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The Possible Association Between Porphyrins and Cancer in Mice*

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Since a method was available (13) that could easily be adapted to a quantitative determination of porphyrins in the harderian glands of mice, it seemed advisable, in view of recent publications by others, to ascertain if there might be any relationship between porphyrin metabolism and the possible causes of the various types of cancer, especially mammary cancer, in mice.

Strong and Figge (18) and Figge and his group (12) have made estimates of the degree of red fluorescence of the porphyrins in the harderian glands of mice, by visual inspection and comparison under near-ultraviolet light. They found that there was a rapid increase in red fluorescence soon after the eyes were open, until a maximum was reached at approximately 100 days of age in female mice. After that age the decrease in the red fluorescence was dependent somewhat upon the strain studied. In mice of the low-cancer JK strain the red fluorescence completely disappeared in middle sexual maturity, and this absence persisted for the remainder of life, whereas those of the cancerous C3H stock showed a slower decline in the intensity with advancing age. The authors stated that, in general, mice of strains with a high incidence of mammary cancer showed the maximal red fluorescence of the harderian glands, although some mice of the cancerous A stock gave readings that were characteristic for mice with intermediate degrees of susceptibility. The authors interpreted their data as evidence for the hypothesis that there is either a direct or an indirect relationship between porphyrin metabolism and inherited susceptibility to mammary cancer. It was concluded that a relatively large production of porphyrins in the harderian glands may be inherited as a dominant.

Figge (11) has further postulated some relationship between excess porphyrins (or a unique metabolism) and the susceptibility to carcinogenic agents.

Following the study of reciprocal hybrids between the cancerous C3H and the low-cancer JK stock, Strong (17) reported the following:

(a) In all groups the degree of red fluorescence increased from the time the eyes opened until early sexual maturity was reached, and then declined gradually.

(b) In all groups the fluorescence intensity for the females was greater than for the males of the same strain.

(c) The fluorescence for mice of the F_1 generation was intermediate in intensity between the two ancestral stocks.

(d) The fluorescence intensity noted in animals of the F_1 generation was closer to that of the female ancestral stock than to that of the paternal strain.

Strong concluded that whether porphyrins have any effect in the etiology of cancer or not must be tested by the suitable administration of porphyrins to experimental animals.

MATERIAL AND METHOD

STOCKS OF MICE

The various strains of mice and their hybrids used in this study, with their approximate incidence of

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spontaneous mammary cancer, are given in Table I. Because of certain points to be discussed later we have indicated whether or not the virgin females of the respective groups have the milk agent, the inherited susceptibility, and the inherited hormonal influence, all of which are generally necessary for the development of spontaneous mammary cancer in virgin females (2, 3, 9, 15, 10). Breeding females of the respective groups would have the same inciting influence and, in addition, the increased hormonal stimulation resulting from the bearing of young.

The animals were fed Purina fox chow and had an unlimited supply of tap water.

The technic described by Figge and his associates (12) for removal of the harderian glands was followed.

TABLE I: INCITING INFLUENCE FOR MAMMARY CANCER IN THE VIRGIN FEMALES OF THE VARIOUS GROUPS USED, WITH THE INCIDENCE OF MAMMARY CANCER

No group is listed as noncancerous, although some have remained free from mammary cancer for several years.

Stock	Matings	Milk agent	Inherited susceptibility	Inherited hormonal influence	Approximate incidence in virgins, per cent	Approximate incidence in breeders, per cent
A	A♀ × A♂	+	+	—	3	90
Ax	Ax♀ × Ax♂	—	—	—	1	1
Z (C3H)	Z♀ × Z♂	+	—	+	63	90
Zb	Zb♀ × Zb♂	—	+	+	1	1
AZF_1	A♀ × Z♂	+	+	+	90	90
ZAF_1	Z♀ × A♂	+	+	+	75	90
$AxZbF_1$	Ax♀ × Zb♂	—	+	+	1	1
$ZbAxF_1$	Zb♀ × Ax♂	—	+	+	1	1
D (dba)	D♀ × D♂	+	+	+	50 *	85 *
B (C57 blk.)	B♀ × B♂	—	? †	—	1	1

* Data incomplete and based on small numbers represent mice from 3 sublines.

† Mammary cancer will develop in some animals with the milk agent, but the inherited susceptibility may vary with the subline.

PORPHYRIN DETERMINATION

The harderian glands [1] from a group of animals, varying in number in different experiments, were weighed wet and then minced and ground in an evaporating dish with a small amount of glacial acetic acid. The method used in extracting protoporphyrin from erythrocytes (13) was employed. Three parts of ethyl acetate were added and thoroughly ground together with the mixture, after which the residue was allowed to settle to the bottom of the dish and the supernatant fluid was decanted through a filter paper into a separatory funnel. The acetic-acid-ethyl acetate extraction was repeated several times, until no more fluorescence was observed.

The combined extracts were washed twice with distilled water and the washings examined carefully for porphyrin. If porphyrin was extracted by the water because of excess acetic acid, it was re-extracted with ethyl acetate, which was then added to the original extract.

The porphyrin was next removed from the ethyl acetate by several extractions with small amounts of 5 per cent HCl. The red fluorescence was quantitated in a Klett fluorophotometer against a 3 mgm. per cent fluorescein standard. The readings for a solution of crystalline protoporphyrin in 5 per cent HCl are given in Table II.

In the tables the readings are expressed either as the number of gamma of porphyrin per 100 mgm. of harderian gland (γ/100 mgm.) or gamma per entire gland (γ/ gland).

From the combined extracts of a large number of glands the protoporphyrin was isolated in the usual way, the crystalline methyl ester melting at 227° C. (corrected). No evidence was found to indicate the presence of any porphyrin other than protoporphyrin.

TABLE II: FLUORESCENCE INTENSITY OF PROTOPORPHYRIN IN 5 PER CENT HCl, READ AGAINST A 3 MGM. PER CENT FLUORESCEIN SOLUTION IN A KLETT FLUOROPHOTOMETER

Per 100 cc.	Klett reading
10	10
20	24
30	38
40	48
50	60
60	70
70	80
80	90
90	100
100	108

The calculation of protoporphyrin concentration in the harderian glands is as follows:

$$\gamma \text{ per 100 cc.} \times \frac{\text{cc. of final 5 per cent HCl solution}}{100} \times \frac{100}{\text{mgm. gland extraction}} = \gamma \text{ protoporphyrin per 100 mgm. of gland.}$$

[1] Direct inspection of the glands in ultraviolet light revealed that the red fluorescence was most intense at the surface but it was evident that some porphyrin was contained in the substance of the gland also.

TABLE III: AVERAGE SIZE AND PORPHYRIN CONTENT OF HARDERIAN GLANDS FROM MICE OF THE Z (C3H) AND A STOCKS IN RELATION TO THE MILK AGENT

Stock		Data from Mice with Milk Agent				Data from Mice without Milk Agent			
		No.	Aver. wt.	γ/100 mgm.	γ/gland	No.	Aver. wt.	γ/100 mgm.	γ/gland
Z (C3H)	Virgin	32	.0182	90.6	16.5	24	.0194	83.5	16.2
A	Virgin	32	.0262	21.8	5.7	47	.0226	17.7	4.0
Average	Virgin	64	.0222	56.2	11.1	71	.0210	50.6	10.1
Z (C3H)	Breeder	21	.0259	169.8	44.0	30	.0248	215.5	53.4
A	Breeder	25	.0319	44.1	14.1	28	.0348	28.9	10.1
Average	Breeder	46	.0289	107.0	29.1	58	.0298	122.2	31.8
Z (C3H) and A	Virgin and Breeder	110	.0256	81.6	20.1	129	.0254	86.4	21.0

EXPERIMENTAL RESULTS

Mice of comparable ages of the A and Z (C3H) stocks, with and without the milk agent, are contrasted in Table III as to the porphyrin content of their harderian glands and the average weight of the glands in grams. Table IV gives similar data for breeding females of the cancerous A and Z stocks, 76 per cent of which had mammary cancer at the time they were used; and for breeding females of these stocks of the same ages, which were free of the milk agent and in which no mammary tumors have been observed during the past 3 years (Ax and Zb lines). Whereas the mice of the A stock with the milk agent showed slightly higher readings than did mice without the agent (Ax line), the opposite was the case for the two lines of the Z stock. The averages for the two groups (virgins and breeders) were approximately the same; mice without the milk agent had slightly higher readings, but the difference was probably not significant.

TABLE IV: DETERMINATIONS OF PORPHYRINS IN HARDERIAN GLANDS OF MICE OF THE A AND Z (C3H) STOCKS WITH AND WITHOUT THE MILK AGENT

Seventy-six per cent of the mice with the agent had mammary cancer.

Stock	No.	Cancerous	Aver. wt. of gland, gm.	γ/100 mgm.	γ/gland
A	20	14	.0388	42.0	16.3
Z (C3H)	22	18	.0251	169.0	42.4
Ax	19	0	.0282	37.7	10.6
Zb	31	0	.0239	221.2	52.9

Since these comparisons did not indicate any relationship between the porphyrin content of the harderian glands and the active milk agent, the data obtained for mice with and without the agent of the A and Z (C3H) stocks and their reciprocal F_1 hybrids have been combined in subsequent tabulations. The porphyrin determinations are presented in Table V according to age. The average weights of the glands in grams and the number of mice

TABLE V: PORPHYRIN DETERMINATIONS FOR MICE OF VARIOUS STOCKS ACCORDING TO AGE EXPRESSED IN GAMMA PER 100 MGM. OF TISSUE OR GAMMA PER GLAND

Stock		Total no.	4-6 wks.		2-7 mos.		8-13 mos.		14-18 mos.		18 + mos.	
			γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland	γ/100 mgm.	γ/gland
Z (C3H)	Vg. ♀	83	27.5	3.3	91.4	17.4	83.0	23.2	155.4	34.2	121.9	28.2
	Br. ♀	75	—	—	164.8	42.0	202.4	48.8	227.6	54.2	—	—
A	Vg. ♀	80	25.8	3.2	22.4	5.5	—	—	—	—	16.7	5.0
	Br. ♀	92	—	—	33.9	11.5	43.9	14.1	26.1	8.6	42.2	15.4
F_1 AxZ	Vg. ♀	74	—	—	—	—	40.5	13.9	76.9	24.5	65.9	19.5
	Br. ♀	70	—	—	97.0	27.5	136.9	42.0	121.6	40.4	165.9	54.1
D (dba)	Vg. ♀	58	5.5	0.9	25.1	5.4	51.8	14.5	—	—	177.0	56.1
	Br. ♀	14	—	—	33.4	5.8	120.8	30.2	105.4	24.0	—	—
B (C57)	Vg. ♀	35	—	—	2.2	0.5	1.3	0.4	—	—	1.4	0.4
	Br. ♀	12	—	—	—	—	5.1	1.9	6.3	2.8	6.2	2.0
F_1 ZxD	Vg. ♀	9	—	—	41.3	9.6	43.6	11.9	—	—	—	—
	Br. ♀	6	—	—	—	—	206.0	63.5	—	—	—	—
A	Spayed ♀	5	—	—	—	—	—	—	—	—	78.8	20.7
ZBC	Spayed ♀	6	—	—	—	—	—	—	—	—	100.2	34.7

killed during each age period are given in Table VI. From the tabulations it is obvious that while the data are not so extensive as might be desired some general conclusions may be drawn.

In virgin females of the Z stock there was a gradual increase in the amount of porphyrin that could be extracted from the harderian glands, from the time the animals were 4 weeks of age until they had attained the age of 18 months. One reading for mice 22 months of age was slightly lower than that for animals 18 months of age, but it was higher than any secured for mice of other ages. From 2 to 18 months of age the breeding females of the Z stock had higher readings than did the virgin females. Breeders of 2 to 7 months of age had heavier glands than did the virgin females, but after 8 months no difference in size was apparent.

There was a slight increase in the harderian gland porphyrin from virgin females of the A stock of 4 weeks to 2 months of age, but by 6 months the amount was approximately the same as in 4 week old animals.[2] Virgin females from 7 to 17 months of age were not available for the study, but those that were 18 to 20 months of age did not show any decrease. Breeding females of the A stock had approximately the same amount of porphyrin during their entire life span, or until the age of 20 months. The harderian glands of the breeders were larger than those of virgins of the A stock and, because of this fact, the porphyrin content of the glands in these groups showed greater differences when the readings were obtained for the entire glands than for 100 mgm. of tissue.

Reciprocal F_1 hybrids between the A and Z stocks, with and without the milk agent, were available as virgins only between the ages of 8 and 24 months. The harderian glands removed from the hybrids were of approximately the same size as in the A stock, and larger than those from mice of the Z strain. Because of the larger glands in the F_1 virgins than in the Z virgins, the differences between the porphyrin content of the entire gland were not so great as the readings for 100 mgm. of tissue. Breeding F_1 females showed higher porphyrin readings at comparable ages than the virgin F_1 females. Because of the larger size of the harderian glands in the F_1 breeders, the porphyrin content of the entire gland was approximately the same as in breeding Z females. However, the hybrid mice attained their high level at a later age than did the Z animals. Based on the porphyrin content for 100 mgm. of gland, the readings for the breeding F_1 females were intermediate between those for A and Z breeders.

At comparable ages the average weight of the harderian glands from virgin females of the D stock was greater, based on small numbers, than the weight of glands from breeding females. From 4 weeks to 7 months of age the porphyrin content of glands from the D stock compared with the readings secured for mice of the A stock. After 8 months of age the increase was more rapid in the breeding females, and one group of virgin females, killed at 22 months of age, had the highest reading for virgins of any strain.

Five determinations were obtained on F_1 hybrids between the Z and D stocks. In virgin females 6 to 12 months of age the averages were about the same

TABLE VI: NUMBER OF MICE OF THE VARIOUS GROUPS KILLED DURING VARIOUS AGE PERIODS, AND AVERAGE WEIGHT OF HARDERIAN GLANDS IN GRAMS

Stock		4-6 wks.		2-7 mos.		8-13 mos.		14-18 mos.		18 + mos.	
		No.	Aver.	No.	Aver.	No.	Aver.	No.	Aver.	No.	Aver.
Z (C3H)	Vg. ♀	16	.0119	38	.0190	3	.0280	6	.0220	20	.0231
	Br. ♀	—	—	24	.0255	43	.0241	8	.0238	—	—
A	Vg. ♀	19	.0125	21	.0246	—	—	—	—	40	.0301
	Br. ♀	—	—	21	.0339	46	.0320	17	.0331	8	.0364
F_1	Vg. ♀	—	—	—	—	5	.0342	21	.0319	48	.0296
	Br. ♀	—	—	5	.0283	24	.0307	21	.0332	20	.0326
D (dba)	Vg. ♀	6	.0161	26	.0215	23	.0280	—	—	3	.0317
	Br. ♀	—	—	4	.0175	7	.0250	3	.0227	—	—
B (C57)	Vg. ♀	—	—	12	.0224	6	.0302	—	—	17	.0283
	Br. ♀	—	—	—	—	6	.0371	2	.0446	4	.0321
F_1 (ZxD)	Vg. ♀	—	—	3	.0233	6	.0272	—	—	—	—
	Br. ♀	—	—	—	—	6	.0308	—	—	—	—
A	Spayed ♀	—	—	—	—	—	—	—	—	5	.0263
ZBC	Spayed ♀	—	—	—	—	—	—	—	—	6	.0348

[2] Four week old animals of the A and Z stocks gave approximately the same readings. The more rapid increase in mice of the Z stock is not apparent in the table because of the small number of mice killed at 6 weeks of age as contrasted with the number killed when they were 4 weeks of age.

as for virgin females of the D stock; in other words, lower than for the Z stock. The single reading for breeding females of the ZDF_1 hybrid generation compared well with the content of glands from breeding females of the Z stock of the same age. The figure for the whole glands was the highest obtained in any group.

While the average weight of the harderian glands from virgin females of the B (C57 black) low-cancer stock was no greater than in A virgins, breeding females of the B stock had the largest glands of any group. While there was a slight increase in the porphyrin content in glands from the breeding females, they had readings that were lower than those for virgin females of any other stock.

With the cooperation of Miss Fern Smith it was possible to obtain readings on 2 groups of ovariectomized mice (16): animals of the A stock that were 20 months of age, and ZBC ($ZbAxF_1$ ♀ x Z♂) females of 23 months. The porphyrin content of the harderian glands from the spayed A females was higher than for any other group, breeder or virgin, of the A stock. The reading for the castrated ZBC females was higher than for any group of AZF_1 virgins, and compared with virgin Z females of 18 months.[3]

DISCUSSION

It is evident that the data submitted above are not adequate to permit a complete analysis of porphyrin metabolism in the different strains of mice. However, from these observations, involving 178 determinations on over 600 mice, some general conclusions may be drawn on the possible role of the porphyrins in the etiology of cancer in mice, and especially of mammary cancer.

We were unable to find any correlation between the active milk agent for mammary cancer and the amount of porphyrin present in the harderian glands.[4] That is, different lines of the same inbred stock, one with and the other without the agent, gave approximately the same reading for mice of comparable ages. No evidence has been obtained that foster nursing of mice of cancerous stocks (2), with the elimination of the active milk agent, will influence the inherited susceptibility for the development of spontaneous mammary cancer (4-7). Also, this susceptibility may be transmitted by males as well as by females of the susceptible stock.

Based on the porphyrin content of 100 mgm. of gland, our findings would tend to confirm those of Figge and his associates (12), and of Strong (17), in that the porphyrin content in hybrids was intermediate between the parental strains. However, when readings for the entire gland were considered the hybrids might compare with the parental stock with the higher amount, except that the maximal level in the hybrids was attained at later ages. This variation in the readings between 100 mgm. of tissue and the entire gland was due primarily to the difference in the average weights of the harderian glands in the mice of the various groups. Although other workers (12) have stated that the degree of red fluorescence was lost at different ages by mice of different stocks, we could find no apparent reduction in the amount of porphyrin that could be extracted from the harderian glands with increasing age. The difference in the method of determination may account for this, since Figge (11) stated: "The extraction of some of the non-red-fluorescence showed that porphyrins were present, but the concentration was not high enough to be detected by fluorescence in near ultraviolet light."

No evidence has been advanced to indicate that mice of the Z or C3H strain are more "susceptible" to the development of mammary cancer than those of the A stock. The breeding females of the two strains employed in the present study have approximately the same incidence (5, 6, 10). While the virgin females of the C3H stock have a much higher incidence than virgin females of the A stock, this difference results primarily from genic control of the hormonal mechanism (9, 15): a condition called the inherited hormonal influence. The genic make-up of the inherited susceptibility is not the same as the inherited hormonal influence (10).

When reciprocal hybrids were produced by mating mice of the cancerous A and Z stocks, the results showed that either the two strains had the same susceptibility for mammary cancer, or, if different genes were transmitted, any combination of these genes would produce susceptibility in the F_2 generations (10). Unless we are able to detect a difference in the inherited susceptibility to mammary cancer in these two strains, for which there is no evidence at present, it would follow that the mice of these two cancerous stocks should have approximately the same porphyrin readings if there be any association between their porphyrins and their inherited susceptibility to spontaneous mammary cancer. This was not the case, and furthermore, because mice of the dilute brown stock, another cancerous strain, behaved quite differently from either of the other two cancerous stocks with respect to the porphyrin content of the

[3] Exophthalmos was noted in the spayed ZBC females, but not in the A animals, and the ZBC mice bled profusely when the harderian glands were removed.

[4] An extract of harderian glands from mice with the milk agent, diluted 1:100, has been shown to have the active agent (unpublished data).

harderian glands, we were unable to find any correlation between the inherited susceptibility to mammary cancer and the porphyrins such as has been postulated by others (11, 17).

This difference of opinion may be due to interpretation of the data in the two experiments. Figge and his associates (11) stated that "one apparent exception to the rule that mice with high susceptibility to spontaneous cancer show maximal red fluorescence of the harderian gland was seen in mice of the A stock." Of the 13 inbred strains of mice that they used in their study only 2, the C3H and the A, showed "high susceptibility" to spontaneous mammary cancer. For this reason their conclusion that a correlation exists does not appear to be warranted.

In every group employed in the present study, where it was possible to make comparisons, the breeding females showed higher readings than did the virgin females of the same stock. From this it would appear that the hormones may influence, in some manner, porphyrin metabolism.

It is of interest to note that the highest readings were observed in mice of stocks that were capable of developing a high incidence of mammary cancer in the virgins. Previous observation (9, 15, 8, 10) have shown that only the virgin females of strains with the inherited hormonal influence have a high incidence of mammary cancer. However, if there is any association between the porphyrins of the harderian glands and the inherited hormonal influence, some physiological effects of which have not yet been determined, the present data would imply that mice of the various stocks with the influence do not respond alike, for in the various groups the maximal readings might be attained at different ages. These differences may be effects of hormonal activity such as are reflected by variation in the average cancer age. On the other hand, if there is any relationship between porphyrin metabolism and the hormones, the high values observed in the 2 groups of spayed females would suggest that at least one of the principal hormones may be involved, the physiological effects of which have not been previously associated with those of the inherited hormonal influence.

These conclusions were made possible only because the readings for virgin females were compared with those of breeding females of the same strain. The other workers (12, 11, 17) have not differentiated between the two groups, although they found that females had higher degrees of fluorescence than males.

While Figge (11) has also assumed that the porphyrins may play some role in reflecting the susceptibility of tissues to various carcinogenic agents, the studies of Andervont (1) and Heston (15), correlated with our findings, would not support such a theory for the induction of lung tumors. As for spontaneous lung cancer, it was common in old mice of the A stock, especially those of the fostered line, in which the readings were low to intermediate; rare in mice of the Z stock with high readings; and common in the F_1 hybrids between the A and Z stocks without the milk agent and with high readings.

Thus it is possible that the primary relationship between the porphyrins and cancer may be an association between the porphyrins and the hormones.

SUMMARY

The extraction of porphyrins from the harderian glands of virgin and breeding females of various inbred stocks of mice and F_1 hybrids would suggest that:

There is no decrease in the porphyrin content of the harderian glands with increasing age.

There is no relationship between the presence of the active milk agent and the porphyrins.

There is no simple correlation between "inherited susceptibility" to mammary and lung cancer and the porphyrins.

In every stock the breeding females showed higher porphyrin concentrations in the harderian glands than did the virgin females. Spayed females may show higher reading than virgin females of the same stock.

The maximal concentrations in the various groups were attained at different ages.

The apparent relationship between the porphyrins and mammary cancer, as described by others, may be due to a correlation of unknown nature between the porphyrins and the hormones. In virgin females this association may involve the "inherited hormonal influence."

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