Retardation of the Rate of Tumor Induction by Hydrolyzing Chlor-Compounds

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The effect on the rate of tumor induction of a group of chlor-compounds which reversibly inhibit the glycolytic process of cells has already been described (5, 6). The compounds used were soluble in water and fats, and were nonhydrolyzable under physiological conditions of pH and temperature. The chemical reactivity of their chlorine atoms was the factor which determined the potency of their biological action. But their influence on tumor induction was not simple. Inhibitory or stimulatory effects could be obtained by a single chlor-compound, the final result being determined by the concentration used and also by the time of its application relative to the treatment with carcinogen.

The most active nonhydrolyzing chlor-compound used was mono-chloracetone. It would have been possible to study the effects of still more active halogen compounds of this type; e.g., the bromine and iodine analogues of mono-chloracetone, had not the irritating properties of such compounds precluded their use.

Chlor-compounds, considered from the point of view of increasing chemical reactivity, can be ranged from the nonhydrolyzing types used in the earlier work to types which are so reactive that contact with aqueous media causes hydrolysis. The acid chlorides are familiar examples of this type, and they can be graded according to their rate of hydrolysis. Berenblum (1-3) has focused attention on mustard gas and related compounds which come under the heading of hydrolyzing halogen compounds. In the work reported here, a series of aliphatic, and one aromatic, acid chlorides has been used.

The experiments demonstrate that all these acid chlorides exert an inhibiting influence on the process of tumor induction in the skin of mice by 3,4-benzpyrene. The degree of inhibition by the different members of this class of compounds varies with their physical and chemical characteristics, molecular size and rate of hydrolysis being the variable factors which probably determine their relative effectiveness.

EXPERIMENTS

1. Using 0.3 per cent 3,4-benzpyrene.—Seven batches of mice, each composed of 15 mixed laboratory stock animals, were epilated in the interscapular region 3 days before the experiment was commenced, but not subsequently. One batch of 15 mice was treated with 0.3 per cent benzpyrene in 98 per cent ether plus 2 per cent liquid paraffin, twice weekly (Mondays and Thursdays). The remaining 6 batches received similar treatment with benzpyrene, and, in addition, were painted with a 1 per cent acid chloride solution, in the same solvent, twice weekly (Tuesdays and Fridays). The hydrocarbon was confined to an area of about 1 sq. cm.; the acid chloride was applied to a considerably larger area to ensure wide overlapping. These applications were continuous over 40 weeks in mice remaining free of tumors. This concentration of acid chloride was chosen quite arbitrarily. Subsequent experiments have shown that considerably lower concentrations act in the same way, though no analysis of the relation between degree of inhibition and concentration applied has been undertaken. Only the qualitative aspect of the experimental findings is emphasized here.

Although most of the mice showed no obvious skin lesions in the painted area, a few suffered from minor wounds as a result of scratching provoked by insect bites. This is unavoidable when applying skin irritants, but in a series of mice treated with the acid chlorides alone, few visible lesions developed, and these healed promptly when the treatment was discontinued. Curves showing the times when papillomas appeared in the various series are reproduced in Fig. 1. The times when malignancy supervened, though noted, cannot be shown by this method of expressing results. They are, in any case, irrelevant for this type of experiment, since the inhibitory action is confined to the latent period. Papillomas, once formed, always grew progressively.

The curves showing the effect of stearyl, acetyl, valeryl, and benzene sulfochlorides only are included in Fig. 1. Those for palmityl and myristyl chlorides are omitted, since they corresponded closely to those for stearyl and valeryl chlorides respectively. Clear inhibitions of the rate of papilloma formation are obvious in all cases, the most pronounced being with benzene sulfonyl chloride. Several factors probably...
determine these differential potencies, including molecular size which reflects the power of diffusion and hence the depth of penetration, and the rate of hydrolysis. No exact correlation with a single physical or chemical characteristic seems possible; e.g., the curves for acetyl and benzene sulfochlorides occupy anomalous positions when molecular size is considered, and the curve for acetyl chloride, the most easily hydrolyzed, is again out of step when rates of hydrolysis are considered. Benzene sulfochloride is hydrolyzed much more slowly than all the other acid chlorides, and is a comparatively small molecule. Its enhanced power of inhibiting tumor induction suggests that further low-molecular acid chlorides, having still slower rates of hydrolysis, may be found to exhibit even greater potency.

Since the mortality rate was very low, and, with one exception, 90-100 per cent of the mice had developed tumors before 40 weeks, significant average induction times for papilloma development can be given (Table 1).

### III. Mode of action of hydrolyzing chlorocompounds

Under test tube conditions in the presence of compounds containing such groups as OH, NH₂, SH, and in strongly alkaline media, the acid chlorides rapidly combine with these active groups. Under physiological conditions of temperature and pH, in Ringer's solution containing, for example, cysteine, manometric experiments have shown that fixation of the thiol group is negligible. Owing to the insolubility of the acid chlorides in aqueous media, a simple hydrolysis predominates.

#### II. Using 0.1 per cent 3,4-benzpyrene

It was thought inadvisable to increase the concentration of the acid chlorides in an attempt to enhance their inhibitory effect. But the converse experiment was carried out. The ratio carcinogen/inhibitor was reduced two-thirds by retaining the same concentration of acid chloride as in Experiment I, and using 0.1 per cent 3,4-benzpyrene. In all other respects the preceding experimental procedure was followed. Details of the result of this experiment are shown in Fig. 2. The curves for palmityl and myristyl chlorides are again omitted from the chart for reasons already given. The curves showing the times of papilloma appearance are here noticeably more spread out along the time axis. The experiment was terminated after 40 weeks when all groups showed almost 100 per cent tumor formation except that treated with benzene sulfochloride.

In this latter group no wart occurred until the 22nd week, at a time when every mouse in the control group bore a tumor. Even after 40 weeks, 43 per cent of the mice treated with benzene sulfochloride were free of papillomas.

### Table I: Average Induction Time for Papillomas in Mice Treated with Benzpyrene and Acid Chlorides

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0.05% benzpyrene</th>
<th>0.1% benzpyrene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzpyrene alone</td>
<td>11 weeks 2 days</td>
<td>14 weeks 1 day</td>
</tr>
<tr>
<td>Benzpyrene + stearyl chloride</td>
<td>14 weeks 5 days</td>
<td>17 weeks 2 days</td>
</tr>
<tr>
<td>Benzpyrene + palmityl chloride</td>
<td>14 weeks 5 days</td>
<td>16 weeks 3 days</td>
</tr>
<tr>
<td>Benzpyrene + myristyl chloride</td>
<td>16 weeks 0 days</td>
<td>22 weeks 6 days</td>
</tr>
<tr>
<td>Benzpyrene + valeryl chloride</td>
<td>17 weeks 3 days</td>
<td>22 weeks 2 days</td>
</tr>
<tr>
<td>Benzpyrene + acetyl chloride</td>
<td>16 weeks 6 days</td>
<td>21 weeks 3 days</td>
</tr>
<tr>
<td>Benzpyrene + benzene sulfochloride *</td>
<td>25 weeks 2 days</td>
<td>26 weeks 4 days</td>
</tr>
</tbody>
</table>

* 43 per cent of surviving mice had not developed papillomas after 40 weeks treatment.

When acid chlorides are applied to the skin of mice, there is probably a twofold effect: first, a simple hydrolysis with liberation of HCl and the corresponding acid; second, a fixation, by chemical combination, of groups containing labile hydrogen atoms. The relative...
extent of these 2 possible reactions is obviously gov-
erned by a variety of factors, the chief of which is the
rate of hydrolysis of the chloride used. Exact data to
decide this point are not available, but evidence from
experiments in vitro, given later when considering the
effect of the acid chlorides upon glycolysis, points
strongly to the probability that simple hydrolysis is
the predominant reaction in the presence of living
cells.

This liberation of HCl within the cells implies pro-
found local changes of pH, with concomitant distur-
bances of all cell equilibria. Such damage, if slight,
is followed by recovery when adequate intervals are
allowed between successive applications. The old
conception that superoptimal “irritation” proves an-
tagonistic towards the process of carcinogenesis has
often been used to explain this retarding effect of non-
specific irritants. Discussion on these lines is sterile
unless the word “irritation” is more precisely defined.
In the experiments previously described, the “irritants”
used were specific inhibitors of glycolysis. Can the
hydrolyzing chlor-compounds be regarded as falling
into the same category?

IV. Effect of acid chlorides on the glycolytic proc-
ess.—The normal technic for studying the effect of
substances on metabolic processes in vitro consists in
adding the substance to the saline medium in which
a thin slice of tissue is suspended. A fall of glycolytic
rate, for example, is observed over a suitable interval,
and on returning the tissue slice to normal physiologi-
cal media, the initial rate of glycolysis returns; i.e., the
action is reversible. All the chlor-compounds used in
earlier work exerted this type of effect, and when the
test was made with tumor tissue, the viability of the
cells was unimpaired after 3 hours of treatment at
suitable concentrations, as proved by 100 per cent
positive transplantations.

When the acid chlorides are tested in this way little
or no inhibition of glycolysis is observed, since the
compounds are rapidly hydrolyzed in the saline
medium, the net result being a lowering of pH to an
extent determined by the amount of chloride used.
Since the curve showing the relation between rate of
glycolysis and the pH of the medium is well known
from the work of Warburg, Posner, and Negelein
(7), it is possible to predict with fair accuracy the
fall in glycolytic rate which will accompany the addi-
tion of known amounts of these hydrolyzing chlor-
compounds to the medium. In fact, the addition of
an equivalent amount of HCl is equally effective in
producing the same degree of inhibition. This result
is conditioned by the fact that little or none of the acid
chloride used comes into direct contact with the
living tissue.

To overcome this difficulty it is possible to add the
chlorides directly to minced tissue, with thorough
mixing. This method was employed by Berenblum
et al. (4) when studying the effect of mustard gas on
cell metabolism. The method has many drawbacks
when highly active compounds are used, because of
uneven distribution of the added agent. The net
change observed in the rate of any metabolic process
can represent only a rough average, and high local
concentrations can introduce destructive effects which
make the concept of specific action meaningless. The
kind of result obtained is illustrated by the following
experiments:

Five grams of Jensen rat sarcoma tissue were finely
minced and 0.08 cc. of myristyl chloride was added
and well mixed by further mincing with scissors. A
suitable amount of this mince was weighed into small
vessels and the anaerobic glycolysis was measured by
the usual manometric methods. During the 20-minute
phase of obtaining temperature and pressure equilib-
rium, the CO2 due to hydrolysis of the chloride was
largely evolved, and afterward the fall in glycolytic
rate was progressive with time; e.g., during 1 hour the
glycolytic rate fell 25-35 per cent; during 2 hours the
glycolytic rate fell 50-60 per cent. When equivalent
amounts of the other acid chlorides used in the animal
experiments were substituted for myristyl chloride,
the degree of inhibition of glycolysis was of the same
order.

If, instead of the organic acid chlorides, an equiva-
 lent of HCl was similarly mixed with the tissue, the
reduction of glycolysis was substantially the same;
e.g., when 0.314 N HCl was mixed with 5 gm. Jensen
rat sarcoma tissue, the glycolytic rate fell, during
half an hour, 35-40 per cent; and during an hour, 50-
60 per cent.

The fact that the biochemical result is approximately
the same whether acid chloride or HCl is mixed with
the tissue strongly suggests that simple hydrolysis
predominates when the compound is in contact with liv-
ing cells, and that there is little necessity to postulate a
fixation of active groupings to account for their mode
of action.

If an equivalent amount of a nonhydrolyzing chlor-
compound; e.g., chloracetone, be similarly minced with
tumor tissue, the cessation of glycolysis is instanta-
aneous, approaching 100 per cent. In this case the
effective concentration greatly exceeds that necessary
to study specific action, but its behavior stands in clear
contrast with that of the hydrolyzing chlor-compounds.

Hence the concept of specific action on metabolic
processes cannot be maintained for hydrolyzing chlor-
compounds. They may be regarded as inflicting gen-
eralized damage to cells, markedly disturbing all cell
processes, and in adequate amounts leading to cell
death. The inhibition of glycolysis was here con-
veniently measured, but other reactions must also be
depressed to an extent dependent upon their nature.

The contrast between the actions of nonhydrolyzing
and hydrolyzing chlor-compounds is emphasized not
only by their respective specific and nonspecific inhi-
bition of glycolysis in vitro but also by their biological
effect. The former type has been shown to affect the
process of carcinogenesis in opposite directions accord-
ing to the conditions of application; all the animal
experiments so far carried out have shown that the
latter type produce inhibitory effects only.

DISCUSSION

The experiments recorded here bring to mind the
anticarcinogenic action of mustard gas and its deriva-
tives, as described by Berenblum (3).

To aid comparison of the two sets of results, the fol-
lowing brief summary of the facts presented in this and
the two previous communications (5, 6) is made.

1. NONH YDROLYZING CHLOR-COMPONDS (Soluble in fats and
   water)
   A. Effect on glycolysis
      Check glycolysis reversibly. Degree of checking is
      proportional to the chemical reactivity of the
      chlorine atom.
   B. Effect on tumor induction.
      (i) Inhibition, when applied in low concentrations over
      the same time periods as the carcinogen. This
      inhibition is annulled when higher concentra-
      tions are applied. Degree of inhibition is pro-
     portional to the chemical reactivity of the
      chlorine atom and therefore also to the degree of
      checking glycolysis.
      (ii) Stimulation, when applied after a preliminary but
      subeffective treatment with carcinogen.

2. HYDROLYZING CHLOR-COMPONDS (Soluble in fats, insoluble
   in water)
   A. Effect on glycolysis
      Checking is nonspecific, due to liberation of HCl.
      In testing the effect on metabolic processes, by
      manometric methods, the result is determined by
      the technic employed:
      (i) If mixed with minced tissue, metabolic rates fall
      as one of the consequences of generalized cell
      damage.
      (ii) If added to the saline medium, the much smaller
      decreases in metabolic rates are conditioned by
      the altered pH of the medium.
   B. Effect on tumor induction.
      Inhibition, in all concentrations, however applied.

Berenblum and his colleagues (3, 4) attempted
unsuccessfully to correlate the rate of hydrolysis, the
inhibition of metabolic processes, and the anticarcino-
genic action of mustard gas and some of its simple
analogues and derivatives. The facts included in the
above summary may suggest a reason for their failure.
These workers made no distinction between hydrolyz-
ing and nonhydrolyzing halogen compounds, thus
comparing the biological effects of substances with
essentially different properties. In view of these dif-
fences correlation is impossible.

Comparison of effects on metabolism.—In studying
metabolic changes induced by mustard gas, etc., Beren-
blum et al. (4) employed 2 separate technics. The con-
trasting results obtained reflected the contrasting ex-
perimental methods of measurement, and not inherent
differences in the properties of the compounds them-
selves. For example, they found that \( \beta\alpha\)-dichloro-
diethylsulfide (mustard gas = M.G.) lowered the
glycolytic rate considerably, while ethylene-bis-\( \beta\)
chloroethylsulfide (E.M.G.) had little effect on this
process, though both compounds were rapidly hydro-
lyzed and were markedly anticarcinogenic. But M.G.
was minced with the tissue and E.M.G. added to the
suspension medium. Both substances belong to the
class of hydrolyzing chlor-compounds, and, by analogy
with the above-mentioned differential results obtained
with a single acid chloride, the different technics would
account for the apparent difference of action. By
reversing the experiments; i.e., mixing E.M.G. with
minced tissue and adding M.G. to the suspension
medium, it is highly probable that the apparent relative
degrees of checking glycolysis would also be reversed.

These two sulfides, insoluble in water and easily
hydrolyzed, cannot be compared in their effect on
glycolysis with the 2 oxidation products of M.G.; viz.,
\( \beta\alpha\)-dichlorodiethylsulfoxide and the correspon-
ding sulfone, which are soluble in water and nonhydro-
lyzable. The sulfone is slowly hydrolyzed under physi-
ological conditions of pH and temperature, but the
effective checking of glycolysis in vitro is certainly due
to the nonhydrolyzed compound. The latter fall into
the category of chlor-compounds described previously
(5), the varying degrees of checking glycolysis ob-
erved being a function of the different reactivities of
their chlorine atoms. The sulfoxide appears to ap-
proximate in activity to a-chlor-diethyl ketone, and the
sulfone to chloracetone, both as inhibitors of glycolysis
and anticarcinogenic agents.

By thus dividing M.G. and its derivatives into 2
classes according to their solubility in water and hydro-
lyzability, they naturally fall in line with the many
types of compounds, unrelated in general structure,
which have been used in this work.

Comparison of effect on tumor induction.—The
problem of the nature of the vesicant action of mustard
gas is probably distinct from that of its anticarcino-
genic action, since the dosage used in the latter case
is small and its effect transitory. This view is also
supported by the fact that the acid chlorides show no
vesicant action and are innocuous in the concentrations
used, in the sense that quick recovery follows any
temporary damage to skin cells. Yet when used with
the chemical carcinogens their qualitative behavior is similar to that of mustard gas.

Though the inhibition of glycolysis in vitro by mustard gas can be explained entirely by its liberation of HCl, this may not entirely account for its anticarcinogenic action, though there seems no reason to postulate any action different, in this respect, from that of the acid chlorides.

An attempt to compare, from the available data, the relative anticarcinogenic potency of mustard gas and the acid chlorides is beset with difficulties. Berenblum (1, 2) used tar and 0.1 per cent mustard gas, and limited his experiments to a time when about 50 per cent of the control animals had developed tumors. There was a high mortality rate in his animals. He expressed his results in a way which precludes the calculation of an average induction time. For the experiments described in this paper 3,4-benzpyrene and 1 per cent acid chlorides were used. There was a low mortality rate, and the experiments were prolonged to 40 weeks; i.e., more than twice the time necessary for 100 per cent of control mice to develop tumors. If the criteria used by Berenblum are applied to the experiments shown in Figs. 1 and 2, it will be seen that at a time when 50 per cent of the surviving control mice had developed papillomas, none had appeared in the mice treated with acid chlorides, except in the case of stearyl chloride, the least potent member used. By ending the experiment at arbitrary times any degree of inhibition may be claimed, from 100 per cent down to the significant percentage given by the average induction time.

It is clear that for a real comparison of relative effectiveness further experiments must be carried out under identical conditions. A similar qualitative action only is emphasized here. The common feature of all these hydrolyzing anticarcinogenic substances is their active halogen atoms. The nature of the molecular residue appears to be of secondary importance.

No doubt the number of compounds biologically active in the way described could be multiplied indefinitely. Apart from the active halogen atom, potency will vary with such physical characters as molecular size and the partition coefficient between fat and water. The toxicity of the compounds or their products of hydrolysis will also make its contribution to the net biological response.

**Summary and Conclusions**

The effect of a series of aliphatic, and one aromatic, acid chlorides on the rate of tumor induction in mice by 3,4-benzpyrene has been investigated. All proved inhibitory. The degree of inhibition varied with the molecular size and rate of hydrolysis, but did not run parallel with any single physical or chemical characteristic. The most effective was benzene sulfochloride, which hydrolyzes less rapidly than the aliphatic acid chlorides.

The action of acid chlorides on the glycolytic process of cells has been studied, and contrasted with that of the specific inhibitors of this process. It is concluded that the acid chlorides impair the activities of cells nonspecifically due to the liberation of HCl, and that the apparent inhibition of glycolysis is merely one expression of this generalized damage.

The work of Berenblum and his colleagues on mustard gas and allied compounds is examined in the light of the above results. The difference in biological action of hydrolyzing and nonhydrolyzing halogen compounds is emphasized.

**References**

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