Carcinogenesis in the Hamster Cheek Pouch

I. Correlation of Histopathology with Soluble Sulfhydryl Groups*

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SUMMARY

Pouches from three experimental and one control animal were excised for analysis at various times during the first week and thereafter each week of carcinogen application (9,10-dimethyl-1,2-benzanthracene) until the 13th week, at which time large tumors were present in all remaining animals. Histologic progression from normal through inflammation, uniform hyperplasia, localized premalignant hyperplasia, to small tumors (7 weeks) and then large squamous-cell carcinomas, was observed. Sulfhydryl group concentration, determined by amperometric titration of acid-soluble fractions of cell-free extracts of the whole pouches of males, showed a biphasic curve with an increase in -SH at 36 hours, followed by a decrease at 60 hours. Pouches from females did not exhibit the early rise, and the decrease was more profound. -SH concentrations remained low until the period of tumor production and then rose slowly in the whole pouches. Tumor tissue contained about 40 per cent of the concentration of soluble SH exhibited by the nontumor-bearing tissue from which it was removed. The latter fell within the range of control tissue.

In 1954, Salley (6) produced the first experimental squamous-cell carcinoma in the cheek pouch of Syrian hamster by painting with 9,10-dimethyl-1,2-benzanthracene. Since that time factors influencing chemical carcinogenesis in that site have been investigated (3–5, 7). It is possible to induce the mucosa of the pouch to undergo changes from normal to malignant in approximately 12 weeks. Tumors develop in all animals with good uniformity of response, and there is no loss of animals due to toxicity. Histopathologic study of biopsy material removed from the pouch after 12 weeks of exposure to the carcinogen reveals tumors which satisfy all the microscopic criteria for malignancy. If application of the carcinogen is then stopped, the animals will survive approximately 4 weeks, at which time metastases are readily demonstrable at autopsy.

The uniform carcinogenic response of the hamster cheek pouch mucosa affords an excellent opportunity for the study of biochemical changes associated with carcinogenesis. This tissue is particularly well suited for biochemical analysis in that it does not contain hair, sebaceous or sweat glands, which complicate the process of carcinogenesis in skin. This investigation was undertaken to correlate the histopathology with the changes in content of acid-soluble sulfhydryl groups during carcinogenesis in the hamster cheek pouch which are reported in this paper and the changes of certain enzymes which are reported in a following paper.

MATERIALS AND METHODS

Animal procedures.—The first pilot experiment included animals of both sexes, but subsequently only one sex was in each series. Syrian albino hamsters were obtained from the Pee Dee Farms, Box 186, Driver, Va. All animals were kept in wire

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mesh cages and fed tap water and Purina chow checkers ad libitum. A 0.5 per cent mineral oil solution of 9,10-dimethyl-1,2-benzanthracene was applied 5 × per week with a #4 camel's hair brush to both cheek pouches of young, adult hamsters weighing from 90 to 170 gm. At periodic intervals, under intraperitoneal nembutal anesthesia, one pouch of an animal was everted and excised distal to black silk sutures which were placed to prevent hemorrhages. In the first series three pouches were removed after 1 week of painting and thereafter weekly—alternating the sexes. In this series and in three later experiments (2, all males, and 4 and 5, all females), pouches from three experimental and one control animal were removed for analysis the day after each of the first six carcinogen applications and thereafter once each week until the 7th or the 18th week. For experiment 3, tissues were removed at 12, 36, and 60 hours, following a single application of the carcinogen to pouches of males. Two series of controls were painted with mineral oil without carcinogen.

A representative portion of the excised pouch was placed in 10 per cent formalin, and the remainder was weighed, immediately frozen in an ice-alcohol bath, and transported to the laboratory for analysis. Hematoxylin- and eosin-stained slides were prepared in the usual manner after paraffin embedding of the formalin-fixed tissue.

**Biochemical procedures.**—The frozen cheek pouches were weighed, then minced with scissors in a cold mortar, and ground with Al₂O₃, weighing 2.5 times the wet tissue weight. The paste was extracted with 5 ml of 0.25 M sucrose per gm. tissue and centrifuged at 10,000 × g for 60 minutes at 4°C. Samples of the supernatant extract were used for analysis of protein content by the method of Botting and Jones (2) and for enzyme analysis (Scott and Lisi [9]).

To one sample was added TPN, glucose-6-phosphate, and nicotinamide, and the mixture was incubated for 15 minutes at room temperature to allow glutathione reductase to oxidize disulfide to -SH by the TPNH produced by glucose-6-phosphate dehydrogenase, both enzymes being present in the extract. From this sample and from an untreated sample of extract the protein was precipitated with cold 4 per cent sulfosalicylic acid and removed by centrifugation. Samples of the supernatant fluid were titrated with 0.001 or 0.0005 M AgNO₃ in the Tris-nitrate buffer by a rotating platinum electrode as described by Benesch, Lardy, and Benesch (1). The half cell consisted of mercury, mercuric oxide, and barium hydroxide according to Samuelson and Brown (8). A Rubicon galvanometer with an Ayrton shunt and a micropipette made a system in which 0.005 μmole of AgNO₃ produced a deflection of 5–10 mm. on the galvanometer. For samples containing 0.01 μmoles -SH or more, duplicate and triplicate determinations were made which did not differ by more than 10 per cent from the mean. This method has been described in detail and evaluated by Stern (11).

Table 1 lists the recoveries of glutathione, which was added to extracts of hamster pouches which had been painted for 5 weeks and were hyperplastic in appearance, before and after precipitation of the protein with sulfosalicylic acid, and the reduction of oxidized glutathione which was added to extracts and was reduced by the addition of TPN and glucose-6-phosphate. There was approximately 50 per cent recovery of 0.1 μmole and no recovery of 0.03 μmole or less of reduced glutathione added to extracts, whereas 0.1 μmole added to the sulfosalicyc acid-soluble fraction was completely recovered. Oxidized glutathione was reduced by the TPNH-glutathione reductase of the extract and the TPNH produced by endogenous glucose-6-phosphate dehydrogenase in the presence of TPN and glucose-6-phosphate. Once the extracts had been acidified there was no greater loss of added glutathione than occurred by the usual slow autooxidation, which was evident even at —20°C.

**RESULTS**

**Gross observations.**—The initial response of the pouch mucosa after application of the carcinogen in this concentration was an inflammation. This was first noticed at the time of the third and fourth painting as a hyperemia and mild edema. In Experiment 5, all very young females, the inflammatory reaction was most acute, with some pouches having bleeding ulcers and all weighing 2–3 times the normal. Although necrosis and suppuration sometimes occurred in the longer experiments, the tissues recovered quickly, and in spite of continued carcinogen application, appeared grossly normal after 2 or 3 weeks. No further changes could be seen until approximately 6 or 7 weeks, at which time initial small tumors were visible. Multiple lesions appeared during the following weeks, and their growth was very rapid. Papillomatous tumors measuring a centimeter in diameter were commonly found at 12 weeks. Frequently these larger lesions showed evidence of necrosis. Accompanying three to five larger tumors were multiple tumors of all sizes, the smaller ones often being too numerous to count. The nontumorous mucosa appeared thickened and had an irregular surface.

**Microscopic observations.**—The normal hamster
pouch mucosa consisted of keratinizing, stratified, squamous epithelium which was three to five cell layers thick, overlying a thin layer of relatively cellular connective tissue (Fig. 1). The remainder of the pouch was composed of striated muscle, the thickness of which decreased slightly as one went from the proximal to the distal portion of the pouch. Salley (7) has described the microscopic changes which the pouch undergoes when exposed to a carcinogen under the conditions used in this experiment. An example of the severe inflammation described above can be seen in Figure 2. The epithelium has become necrotic and has sloughed away, and inflammatory cells can be seen surrounding the muscle bundles. It should be pointed out that this illustrates an extreme involvement and that the epithelium was not invariably nor uniformly sloughed. The pouch of Figure 2 was from a female of Experiment 5; these hamsters were younger than those in other series, weighing 80–100 gm. Also, inflammation is not an inherent phase of pouch mucosal carcinogenesis. No inflammation has been observed when tumors were induced with weaker concentrations of the carcinogen (3).

After recovery from the inflammation, regeneration and hyperplasia of the epithelium occurred. This was first seen as a uniform hyperplasia resulting in an epithelium which was eight to ten cell layers in thickness (Fig. 3). The keratin layer might be somewhat increased at this stage, but it was not a conspicuous finding.

The earliest indication that a tumor was going to develop was seen as a proliferative downgrowth of the epithelium (Fig. 4). We have termed this preneoplastic hyperplasia in contrast to the uniform hyperplasia from which it originated. A squamous-cell carcinoma can be seen in Figure 5 in which pleomorphism, mitoses, and invasion of connective tissue and muscle are evident.

In the dynamic carcinogenic process, overlapping of the described stages occurred. For example, after approximately the 11th week the most important microscopic finding was that of malignant tumors of varying size. Much of the mucosa, however, was in the stage of preneoplastic hyperplasia. The weeks from initial painting at which the described stages were most conspicuous and dominant are as follows: 1–2, inflammation; 3–5, uniform hyperplasia; 7–9, preneoplastic hyperplasia; 11–13, malignant tumors. The histological investigation of control pouches painted with mineral oil showed no significant changes.

In Experiment 4 with females, no pouches con-

### Table 1

<table>
<thead>
<tr>
<th>Extract</th>
<th>Additions (µMole)</th>
<th>TPN+ G-6-P</th>
<th>Titrated total-SH (µMole)</th>
<th>Calculated total-SH (µMole)</th>
<th>Difference total-SH (µMole)</th>
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<tr>
<td>No.</td>
<td>M1.</td>
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<td>GSII 0.020</td>
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<tr>
<td>Mixed acid-soluble</td>
<td>GSII 0.090</td>
<td>0.065</td>
<td>0.156</td>
<td>0.155</td>
<td>0</td>
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</table>

* GSSG, calculated as GSH equivalents.
tained tumors at 44 days, two of the three pouches at 51 days, and all thereafter.

One pouch of a male in Experiment 2 had a small tumor as early as 9 days, and tumors were found in all three at 43 days and thereafter, except for one tumor-free pouch at 59 days. There was some difference in time of response to the carcinogen, both in degrees of inflammation or hyperplasia and in the time of appearance of tumors.

In Experiment 1, two of the three pouches of females had at least small tumors at 49 days, and all at 56 days and thereafter; two-thirds of the males had tumors at 49 days and all thereafter, except one pouch which had no grossly visible tumor at 77 days.

Sulfhydryl determinations.—Controls: The means, range, and standard deviations of the sulfhydryl titrations on the acid-soluble fraction of the extracts of unpainted cheek pouches as controls are shown in Table 2. By direct titration the means for males and females were the same. The higher value for total -SH concentration after reduction of the male compared with female pouches is significant with $P < .05$ and $>.02$.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Direct (μmoles/gm protein)</th>
<th>Total after reduction (μmoles/gm protein)</th>
<th>Per cent reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Mean: 21.2 (96)*</td>
<td>29.0 (16)</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>Range: 14–34</td>
<td>± 5.8</td>
<td>± 5.8</td>
</tr>
<tr>
<td></td>
<td>S.D.: ± 1.17</td>
<td>± 1.48</td>
<td>± 1.48</td>
</tr>
<tr>
<td>Female</td>
<td>Mean: 20.8 (12)</td>
<td>24.2 (14)</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Range: 18–26</td>
<td>± 4.2</td>
<td>± 4.2</td>
</tr>
<tr>
<td></td>
<td>S.D.: ± 1.33</td>
<td>± 1.40</td>
<td>± 1.40</td>
</tr>
</tbody>
</table>

* In parentheses are the numbers of individual pouches examined.

In Table 3 are listed the mean values of the -SH titrations of extracts of individual pouches painted with mineral oil only as controls. At different times after one painting the sulfhydryl contents lay within the range of the unpainted controls. Two of the three pouches which had been painted 3 times weekly for 9 weeks had significantly lower sulfhydryl values than the controls.

Experimental: The pouch wet weights were found to be increased during the inflammatory period from 8 to 15 days, decreased from 3 to 6 or 7 weeks, and increased again during the period of preneoplastic hyperplasia and tumor formation. The protein concentration of the extracts ranged from 2.6 to 14.8 mg/ml. The young females of Experiment 5 had a high concentration of protein (7.0–8.8) in the initial pouch extracts; this was increased after 5 days (three paintings) to 3.3–12.6 at the beginning of inflammatory reaction. In all experiments the total soluble protein per pouch was increased during the inflammatory period and the preneoplastic period and thereafter, to 2–4 times the control values at the time of appearance of tumors.

The sulfhydryl concentrations of the three painted pouches taken at any one time showed greater variation among themselves than those of the controls. In Charts 1 and 2 are plotted the means of the -SH concentrations as μmoles of -SH per gm. of protein of the extracts of the three individual pouches at specified times after the initiation of painting with dimethyl benzanthracene.

The -SH by direct titration of the acid-soluble fraction of the male pouches reacted to painting with carcinogen with an early rise which was significantly higher than that of the controls in five of six animals at 24 and 36 hours after one painting (Chart 1). With further paintings the values fell below the control mean and in 14/18 animals was significantly low ($P = <.05$) during the 2d, 3d, and 4th week. The -SH concentrations increased thereafter, but 11/15 were still below the mean control for the remaining period of painting—that is, during the time of hyperplasia and tumor formation.

The direct titration of the acid-soluble fraction
tained tumors at 44 days, two of the three pouches at 51 days, and all thereafter.

One pouch of a male in Experiment 1 had a small tumor as early as 9 days, and tumors were found in all three at 413 days and thereafter, except for one tumor-free pouch at 59 days. There was some difference in time of response to the carcinogen, both in degrees of inflammation or hyperplasia and in the time of appearance of tumors.

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<table>
<thead>
<tr>
<th>Sex</th>
<th>Direct (µmoles/gm protein)</th>
<th>Total after reduction (µmoles/gm protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Mean: ~1.2 (±26)* 29.0 (±16)</td>
<td>S.D.: ±5.8  S.E.M.: ±1.17</td>
</tr>
<tr>
<td>Females</td>
<td>Mean: 20.8 (±12) ±4.2 (±14)</td>
<td>Range: 14-34  S.D.: ±4.6  S.E.M.: ±1.33</td>
</tr>
</tbody>
</table>

Per Cell?
73
86

* In parentheses are the numbers of individual pouches examined.

In Table 3 are listed the mean values of the -SH titrations of extracts of individual pouches painted with mineral oil only as controls. At different times after one painting the sulfhydryl contents lay within the range of the unpainted controls. Two of the three pouches which had been painted 13 times weekly for 9 weeks had significantly lower sulfhydryl values than the controls.

Experimental: The pouch wet weights were found to be increased during the inflammatory period from 8 to 15 days, decreased from 13 to 6 or 7 weeks, and increased again during the period of preneoplastic hyperplasia and tumor formation. The protein concentration of the extracts ranged from ~.6 to 14.8 mg/ml. The young females of Experiment 5 had a high concentration of protein (7.0-9.8) in the initial pouch extracts; this was increased after 5 days (three paintings) to 8.3-11.6 at the beginning of inflammatory reaction. In all experiments the total soluble protein per pouch was increased during the inflammatory period and the preneoplastic period and thereafter, to ~4 times the control values at the time of appearance of tumors.

The sulfhydryl concentrations of the three painted pouches taken at any one time showed greater variation among themselves than those of the controls.

Chart 1.—Concentration of acid-soluble sulfhydryl of pouches of male hamsters.

Each symbol is the mean sulfhydryl concentration of three pouches taken at the time indicated after initiation of painting with 9,10-dimethyl-1,2-benzanthracene. The filled symbols and unbroken lines are concentrations of the acid-soluble sulfhydryl of untreated extracts of pouches. The open symbols and broken lines are concentrations of the acid-soluble sulfhydryl of extracts which have been incubated with TPN + G-6-P. The inverted triangles ▼ are Exp. 1, the triangles ▲ are Exp. 3, and the circles ○ are Exp. 2. The horizontal lines indicate the mean sulfhydryl concentration of control (untreated pouches).

Chart 2.—Concentration of acid-soluble sulfhydryl of pouches of female hamsters.
The symbols are the same as in Chart 1, except that X's indicate the direct titrations of acid-soluble extracts of Exp. 1, the triangles ▲, △ are Exp. 4, and circles ●, ○ Exp. 5.
Chart 3.—Concentration of acid-soluble sulfhydryl of extracts of tumors and of the residual pouches.

The -SH concentrations of tumors and of the residual pouches are represented by solid bars and crosshatched bars, respectively. The dotted bars represent whole pouches, either containing tumors or tumor-free as indicated. The mean and the ranges of -SH concentrations of extracts of control pouches are shown at the left.

Chart 4.—Total acid-soluble sulfhydryl of pouches of female hamsters.

Each symbol represents the mean value of sulfhydryl concentration per ml. times the total volume of extract of one pouch taken at the time indicated after initiation of painting with carcinogen. The heavy lines connect the mean content at each time. The filled circles, ●, are the sulfhydryl content of the acid-soluble fraction of unincubated extracts; and the crosses, X, are the sulfhydryl content of the acid-soluble fraction of extracts which have been incubated with TPN + G-6-P.
of extracts of the painted pouches of females (Chart 2) did not show the initial rise evident in the males. After the 3d day and the beginning of the inflammatory reaction and until the 7th week, all but one pouch had significantly low sulfhydryl concentrations. Thereafter, the values rose—after 6 weeks in Experiment 4, or 8 weeks in Experiment 1. The tumor-containing pouches were all within normal range.

The -SH concentration after reduction in the extracts of pouches of males showed an increase at 37 hours and was normal at 60 hours after one painting. It was not determined during the inflammatory period, was low at 4 weeks, and recovered to the control range during the rest of the experiment (Chart 1).

In female pouches, especially those of the young animals of Experiment 5, the -SH concentration after reduction was significantly low at 5 days—i.e., after three paintings, and all during the inflammatory reaction. In Experiment 4 the inflammation and the low total -SH occurred at 8 days—i.e., after four paintings.

The lines have been drawn to connect points in two later experiments, one for each sex. The values from the first experiment are also in the chart and illustrate the variability between experiments, although the over-all pattern is not different.

When large tumors had become evident, the grossly visible tumor masses were separated and analyzed separately from the residual pouch, and the analytical results are shown in Chart 3. A significant difference is evident between the low values of the tumors and the high values of the residual tissue.

This difference is greater for the tumors from females than from males. Low -SH content was found in two pouches from males which were filled with tumors and very necrotic. Normal values were found in two pouches of females in which painting was discontinued when gross tumors were seen; at the time when the pouches were taken, no tumors were grossly visible. In two more pouches on which painting had been discontinued, small tumors were found on the ends of the pouches when they were removed. These were considered as partially regressed; the -SH content was lower than the normal range but not as low as the other tumors of females.

DISCUSSION

The results reported above have been in terms of concentrations and do not take into consideration the greatly increased size of the pouches and the higher protein content of the extracts during the inflammatory period and again during the latter period of hyperplasia and tumor formation. Therefore, the total -SH content of each pouch has been calculated, and the results for the two series of females are shown in Chart 4. The range of contents of the three pouches at each time period has been shaded, and the mean is shown by the solid lines—with circles for the direct -SH and crosses for the total after reduction. In this chart the difference between the -SH contents of TPNH-treated and untreated extracts is very evident. The direct -SH fell as low as 25 per cent of the total during the severe inflammatory reaction and was increased later. The total titrated after reduction increased discontinuously, instead of decreasing. When tumors started to form, both total -SH contents were above the control.

These patterns of changes in sulfhydryl content of the cheek pouch of the hamster during painting with carcinogen were similar to the changes in the rat uterus during the hyperplastic response to estradiol (Scott and Pakoskey [10]). With extracts of the uterus the titrable -SH could pass from the acid-soluble to the acid-precipitable form, with the sum remaining constant, and also on addition to the extract of TPN and glucose-6-phosphate some of the increased -SH in the acid-soluble fraction seemed to be derived from the previously acid-insoluble fraction. Ungar and Romano (19) reported that when nerve was electrically stimulated, -SH groups were uncovered in protein and some -SH went from a nondialyzable to a dialyzable form. The -SH content of the acid-insoluble protein was not determined in the hamster cheek pouch, so it is not possible to ascertain whether substances carrying -SH passed from one state to the other.

Whether, in these studies, changes in acid-
soluble -SH were causative or merely accompanied
by hyperplasia is not evident from the data. It is
possible that both the pyridine nucleotides and
 glutathione are involved in reactions which de-
 toxify the carcinogen or react with the estrogen,
resulting in decreased glutathione concentration
and increased proportion of oxidized TPN. Perhaps
some other member of the chain on the way to
oxygen--such as flavoprotein or a hemeprotein--
has reacted with the carcinogen or with products
of cell destruction, and the oxidized flavoprotein
or cytochrome c has oxidized the TPN, thus de-
creasing the TPNH available for keeping the glu-
tathione and the protein -SH reduced. We expect
to investigate these alternative possibilities.

The difference between the sexes in the changes
of -SH in response to the carcinogen, both during
the early inflammatory reaction and the later
period of tumor formation, is striking. This effect
may be related to the differing action of sex hor-
mones.

It is recognized that analysis of the whole pouch
cannot give specific information about the epi-
thelial tissues in which the tumors appear. IIow-
ever, except for the most violent inflammatory
reactions, no change in the morphology of the
underlying connective tissue and muscle strands
has been seen. Therefore, we suggest that the
sulfhydryl changes in the total tissue may be
correlated with the morphological changes in the
epithelial layer. Methods of treating the tissue to
separate the epithelial layer from connective layer
are being investigated, with the hope that some
treatment will be found which will neither change
the sulfhydryl values nor inactivate the enzymes.

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Fig. 4.—Preneoplastic hyperplasia of hamster cheek pouch mucosa after 24 thrice-weekly applications of carcinogen. H. & E., ×150.

Fig. 5.—Squamous-cell carcinoma arising in the hamster cheek pouch after 36 thrice-weekly applications of carcinogen. H. & E., ×150.
soluble -SH were causative or merely accompanied by the hyperplasia is not evident from the data. It is possible that both the pyridine nucleotides and glutathione are involved in reactions which detoxify the carcinogen or react with the estrogen, resulting in decreased glutathione concentration and increased proportion of oxidized TPN. Perhaps some other member of the chain on the way to oxygen--such as flavoprotein or a hemeprotein--has reacted with the carcinogen or with products of cell destruction, and the oxidized flavoprotein or cytochrome c has oxidized the TPN, thus decreasing the TPNH available for keeping the glutathione and the protein -SH reduced. We expect to investigate these alternative possibilities.

The difference between the sexes in the changes of -SH in response to the carcinogen, both during the early inflammatory reaction and the later period of tumor formation, is striking. This effect may be related to the differing action of sex hormones.

It is recognized that analysis of the whole pouch cannot give specific information about the epithelial tissues in which the tumors appear. However, except for the most violent inflammatory reactions, no change in the morphology of the underlying connective tissue and muscle strands has been seen. Therefore, we suggest that the sulfhydryl changes in the total tissue may be correlated with the morphological changes in the epithelial layer. Methods of treating the tissue to separate the epithelial layer from connective layer are being investigated, with the hope that some treatment will be found which will neither change the sulfhydryl values nor inactivate the enzymes.

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