Adenocarcinoma of the Pancreas in Azaserine-treated Rats

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Summary

Development of a model of carcinoma of the pancreas in rats was approached by attempting to identify chemicals that (a) behave as mutagens and (b) localize in the pancreas following systemic administration; and then to study the effects of long-term administration. Azaserine was selected because it behaves as a direct-acting mutagen in two bacterial test systems and because tissue distribution studies showed concentration especially in kidney and pancreas. Groups of rats have been given i.p. injections once or twice weekly for 6 months, and rats have been autopsied after 6 to 18 months. During the first year pancreases developed (a) nodules of atypical exocrine cells which seem to represent hyperplastic foci and (b) encapsulated adenomas. After 1 year most pancreases from treated rats are diffusely abnormal and contain many hyperplastic nodules and adenomas, while more than one-quarter have had pancreatic adenocarcinoma. Metastases have been observed in lymph nodes, liver, and lung. No carcinomas or adenomas have been observed in control rats. No other organ shows as high an incidence of involvement as pancreas, but renal neoplasms were frequent.

Studies with another chemical O-(N-methyl-N-nitroso-β-alanyl)-L-serine, are at an earlier stage. The tissue distribution of radioactivity following injection of a 14C-labeled sample is similar to that of azaserine; however, this compound is not a direct-acting bacterial mutagen. Rats treated for 6 months twice weekly i.p. have a higher incidence of nodules of atypical acinar cells than did controls, although the number of nodules per rat is few. No adenomas or carcinomas have been found during 13 months of the study.

We conclude that azaserine is a carcinogen in rats and causes major abnormalities of growth and differentiation of the exocrine pancreas, including adenocarcinoma in some rats. O-(N-Methyl-N-nitroso-β-alanyl)-L-serine had less effect than azaserine on pancreatic growth and differentiation.

Introduction

Investigation of possible causes of carcinoma of the pancreas is receiving increased attention (8, 12), and there has been recent success in the development of new animal models of pancreatic neoplasms (3, 5, 9, 11). Experimental induction of adenocarcinoma of the exocrine pancreas in rats is the goal of work in progress in our laboratory. Our approach has been to study the long-term effects of chemicals (a) that are mutagenic or carcinogenic and (b) that localize in the pancreas following systemic administration. We initially selected azaserine (NSC 742) as a potential pancreatic carcinogen because (a) it is a bacterial mutagen and (b) it has a high affinity for pancreas following i.v. injection. We have also studied alanoser, a newly synthesized compound that localized similarly to azaserine following injection. Alanoser is not a direct-acting bacterial mutagen.

In studies still in progress, pancreatic adenocarcinomas have developed in azaserine-treated rats but not in rats treated with alanoser, although the latter has induced a higher incidence of hyperplastic nodules of acinar cells than is observed in control rats. These experiments are described here.

Materials and Methods

Wistar rats (Charles River Breeding Laboratories, Wilmington, Mass.) fed Purina rat chow ad libitum and caged in groups of 2 to 5 were used in all experiments. Azaserine (Calbiochem, La Jolla, Calif.) dissolved in 0.9% NaCl solution was injected i.p. at a dose of 5 mg/kg once or twice weekly for 6 months (Table 1). The higher dose, 5 mg/kg 2 times/week proved to be close to a chronic LD10.

Alanoser, synthesized by a method which will be described in a separate publication, was injected i.p. twice weekly at a dose of 10 or 50 mg/kg (Table 1). Preliminary toxicity studies showed that rats tolerated up to 1-g/kg doses of alanoser injected i.p. without morbidity or mortality. Solutions of both agents were made immediately prior to injection, and solutions appear to be stable for at least 1 day. The structures of azaserine and alanoser are shown in Chart 1.

Puromycin (Nutritional Biochemicals Corp., Cleveland, Ohio), 40 mg/kg, was given in courses of 4 hourly doses in the 3rd and 4th months to one-half of the rats in Groups 2 and 3 (Table 1) to cause cycles of pancreatic acinar cell necrosis and regeneration (6).
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Table 1
Summary of treatment regimens

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>Dose/Injection (mg/kg)</th>
<th>Injections/wk</th>
<th>No. of rats*</th>
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<tbody>
<tr>
<td>1</td>
<td>Azaserine</td>
<td>5</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>Azaserine</td>
<td>5</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>0.9% NaCl solution</td>
<td>5*</td>
<td>2</td>
<td>60</td>
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<tr>
<td>4</td>
<td>Alanoser</td>
<td>5</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>0.9% NaCl</td>
<td>2</td>
<td>3</td>
<td>30</td>
</tr>
</tbody>
</table>

* All rats were treated for 6 months. All injections were i.p.
* One-half of each group were female.
* One-half of Groups 2 and 3 received, in addition, 2 series of 4 hourly 40-mg/kg injections of puromycin given at 3.5 and 4.5 months during azaserine treatment.
* During Weeks 2 to 6 of treatment, injections were 10 mg/kg for 9 doses because of short supply of alanoser.
* Group 3 were controls for Groups 1 and 2. Group 5 were controls for Group 4.

\[
\begin{align*}
\text{N}_2\text{CHCO}-\text{OCH}_2\text{CHCO}_2^- \\
\text{NH}_3 \\
\text{AZASERINE}
\end{align*}
\]

\[
\begin{align*}
\text{CH}_3\text{NCH}_2\text{CH}_2\text{CO}-\text{OCH}_2\text{CHCO}_2^- \\
\text{NO} \\
\text{NH}_3 \\
\text{ALANOSER}
\end{align*}
\]

Chart 1. Formulas of azaserine and alanoser.

Rats were killed and autopsied at 6, 9, 12, and 18 months following 1st treatment and, in addition, whenever (a) there was weight loss of 15% or 100 g, or (b) when there was obvious morbidity so that survival was questionable. The pancreas was weighed, fixed in toto, and embedded as slices 1 to 2 mm thick. Representative sections of liver, kidney, intestine, stomach, testis or ovary, and salivary glands were routinely taken. Thymus, prostate, lung, pituitary, breast, and obvious lesions in other tissues were sampled in selected rats. Tissues were fixed in Susa’s solution, embedded in paraffin, and routinely stained with hematoxylin and eosin. Prior to sacrifice, selected rats were laparotomized under ether anesthesia, and sections of pancreas, liver, or kidney were excised and fixed for electron microscopy in 4% cacodylate-buffered glutaraldehyde, pH 7.4. These tissues were postfixed in 2% osmium tetroxide in cacodylate buffer and prepared for electron microscopy as has been previously described (6). Sections were examined in a Siemans-Elmiskop 1A electron microscope or Philips EM 201 with 60 kV.

The mutagenic activity of azaserine and alanoser was evaluated in 2 bacterial test systems (1, 7). Salmonella typhimurium test strains were supplied by Dr. Bruce Ames.

In addition, a sample of alanoser was submitted to Herbert S. Rosenkranz, Department of Microbiology, Columbia University, for evaluation in the bacterial systems.

Tissue localization studies were done using \[^{1}H\]azaserine, \[^{14}C\]alanoser, and \[^{14}C\]serine (New England Nuclear, Boston, Mass.). The first was obtained by submitting a 500-mg sample of azaserine for commercial tritium gas exposure labeling. The product, returned in aqueous solution, was purified by recrystallizing from 90% ethanol after adding unlabeled azaserine, followed by preparative thin-layer chromatography on silica gel plates using a butanol-acetic acid-water solvent system. The zone coinciding with azaserine was extracted with water. An analytical thin-layer chromatogram of the final product was scanned in a radiochromatogram scanner and showed more than 90% of radioactivity migrating in the position of azaserine. \[^{14}C\]-Alanoser was synthesized from commercially obtained \[^{14}C\]serine. A thin-layer radiochromatogram showed greater than 96% radiochemical purity.

Radioactivity in trichloroacetic acid-soluble and -insoluble fractions of several tissues was measured following i.v. injection of the agents. We followed our previously described procedures (4) except that we omitted the hydrolysis of nucleic acids in preparation of the acid-insoluble fraction so that these should contain both nucleic acids and proteins.

**Results**

**Mutagenicity.** We have previously reported that azaserine behaves as a direct-acting mutagen in the Escherichia coli repair test but that alanoser does not (7). Data in Table 2 indicate that azaserine is also a direct-acting mutagen in the S. typhimurium reversion test system whereas alanoser is not.

**Distribution Studies.** Radioactivity levels found in tissues following administration of \[^{1}H\]azaserine or \[^{14}C\]-alanoser are summarized in Table 3. The kidney consistently contained the highest levels of acid-soluble

<table>
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<tr>
<th>Chemical</th>
<th>(\mu g)</th>
<th>(n)</th>
<th>Strain 1535</th>
<th>Strain 1536</th>
<th>Strain 1537</th>
<th>Strain 1538</th>
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<td>10</td>
<td>0</td>
<td>5</td>
<td>9</td>
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<td>22</td>
<td>14</td>
<td>61</td>
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<td>2</td>
<td>189</td>
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<td>2</td>
<td>98</td>
<td>94</td>
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<td>2</td>
<td>14</td>
<td>10</td>
<td>20</td>
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<td>1</td>
<td>9</td>
<td>0</td>
<td>4</td>
<td>13</td>
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* Direct activity. No microsomal activation system was used. Counts of fewer than 50 revertants/plate are regarded as negative for mutagenic activity. The count reported in the average of \(n\) plates.
### Table 3
Specific radioactivity of trichloroacetic acid-soluble and -insoluble fractions of tissues from rats following injections of [\(^{1}H\)]azaserine, [\(^{14}C\)]alanoser, and [\(^{14}C\)]serine

<table>
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<tr>
<th></th>
<th>No. of rats</th>
<th>Time (min)</th>
<th>Dose (mg/kg)</th>
<th>Rat wt (g)</th>
<th>Route</th>
<th>Pancreas</th>
<th>Kidney</th>
<th>Liver</th>
<th>Intestine</th>
<th>Spleen</th>
<th>Testis</th>
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<td><strong>Soluble fractions</strong></td>
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<tr>
<td>Group 1</td>
<td>4</td>
<td>15</td>
<td>2.6</td>
<td>8</td>
<td>160</td>
<td>i.v.</td>
<td>71,892</td>
<td>295,473</td>
<td>26,642</td>
<td>27,393</td>
<td>28,703</td>
<td>13,947</td>
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<td>2,180</td>
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<td>3</td>
<td>15</td>
<td>5.2</td>
<td>16</td>
<td>161</td>
<td>i.v.</td>
<td>139,070</td>
<td>548,977</td>
<td>51,738</td>
<td>57,134</td>
<td>51,485</td>
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<td>3,233</td>
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<td>Group 3</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>281</td>
<td>i.v.</td>
<td>249,255</td>
<td>603,293</td>
<td>31,253</td>
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<td>10,341</td>
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<td>990</td>
<td>1,266</td>
<td>605</td>
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<td>Group 4</td>
<td>4</td>
<td>15</td>
<td>2</td>
<td>3</td>
<td>288</td>
<td>i.p.</td>
<td>97,647</td>
<td>311,647</td>
<td>28,326</td>
<td>18,859</td>
<td>29,229</td>
<td>8,775</td>
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<td>17,713</td>
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<td>3,690</td>
<td>3,535</td>
<td>4,281</td>
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<td>(^{14}C)Serine</td>
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<td>Group 5</td>
<td>5</td>
<td>15</td>
<td>2</td>
<td>0.02</td>
<td>83</td>
<td>i.v.</td>
<td>55,815</td>
<td>47,789</td>
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<td>2,623</td>
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<td>5</td>
<td>15</td>
<td>2</td>
<td>0.01</td>
<td>165</td>
<td>i.p.</td>
<td>89,622</td>
<td>33,408</td>
<td>60,403</td>
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<td><strong>Insoluble fractions</strong></td>
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<td></td>
<td>18.2 ± 2.4</td>
<td>9.8 ± 0.4</td>
<td>2.2 ± 0.6</td>
<td>7.6 ± 0.5</td>
<td>7.4 ± 3.5</td>
<td>1.4 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>2.5 ± 0.8</td>
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<td>19.7 ± 2.5</td>
<td>17.2 ± 0.5</td>
<td>3.0 ± 0.2</td>
<td>10.1 ± 0.7</td>
<td>5.6 ± 3.8</td>
<td>1.7 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>2.6 ± 0.1</td>
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<td>17 ± 1</td>
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<td>13 ± 1</td>
<td>12 ± 3</td>
<td>43 ± 4</td>
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<td></td>
<td>314 ± 68</td>
<td>115 ± 2</td>
<td>120 ± 27</td>
<td>312 ± 76</td>
<td>178 ± 30</td>
<td>33 ± 4</td>
<td>8 ± 1</td>
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<td>532 ± 80</td>
<td>45 ± 5</td>
<td>129 ± 30</td>
<td>177 ± 31</td>
<td>96 ± 16</td>
<td>56 ± 22</td>
<td>7 ± 1</td>
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</table>

* Interval from injection to killing.

* Approximate dose of azaserine, alanoser, or serine.

* Average weight/rat. Within groups the weight range was 10 to 20 g.

* Data have been corrected for varying weight of rats by the formula:

\[
\text{Observed cpm/g} \times (\text{wt of rat}/100) = \text{"corrected" cpm/g}
\]

* Mean ± S.E.

* Experimental data for all groups under "Insoluble fractions" are the same as those for corresponding groups under "Soluble fractions."
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radioactivity, and the pancreas consistently ranked 2nd with both compounds. The radioactivity of pancreas-derived acid-insoluble fractions from [3H]azasenine-treated rats exceeded that in all other tissues, suggesting that macromolecules were alkylated or that azaserine or [3H]serine derived from [3H]azasenine had entered into protein synthesis.

The acid-insoluble fraction from pancreas ranks 3rd in radioactivity at 5 min and 2nd at 15 min following [14C]alanoser. Since the molecule was labeled in serum, the acid-insoluble radioactivity may reflect hydrolysis, release of [14C]serine, and its incorporation into protein. We collected urine from these rats at sacrifice and found increasing radioactivity at 5, 15, and 60 min after injection. Thin-layer chromatograms of the urine showed more radioactivity in the alanoser spot (Rf 0.66) than in the serine spot (Rf 0.27) at all intervals.

The distribution of [14C]serine is also given in Table 3. With the natural amino acid, the levels of both acid-soluble and acid-insoluble radioactivity in pancreas exceed those in the kidney as well as in other tissues in both groups. The specific radioactivity achieved in the acid-insoluble fraction exceeds that found following either alanoser or azasenine suggesting that the latter are not readily incorporated into proteins.

Rat Autopsy Findings. Hyperplastic nodules of acinar cells such as we have previously described have been found in the pancreas of all azaserine-treated rats (Table 1, Groups 1 and 2) autopsied to date. Pancreases from at least 25% of the rats have contained small encapsulated tumors (adenomas) of acinar cell origin. However, beginning 12 months after treatment, increasing numbers of rats have had diffusely abnormal pancreases with multiple nodules such as that illustrated in Fig. 1. The mean weight of 12 such pancreases was 2.94 ± 0.48 (S.E.) g, whereas the mean weight of 5 pancreases from similar-sized control rats was 1.35 ± 0.19 g. Individual nodules measure from 2 to 10 mm in diameter, and they are found in all portions of the pancreas. This change in pancreas seems to merit a descriptive designation of diffuse adenomatous hyperplasia. It seems fatuous to designate individual nodules in such pancreases as "adenomas" although they might be several mm in diameter and encapsulated. A representative histological field from such a diffusely abnormal gland is shown in Fig. 2. Some foci in such pancreases clearly become malignant as evidenced by lymph node invasion, vascular invasion (Figs. 3 and 4), or metastasis to liver (Fig. 5) or lung (Figs. 6 and 7). In other diffusely abnormal pancreases, individual nodules or adenomas become large and exhibit marked changes in cellular differentiation that are like those seen in the clearly malignant tumors; however, no vascular or lymphatic invasion or metastatic spread has been detected in several such rats. We have designated these as "probable carcinomas."

Individual malignant tumors have measured as much as 6.5 cm in greatest diameter. The weight of the total pancreas in this rat was 37 g. Malignant tumors have been found in all portions of the pancreas [head, body, tail (Fig. 8)].

Of the 23 Group 1 azaserine-treated rats autopsied 12 or more months after initial treatment, 9 had had adenocarcinomas of the exocrine pancreas and 3 more had tumors that were probably carcinomas. Of 18 Group 2 azaserine-treated rats, 5 had adenocarcinomas and 1 had probable carcinoma. Five of the 14 rats with pancreatic carcinomas had metastases in the liver, 1 had diffuse spread in peritoneal fat, and 1 had a pulmonary metastasis. In general, the metastatic foci were similar in differentiation to the most abnormal tumor nodule in the pancreas, e.g., Figs. 4 and 5, or 6 and 7. Of the 18 rats with malignant or probably malignant pancreatic neoplasms, 16 were males. Among Group 2 rats, only 1 of 5 with pancreatic carcinoma had been treated with puromycin.

Differentiation. Cells in hyperplastic nodules have retained acinar cell differentiation with varying degrees of zymogen production and marked variation in nuclear size as had been noted before (5). Cells often remain organized into acini with central lumens (Fig. 8).

The adenomas are characteristically less differentiated than nodules, but they usually retain evidence of zymogen production. Duct-like areas appear within some adenomas and nodules in the diffusely abnormal pancreases (Fig. 10). Cystic tumors were found that seem to merit designation as cystadenomas (Fig. 11).

The adenocarcinomas (and the tumors designated as probable carcinomas) have varied widely in differentiation. The most highly differentiated clearly produce zymogen (Fig. 14), even in metastatic sites (Figs. 12 and 13); whereas poorly differentiated tumors, although clearly epithelial, have lost all glandular pattern and show no zymogen production (Figs. 2, 4, and 6). Electron microscopy of several such tumors has failed to reveal evidence of zymogen production. Some carcinomas have contained duct-like structures (Fig. 15), but none has been exclusively a ductal carcinoma.

Ultrasrructural Characteristics. The most highly differentiated cells in the hyperplastic nodules retain ultrastructural appearance and organization of normal acinar cells. Cells in some nodules characteristically have enlarged nuclei as has been apparent by light microscopy. In less-differentiated tumors there is decreased zymogen production and loss of cell polarity coincident with loss of acinar organization. Even cells with little or no zymogen tend to retain moderate amounts of rough endoplasmic reticulum and prominent Golgi vesicles. Zymogen granules sometimes assume bizarre cylindrical or angular shapes or are smaller than normal. In the poorly differentiated tumors, we have encountered cytoplasmic inclusions such as annulate lamellae and what appear to be aggregates of microtubules.

Tumors in Other Tissues of Azaserine-treated Rats. Neoplasms encountered in other tissues are summarized in Table 4. No effort has been made to classify the renal tumors as benign or malignant. Papillary epithelial tumors, clear-cell tumors, and tubular adenomas have been seen. Only one had metastasized (to liver). The smallest were microscopic, and the largest weighed 44 g. The renal tumors will be more completely described in a separate report. Nor have we classified the breast tumors regarding...
state of malignancy. Some were fibroadenomas and some
appeared to be adenocarcinomas, although none had me-
tastasized. There was no evidence of metastatic spread of
any of the other tumors, and they are classified by histo-
logical appearance. Animals survive in all groups so that
incidence was 2 of 9. No adenomas, glands with diffuse
adenomatous hyperplasia. This alteration is evidenced as early as 2
months (D. S. Longnecker, unpublished observations) after
treatment by the presence of nodules of atypical acinar
cells. Apparently, some such foci represent clones that will
ultimately grow to become "adenomas," whereas a few
have or acquire malignant growth potential.

In the experiment reported here, one-half of the Group 2
azaserine-treated rats received 2 courses of puromycin
injections as a stimulus for promotion, since it has been
shown that such treatment induces a cycle of cell regeneration
in acinar cells (6). At this point there is no indication
that this treatment has increased the incidence of pancreatic
cancer in the group that received it.

The spectrum of changes encountered in the pancreases
of azaserine-treated rats leaves no question regarding the
likely cell of origin for most tumors; it is the acinar cell.
Many adenomas and some carcinomas retain a high degree
of acinar cell differentiation. Thus, it is clear that this model
of chemical carcinogenesis in rat pancreas does not simul-
ate the majority of spontaneously occurring human neo-
plasms, which appear to be ductal.

Azaserine, which is not a direct-acting bacterial mutagen,
haves a much smaller effect on pancreatic growth and
differentiation than does azaserine. At this point, it seems
unlikely that azaserine will prove to be a carcinogen,
although it does seem to induce a few nodules of atypical
acinar cells. We have reported small zones of inhibition of a
DNA polymerase-deficient *E. coli* mutant in the presence of a
liver microsomal system, *i.e.*, evidence that azaserine may
be mutagenic following metabolic activation (7). However,
subsequently, it has been found that azaserine is apparently
nonmutagenic for *S. typhimurium* strains in the Ames
system in the presence of a liver microsomal activating
mixture (H. S. Rosenkranz, personal communication).

We believe that additional amino acid derivatives that are
direct- or indirect-acting bacterial mutagens should be
evaluated with regard to their effect on pancreatic growth.

**References**

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Pancreatic Carcinoma in Azaserine-treated Rats

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Adenocarcinoma of the Pancreas in Azaserine-treated Rats

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