.57	94.3
	15B	6.36	478	13.0	9.6	3.57	98.8
	15C	6.63	478	11.6	9.6	3.57	88.2
	15D	7.00	478	12.7	9.6	3.57	96.6
24 paintings, methylcholanthrene	16	6.36	206	10.6	6.4	3.57	106
Squamous-cell carcinoma	17	6.36	69.6	6.0	2.5	3.57	99.0

* Citric acid content of final aliquot of recovery sample.
† Citric acid content of final aliquot of nonrecovery sample.
‡ Final aliquot of amount of citric acid added to recovery sample.

After the addition of a suitable amount of sulfuric acid from a burette, the solution was shaken and placed for about 5 minutes in a water bath maintained at from 15° to 18° C. Then the following solutions were added from a burette: 0.5 ml. of 40 per cent manganese sulfate, 0.5 ml. of bromide-bromate solution (9), and 1.5 ml. of 5 per cent potassium permanganate. The mixture was shaken thoroughly and allowed to stand for about 30 minutes in the water bath maintained at from 15° to 18° C., after which time suction was applied to remove the bromine fumes. Four ml. of 40 per cent ferrous sulfate (freshly prepared) was added, and the mixture was shaken and was allowed to stand for about 3 minutes, after which time suction was again applied as an added precaution. Seven ml. of normal heptane was added from a burette, and the mixture was shaken in the Aloe shaking machine for 6 minutes. The contents were poured into a separatory funnel and washed about 5 times (to neutrality) with doubly distilled water. Upon complete separation of the two layers, 5 ml. of the heptane layer was pipetted into a 30-ml. glass-stoppered Pyrex bottle.

Three and one-half ml. of buffered thiourea solution (9) was delivered from a burette into the bottle, which was then stoppered and shaken in the

Aloe shaking machine for 6 minutes. The contents of the bottle were poured into a 12-ml. centrifuge tube and centrifuged for 5 minutes at 2,000 r.p.m. The aqueous layer was then pipetted into a 10-mm. Coleman cuvette and read at a wave length of 445 mμ in the Coleman spectrophotometer.

The formula used for the calculation of the citric acid levels of tissue samples is as follows:

$$T = \frac{F \times K \times V_1 / V_2}{W}$$

where T is the citric acid content of the tissue (γ/gm), F the citric acid content of the final aliquot (γ), K (1.40) the proportion of the original aliquot used, V_1 the volume of the original tissue filtrate (ml.), V_2 the volume of filtrate used in the analysis (ml.), and W the weight of the tissue (gm.).

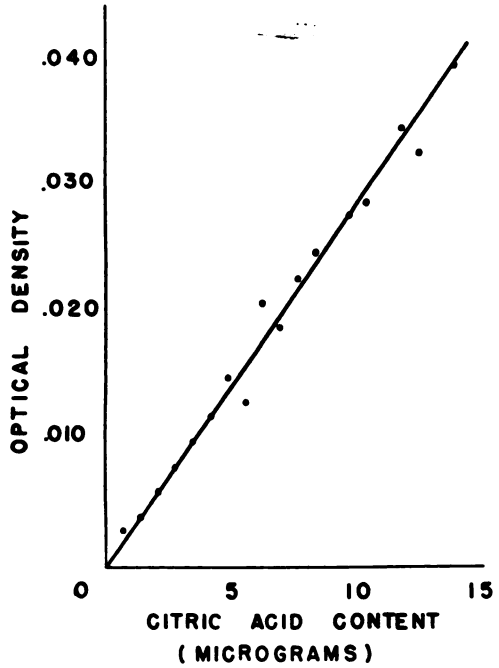

FIG. 1.—Standard curve

Twelve samples, including one blank and two standards, were run simultaneously, although this number was sometimes varied. However, one blank and at least one standard were always run with tissue samples. A satisfactory standard curve was plotted from the results of the analyses of 27 standard solutions (Fig. 1).

RESULTS

Greenstein's observation (6) that "no matter how, or from which tissues tumors arise, they more nearly resemble each other chemically than they do normal tissues or than normal tissues resemble each other" has received further support from this present work. The citric acid contents of the tumors analyzed were more alike than were the individual tumors in respect to their normal homologs. Although a great variation was shown between the various normal tissues that were analyzed, a relatively small variation was found between the citric acid contents of the tumors (Tables 2 and 3 and Fig. 2). With the exception of epidermis, a marked increase in the citric acid contents of all tumors, as compared with values for normal tissues, was consistently displayed. The citric acid content of the squamous-cell carcinoma, however, presented a very marked decrease from the normal values. Thus, all tumors analyzed tended to attain an almost identical citric acid content, irrespective of the citric acid level of their normal homologs.

TABLE 2

THE CITRIC ACID CONTENT OF LIVER AND MUSCLE TUMORS AND THEIR NORMAL HOMOLOGS (NORMALITY OF SULFURIC ACID, 7.24)

Tissue	Number of mice	Average citric acid concentration (γ/gm., wet wt.)
C strain liver	6	11.2
C strain hepatoma	4	130.5
C strain blood	about 20	29.1
Leaden strain liver	6	37.9
Leaden strain hepatoma	4	64.8
Necrotic portion of leaden strain hepatoma	1	53.1
C3H strain muscle	3	30.0
C3H strain rhabdomyosarcoma	4	68.0
Necrotic portion of rhabdomyosarcoma	2	91.6

Analyses of the necrotic portions of the Leaden strain hepatoma, squamous-cell carcinoma, and rhabdomyosarcoma indicated slight differences in the citric acid contents of these areas as compared to the viable zones (Tables 2 and 3). The slight decrease shown by the necrotic portion of the Leaden strain hepatoma is certainly not significant, while the slight increases shown by the rhabdomyosarcoma and squamous-cell carcinoma are unquestionable, since there is no overlapping of values. Certainly, however, the results of this work cannot explain the diametrically opposed findings of Dickens (5) and Haven, Randall, and Bloor (8) on the citric acid contents of necrotic tissue. One possibility, however, is that the citrate contents of necrotic areas analyzed by these investigators were affected by bacterial contamination. The tumors used in this present work were probably not contaminated, as revealed by cultures made from several of these tissues. Neglecting the possibility of contamination, it would seem

TABLE 3

CITRIC ACID IN EPIDERMAL CARCINOGENESIS
(NORMALITY OF SULFURIC ACID, 6.36)

No. of mice	No. of paintings	Citric acid content (γ/gm., wet wt.)
	Normal epidermis	
4		539
4		375
4		463
3		482
Total: 15		Average: 465
	Benzene-treated mice	
9	3	417
5	3	503
5	3	400
9	3	385
Total: 28		Average: 426
4	6	275
3	6	155
3	6	377
3	6	529
Total: 13		Average: 334
4	12	305
5	12	399
6	12	393
7	12	389
8	12	478
Total: 30		Average: 393
4	24	279
5	24	321
6	24	336
7	24	226
8	24	277
Total: 30		Average: 288
	Methylcholanthrene-treated mice	
5	3	206
4	3	242
8	3	205
7	3	196
Total: 24		Average: 212
4	6	250
4	6	163
3	6	256
3	6	195
Total: 14		Average: 216
3	12	272
5	12	218
6	12	196
7	12	203
8	12	192
Total: 29		Average: 216
3	24	352
5	24	208
6	24	240
10	24	206
Total: 24		Average: 252
	Squamous-cell carcinoma	
4		69.6
2		70.8
3		81.2
Total: 9		Average: 73.9
	Necrosis from squamous-cell carcinoma	
Total: 1		110

at present that there is no set pattern for the citrate content in necrotic portions of tumors with respect to the non-necrotic portions of the same tumors. Obviously, more work is needed to settle the issue on this particular side line.

The application of methylcholanthrene resulted in a very marked initial decrease in the epidermal citric acid content (Fig. 3). This diminution was maintained throughout hyperplasia following a number of treatments with the carcinogen. A second noteworthy decrease may also be noted between hyperplastic epidermis and the squamous-cell carcinoma.

Benzene-treated epidermis also decreased in citrate content, but not so markedly as the methylcholanthrene-treated epidermis (Table 3). No comparable decrease in benzene controls was found in epidermal calcium analyses (2, 10, 11).

DISCUSSION

The results of this present work indicate that some very definite, clear-cut changes take place in the citric acid contents of tumor tissues as com-

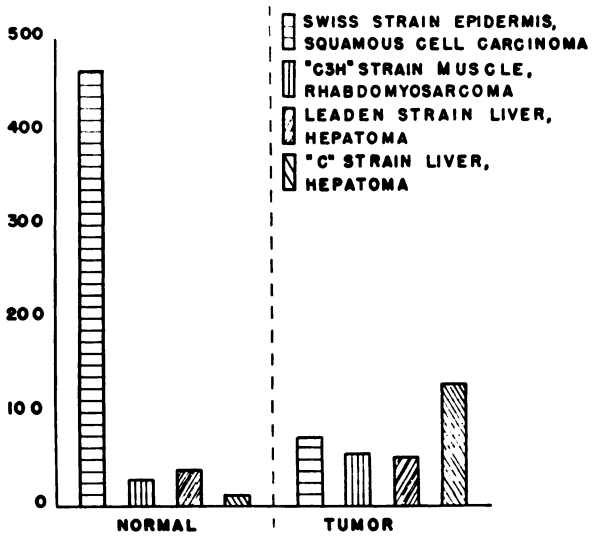

FIG. 2.—The citric acid content of various tumors and their normal homologs (γ/gm.).

pared to their normal homologs. Such changes probably reflect some metabolic alterations in the biochemistry of tumor tissue. The nature of these metabolic processes, however, is uncertain.

Body needs for citric acid are probably obtained by processes of intermediate metabolism (4). Certainly, citric acid cannot be directly derived from nutritional sources in the same manner that calcium and other minerals are obtained. Bone also appears to be an unlikely source (4), despite the high content of citrate in its substance (5). It is, therefore, most probable that the source of citric

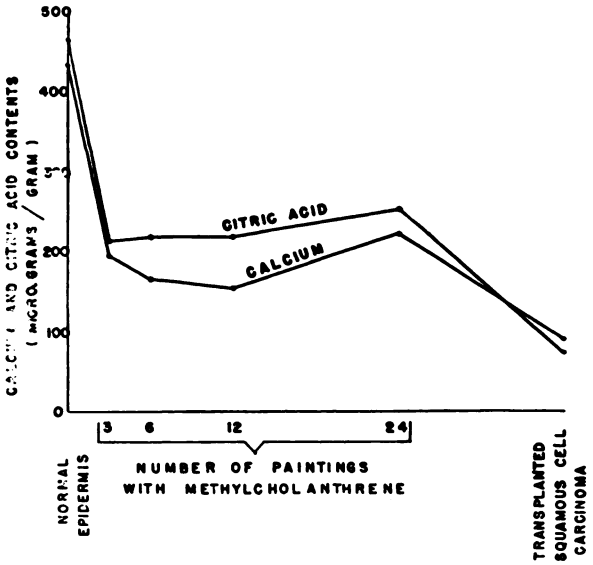

FIG. 3.—Calcium and citric acid in epidermal carcinogenesis.

acid is from the aerobic oxidation of carbohydrates, since citric acid is known to be formed as a by-product of the tricarboxylic acid cycle. Thus, it might be possible to interpret the present data on the basis of changes taking place in the aerobic oxidation of carbohydrates. Much further work is necessary in order to investigate these possibilities.

The correlations between the calcium and citric acid metabolisms of normal and hyperplastic epidermis and a transplanted squamous-cell carcinoma reveal some interesting data (Fig. 3). The changes in the epidermal contents of these two substances are similar in the carcinogenic processes, suggesting the possibility of the two compounds being bound together, probably in the form of a diffusible complex, throughout the process. It is also conceivable that calcium is removed from the site of hyperplasia to a great extent by citric acid, the two substances passing together into the tissue fluids and, hence, into the blood stream. It, therefore, seems very likely that there is some correlation between the present analyses and those of calcium (2, 10, 11).

A probable formula for calcium citrate (Ca_3Cit_2) has been presented by several investigators (7). Calculating the mole ratios between calcium and citric acid in the various epidermal tissues analyzed (Table 4) clearly indicates that, according to this or any other probable formula, only a part of the calcium present in the epidermis or squamous-cell carcinoma can theoretically be bound to citric acid. This fact, however, does not in any way destroy the possibility that citric acid plays an important role in calcium removal. Other substances, such as nucleoproteins and possibly other tricarboxylic acids, undoubtedly

TABLE 4

THE MOLE RATIOS BETWEEN CALCIUM AND CITRIC ACID IN EPIDERMAL CARCINOGENESIS (EXPRESSED AS NUMBERS OF MOLES OF CALCIUM/MOLE OF CITRIC ACID)

Number of paintings with methylcholanthrene	Moles of calcium/mole of citric acid
Normal, untreated epidermis	4.51
3 paintings	4.32
6 paintings	3.63
12 paintings	3.42
24 paintings	4.20
Squamous-cell carcinoma	5.84

share in the functioning of such a mechanism, if it exists at all.

Morphological evidence presented by Banyen[4] may necessitate changes in the interpretations of chemical data from the idea that the individual cell changes chemically in carcinogenesis to the concept that chemical changes are merely due to changes in cell types. It was shown by this investigation that basal cells remain fairly constant in hyperplasia, while granular and spinous cells increase greatly in number but always are present in about the same proportion. The interpretation of calcium and citric acid analyses in epidermal hyperlasia may therefore be modified as follows: The contents of these substances in normal epidermis are very high in the basal layer and much lower in the granular and spinous layer. In epidermal hyperplasia, an increase in the number of granular and spinous cells would serve to decrease the amounts of calcium and citric acid per unit weight of epidermis. Therefore, changes in these substances can be explained on the basis of cell ratios, and not on the basis of an alteration in the

[4] D. Banyen, Study of Cell Types in Mouse Epidermis during Methylcholanthrene Carcinogenesis (in preparation).

chemistry of the individual cell. This concept, of course, is purely hypothetical, but certainly worthy of our attention. It may be that the two conflicting hypotheses, chemical and morphological, each play a definite integrated role in the dynamics of carcinogenesis.

SUMMARY

1. A method for the determination of citric acid in tissues has been outlined.

2. It was found that each normal tissue examined had a content of citric acid characteristic for that tissue, while the tumors, regardless of derivation or source, showed very similar levels of citric acid.

3. An attempt has been made to correlate the results in terms of calcium and carbohydrate metabolism and cell ratios.

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