EVALUATION OF THE HEALTH ASPECTS OF SOY PROTEIN ISOLATES AS FOOD INGREDIENTS

1979

Prepared for

Bureau of Foods
Food and Drug Administration
Department of Health, Education, and Welfare
Washington, D.C.

Contract No. FDA 223-75-2004
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Life Sciences Research Office
Federation of American Societies
for Experimental Biology
9650 Rockville Pike
Bethesda, Maryland 20014
NOTICE

This report is one of a series concerning the health aspects of using the Generally Recognized as Safe (GRAS) or prior sanctioned food substances as food ingredients, being made by the Federation of American Societies for Experimental Biology (FASEB) under contract no. 223-75-2004 with the Food and Drug Administration (FDA), U.S. Department of Health, Education, and Welfare. The Federation recognizes that the safety of GRAS substances is of national significance, and that its resources are particularly suited to marshalling the opinions of knowledgeable scientists to assist in these evaluations. The Life Sciences Research Office (LSRO), established by FASEB in 1962 to make scientific assessments in the biomedical sciences, is conducting these studies.

Qualified scientists were selected as consultants to review and evaluate the available information on each of the GRAS substances. These scientists, designated the Select Committee on GRAS Substances, were chosen for their experience and judgment with due consideration for balance and breadth in the appropriate professional disciplines. The Select Committee's evaluations are being made independently of FDA or any other group, governmental or nongovernmental. The Select Committee accepts responsibility for the content of each report. Members of the Select Committee who have contributed to this report are named in Section VII.

Tentative reports are made available to the public for review in the Office of the Hearing Clerk, Food and Drug Administration, after announcement in the Federal Register, and opportunity is provided for any interested person to appear before the Select Committee at a public hearing to make oral presentation of data, information, and views on the substances covered by the report. The data, information, and views presented at the hearing are considered by the Select Committee in reaching its final conclusions. Reports are approved by the Select Committee and the Director LSRO, and subsequently reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent society of FASEB) under authority delegated by the Executive Committee of the Federation Board. Upon completion of these review procedures the reports are approved and transmitted to FDA by the Executive Director of FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of all of the individual members of its constituent societies.

[Signature]
Kenneth D. Fisher, Ph.D., Director
Life Sciences Research Office
FASEB
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I. INTRODUCTION

This report concerns the health aspects of using soy protein isolates as food ingredients. It has been based partly on the information contained in a scientific literature review (monograph) furnished by FDA (1), which summarizes the world's scientific literature from 1920 through 1974*. To assure completeness and currency as of the date of this report this information has been supplemented by searches of over 30 scientific and statistical reference sources and compendia that are generally available; use of new, relevant books and reviews and the literature citations contained in them; consideration of current literature citations obtained through computer retrieval systems of the National Library of Medicine; recent literature searches by the Toxicology Information Response Center, Oak Ridge, Tennessee; searches for relevant data in the files of FDA; and by the combined knowledge and experience of members of the Select Committee and the LSRO staff. In addition, announcement was made in the Federal Register on June 16, 1978 (43 FR 26132) that opportunity would be provided for any interested person to appear before the Select Committee at a public hearing to make oral presentation of data, information and views on the health aspects of using soy protein isolates as food ingredients. Five requests were received, but all were subsequently withdrawn. However, since the requests were withdrawn after public announcement of the hearing in the Federal Register on September 15, 1978 (43 FR 41276), a hearing was held as announced on September 26, 1978; details are given on page 44.

As indicated in the Food, Drug, and Cosmetic Act [21 USC 321 (s)], GRAS substances are exempt from the premarketing clearance that is required for food additives. It is stated in the Act and in the Code of Federal Regulations (2) [21 CFR 170.3 and 170.30] that GRAS means general recognition of safety by experts qualified by scientific training and experience to evaluate the safety of substances on the basis of scientific data derived from published literature. These sections of the Code also indicate that expert judgment is to be based on the evaluation of results of credible toxicological testing or, for those substances used in food prior to January 1, 1958, on a reasoned judgment founded in experience with common food use, and is to take into account reasonably anticipated patterns of consumption, cumulative effects in the diet, and safety factors appropriate for the utilization of animal experimentation data. FDA (2) recognizes further [21 CFR 170.30] that it is impossible to provide assurance that any substance is absolutely safe for human consumption.

*The document (PB-241 954/7) is available from the National Technical Information Service, U.S. Department of Commerce, P.O. Box 1553, Springfield, Virginia 22161.
The Select Committee on GRAS Substances of LSRO is making its evaluations of these substances in full recognition of the foregoing provisions. In reaching its conclusions on safety, the Committee, in accordance with FDA's guidelines, is relying primarily on the absence of substantive evidence of, or reasonable grounds to suspect, a significant risk to the public health. While the Committee realizes that a conclusion based on such reasoned judgment is expected even in instances where the available information is qualitatively or quantitatively limited, it recognizes that there can be instances where, in the judgment of the Committee, there are insufficient data upon which to base a conclusion. The Committee, is aware that its conclusions will need to be reviewed as new or better information becomes available.

In this context, the LSRO Select Committee on GRAS Substances has reviewed the available information on soy protein isolates and submits its interpretation and assessment in this report, which is intended for the use of FDA in determining the future status of these substances under the Federal Food, Drug, and Cosmetic Act.
II. BACKGROUND INFORMATION

Soybeans are the seed of *Glycine max* (L.) Merr. (*G. soya, G. hispida* or *Soja hispida*) legumes native to eastern Asia. Although introduced into the United States during the nineteenth century, they were planted sparsely until the 1920's. Soybeans are now a major crop that is processed primarily for edible oil and meal, the latter being used principally as a protein source in animal feeds. In recent years, however, increasing quantities of extracted soybean flakes have been processed into products for food use (3).

The approximate composition of whole soybeans on a dry-weight basis is 40 percent protein (N X 6.25), 34 percent carbohydrate, 21 percent fat and 4.9 percent "ash" (3). The beans are cleaned and cracked and the cotyledons separated from the hull and hypocotyl. The cotyledons are then flaked and the oil extracted for salad oil, cooking oil, shortening and margarine. The defatted flakes provide defatted meals, flours and grits (40 to 60 percent protein), protein concentrates (70 percent protein) and isolates (90 to 98 percent protein). Meals, flours and grits may also be prepared from flaked soybean cotyledons without prior fat extraction or with extraction of only part of the fat and, in that case, contain lesser percentages of protein. Soy protein isolates are prepared by removing water-insoluble polysaccharides as well as water-soluble sugars and other minor constituents.

Soy protein isolates are generally recognized as safe (GRAS) under the provisions of the Code of Federal Regulations (2) as substances migrating to food from paper and paperboard products used in food packaging [21 CFR 182.90]. The Food and Drug Administration also regards soy protein isolates as direct GRAS food ingredients (4). Soy protein isolates are accepted by the U.S. Department of Agriculture [CFR 9 Part 319] as binders or extenders in sausages, nonspecific meat loaves, chile con carne, pork or beef with barbecue sauce, soups and stews with limitations on the level of addition (5). This report considers the health aspects of using soy protein isolates as direct food ingredients and as components of paper and paperboard products used in food packaging. Soy protein isolates, used in this report, is synonymous with soy protein isolated (1, 2, 4, 5).

First commercial use of soy protein isolates developed in the 1930's was for industrial purposes, principally as binders in paper coatings (6). Edible soy protein isolates for food uses appeared about 1957 as a major article of commerce. The products for industrial and food uses differ in their preparation and properties. Both are prepared from solvent extracted soybean flakes by alkaline aqueous extraction, clarification of the extract by removal of flake residues, and precipitation of the protein in the clarified liquor by acidification. For edible isolated protein production, extraction is usually carried out at pH below 9 to avoid hydrolytic or rheologic changes. The pH of the clarified extract is lowered to 4 to 5 by addition of a food grade
acid such as sulfuric, hydrochloric, phosphoric or acetic and the precipitated protein removed by centrifugation or filtration, followed by a washing step. This procedure yields acid-precipitable globulins (isoelectric protein). The curd may be neutralized with food grade alkali to form the sodium proteinate before drying. In current commercial drying practice, a concentrated solution of the protein isolate is spray-dried in equipment heated by direct-fired burners. Soy protein isolate dried in this type equipment may contain up to about 50 ppm nitrite. The nitrite appears to be formed in the drying process in which the product comes in contact with combustion gases (7-11). Nitrite also has been reported in spray-dried caseinate, eggs and egg white (9) and in spray-dried nonfat dry milk (11) processed in direct gas-fired dryers. Analysis of spray-dried soy protein isolate containing up to 69 ppm nitrite gave negative results for N-nitrosamines, compounds that may be formed by the reaction of nitrite with certain nitrogen compounds (12).

Soy protein isolates prepared for food use vary from off-white to light tan; their flavor is characterized as "bland" to "mild cereal" and their odor as none to "mild cereal" (13). The manufacturers strive to produce colorless, odorless and flavorless products. The ranges of analytic characteristics claimed by manufacturers of food grade soy protein isolates are indicated in Table I. However, soy protein isolates also have been demonstrated to contain materials extractable with aqueous alcohols, including phosphatides, saponins, β-sitosteryl glucoside, genistein, triglycerides, and unidentified compounds (14). The mean and range of concentrations of seven essential amino acids of commercially available food grade soy protein isolates are presented in Table II (13).

In the preparation of soy protein isolates for use as sizing and coating adhesives, the process for food grade isolates is modified to give a product having high solubility, adhesive strength and desirable viscosity characteristics (15). The extraction step may be carried out at higher pH and the precipitated protein is redissolved and modified by digestion with alkali at high pH for several hours to produce the desired characteristics. Sulfur dioxide is generally used for precipitating soybean protein because of its bleaching action and its preservative properties.

Treatment with alkali has been shown to modify the amino acid composition of proteins. Bohak (16) reported that treatment of proteins at pH 12.2 and 25°C or pH 8 or above in boiling water leads to the formation of cross-links in the protein. The cross-linked substances were characterized, after hydrolysis of the treated proteins, as an amino acid, lysinoalanine [N-epsilon-(DL-2-amino-2-carboxyethyl)-L-lysine]. Lysinoalanine has been implicated as a renal toxic factor in rats (17). De Groot and Slump (18) investigated the effect of different conditions of alkaline treatment on the amino acid composition of soy protein isolate. The pH was varied between 7 and 12.2, temperature between 23° and 80°C and duration of treatment
TABLE I

Ranges of Analytic Characteristics Claimed by Manufacturers of Food Grade Soy Protein Isolates

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (N x 6.25)</td>
<td>90.0 - 97.7</td>
</tr>
<tr>
<td>Fat</td>
<td>0.2 - 1.2</td>
</tr>
<tr>
<td>Moisture</td>
<td>3.9 - 7.0</td>
</tr>
<tr>
<td>Ash</td>
<td>2.5 - 4.5</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>0.01 - 0.2</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>0.14 - 0.39</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.8 - 0.9</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.15 - 1.5</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.07 - 1.0</td>
</tr>
</tbody>
</table>

* Modified slightly from Mattil (13).

b Eleven products analyzed. Concentration of heavy metals, 0.3 ppm.

TABLE II

Concentration of Seven Essential Amino Acids of Commercially Available Food Grade Soy Protein Isolates

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Concentration (g/16 g N)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td></td>
<td>5.5</td>
<td>5.4 - 5.7</td>
</tr>
<tr>
<td>Threonine</td>
<td></td>
<td>3.3</td>
<td>3.0 - 3.5</td>
</tr>
<tr>
<td>Valine</td>
<td></td>
<td>4.6</td>
<td>4.2 - 5.0</td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td>1.1</td>
<td>0.9 - 1.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td></td>
<td>4.4</td>
<td>4.3 - 4.6</td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td>7.6</td>
<td>7.3 - 7.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td></td>
<td>5.1</td>
<td>4.9 - 5.2</td>
</tr>
</tbody>
</table>

* Modified slightly from Mattil (13). Based on analyses of six products. Tryptophan was not reported; other investigators have reported values ranging from 1.0 to 1.5 g/16 g N (6).
from 1 to 8 hours. Lysinoalanine was present in all samples treated at pH 12.2 and also in the one sample treated at pH 10 at 40°C for 4 hours.

Lysinoalanine content after treatment at pH 12.2 at 40°C for 1, 2, and 4 hours was 0.42, 0.68 and 0.83 percent, respectively, in the recovered protein; this was increased to 2.08 percent after treatment at 80°C for 4 hours. Cystine was decreased about 50 percent or more in all samples treated at pH 12.2; lysine was decreased to a lesser extent and serine was distinctly decreased only after exposure at pH 12.2 at temperatures above 40°C.

Woodard and Short (17) reported the amino acid composition of (a) a commercial soybean protein isolate prepared for paper and paperboard and similar industrial applications, (b) another commercial soybean protein isolate marketed for edible purposes, and (c) the edible soy isolate after treatment in 0.1 NaOH (100 g per liter) for 8 hours at 60°C. As may be seen from Table III, lysinoalanine was present in the industrial protein product and the alkali-treated edible isolates; both proteins had lower contents of cystine than the edible soybean protein isolate. Alkali treatment of the edible protein also resulted in marked changes in the contents of glutamic acid, leucine and phenylalanine. The investigators attributed these compositional changes to the hydrolytic action of alkali and the failure of small peptides to precipitate on neutralization with acid. However, De Groot et al. (19) reported little change in the content of these three amino acids of soy protein after similar treatment (Table IV).

The spinning processes for making fibers from soy protein isolate that are used in the preparation of meat analogs consist of dispersing soy protein isolate at a concentration of 12.5 to 18 percent in sodium hydroxide at pH 10 to 12, resulting in a viscous dope which is extruded through spinnerettes into a coagulating bath containing acid and salts to neutralize and precipitate the fibers (20-22). Protein concentration, pH, aging time and additives incorporated in the spinning dope vary with the specific process and the extent of lysinoalanine formation may be expected to vary accordingly. Lysinoalanine content of a commercial sample of food grade spun soy fiber was reported to be 0.036 percent, as is basis (78.6 percent moisture) or 0.168 percent, dry basis (23). Values for more recent production of the same manufacturer are reported to be 0.02 percent, as is basis (24).

Sternberg et al. (25) recently reported that lysinoalanine is generated in a variety of proteins when heated under nonalkaline conditions. They found, for example, 1,700 µg per g of protein in casein after heating at pH 6 for 1 hour at 120°C, 270 µg per g protein in egg white boiled 10 minutes, 150 µg per g protein in charcoal broiled frankfurters, 150 to 640 µg per g protein in milk based infant formulas from three manufacturers, 430 to 6,900 µg per g protein in sodium caseinate and similar concentrations in a variety of food products or ingredients. Values for 45 samples of commercial soy protein isolate from two manufacturers ranged from 0 to 370 µg per g protein.
TABLE III
Amino Acid Composition of Soybean Protein Isolates

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Commercial alkali-treated protein(^b)</th>
<th>Edible protein(^e)</th>
<th>Edible protein(^c, d) after alkali treatment percent of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>4.8</td>
<td>5.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.0</td>
<td>2.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.8</td>
<td>5.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>12.3</td>
<td>12.6</td>
<td>10.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.9</td>
<td>3.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Serine</td>
<td>6.3</td>
<td>6.2</td>
<td>2.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>19.1</td>
<td>20.3</td>
<td>10.7</td>
</tr>
<tr>
<td>Proline</td>
<td>5.8</td>
<td>5.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.0</td>
<td>7.0</td>
<td>6.6</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.7</td>
<td>5.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Valine</td>
<td>5.7</td>
<td>5.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.2</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.8</td>
<td>4.7</td>
<td>6.4</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.2</td>
<td>7.8</td>
<td>17.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.6</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.2</td>
<td>3.8</td>
<td>7.0</td>
</tr>
<tr>
<td>Half cystine</td>
<td>trace</td>
<td>0.4</td>
<td>trace</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>0.3</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Carboxy met. cyst.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Lysinoalanine</td>
<td>0.6</td>
<td>---</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\(^a\) Woodard and Short (17).
\(^b\) A commercial soybean protein isolate marketed for sizing and coating of paper and paperboard.
\(^c\) A commercial soybean protein isolate marketed for use as a food ingredient.
\(^d\) The edible protein was suspended in 0.1N NaOH (100g protein per liter) and incubated with stirring at 60\(^\circ\)C for 8 hours. The protein was precipitated at pH 4.5, centrifuged, washed and dried.
TABLE IV
Amino Acid Composition of Isolated Soybean Protein and Casein
Before and After Alkali Treatment (19)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Soybean protein</th>
<th>Casein</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>untreated</td>
<td>alkali treated&lt;sup&gt;a&lt;/sup&gt;</td>
<td>untreated</td>
<td>alkali treated&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>g/16gN</td>
<td>g/16gN</td>
<td>g/16gN</td>
<td>g/16gN</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.0</td>
<td>5.1</td>
<td>5.9</td>
<td>4.6</td>
</tr>
<tr>
<td>Alloisoleucine</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.5</td>
<td>7.7</td>
<td>10.2</td>
<td>8.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.7</td>
<td>5.7</td>
<td>8.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.6</td>
<td>4.1</td>
<td>6.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.3</td>
<td>5.5</td>
<td>5.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.87</td>
<td>&lt;0.5</td>
<td>0.4</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.25</td>
<td>1.2</td>
<td>3.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.5</td>
<td>3.9</td>
<td>4.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Valine</td>
<td>5.0</td>
<td>5.2</td>
<td>7.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>7.7</td>
<td>7.4</td>
<td>4.2</td>
<td>3.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.3</td>
<td>2.6</td>
<td>2.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.7</td>
<td>4.4</td>
<td>3.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.7</td>
<td>11.7</td>
<td>7.5</td>
<td>6.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>20.9</td>
<td>19.1</td>
<td>23.4</td>
<td>19.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.0</td>
<td>4.1</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Proline</td>
<td>5.0</td>
<td>5.4</td>
<td>11.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Serine</td>
<td>5.6</td>
<td>5.3</td>
<td>6.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Lysinoalanine (total)</td>
<td>0.0</td>
<td>0.96</td>
<td>0.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Lysinoalanine (free)</td>
<td>0.0</td>
<td>&lt;5.0 x 10^-4</td>
<td>0.0</td>
<td>&lt;1.0 x 10^-4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Soy protein isolate was treated in 0.1N NaOH (100 g per liter) for 8 hours at 60°C. The protein was precipitated at pH 4.5, centrifuged, but not washed, and dried.

<sup>b</sup> 200 g casein and 37 g Ca(OH)<sub>2</sub> in 400 ml water were heated for 1 hour at 105°C. The protein was precipitated at pH 4.5, filtered, washed with deionized water and dried.
III. CONSUMER EXPOSURE DATA

A National Research Council (NRC) subcommittee survey of food manufacturers (26) did not request information on level of addition of soy protein isolates to foods; however, some manufacturers volunteered this information. Based on information supplied by manufacturers who reported adding the substances to at least one food in a category, weighted means were calculated for the usual level of addition to the food category (Table V). Some of the values in Table V appear not to be representative of commercial products. In case of baby formulas, the level of addition listed in Table V is higher than that (1.8 to 2.7 g per 100 ml) reported in the Physicians' Desk Reference (27). Also, it is probable that addition of soy protein isolate to processed fruits for babies is limited to a few specialty products. The level given for reconstituted vegetable proteins appears low since soy protein isolate in the form of the spun fiber may be used at a level of 20 percent or more in fabricated meat analogs (6).

The NRC subcommittee estimated possible daily average intakes of food grade soy protein isolates from Market Research Corporation of America data on the mean frequency of eating foods by food category and age, U.S. Department of Agriculture data on mean portion size of foods in these categories and the assumption that all food products within a category contain soy protein isolates at the level shown in Table V. Such an assumption is likely to lead to overestimates of intake. The NRC subcommittee has recognized that in the case of most such calculations, estimates of intakes of the substance in question are overstated, often by considerable margins. Because of factors detailed in Section XI of the subcommittee's report (26), the possible average dietary intakes estimated in this manner are likely to be much greater than would be the intakes achieved through consumption of a diet consisting totally of processed foods to which the substances had been added at the maximum levels.

In the case of soy protein isolates, the estimates made in this manner do appear to be high. For individuals over 2 years of age in the United States, the NRC subcommittee estimated the possible average daily intakes to be more than 1 g each from four food categories (breakfast cereals; milk and milk products; frozen dairy desserts, mixes; meat products) and more than 0.5 g from each of three other food categories. However, the total usage of soy protein isolates employed in formulating food products in 1970 was estimated to be about 20 to 25 million pounds (28). Assuming a population of 205 million in 1970, use of 25 million pounds in foods suggests a maximum per capita daily intake of about 150 mg. The Select Committee believes that this per capita intake is more probable than the estimates of the NRC subcommittee which suggest an average daily intake of several grams based on calculations employing the data in Table V. However, the increasing use of soy protein products as replacements for animal proteins is likely to increase intake.
TABLE V

Level of Addition of Soy Protein Isolate
To Foods by Food Category (26)

<table>
<thead>
<tr>
<th>Food category</th>
<th>Soy protein isolate Weighted mean percent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baked goods, baking mixes</td>
<td>0.48</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>4.40</td>
</tr>
<tr>
<td>Milk, milk products</td>
<td>3.50</td>
</tr>
<tr>
<td>Frozen dairy desserts, mixes</td>
<td>3.00</td>
</tr>
<tr>
<td>Meat products</td>
<td>2.02</td>
</tr>
<tr>
<td>Poultry products</td>
<td>1.23</td>
</tr>
<tr>
<td>Gelatins, puddings, fillings</td>
<td>2.00</td>
</tr>
<tr>
<td>Snack foods</td>
<td>14.88</td>
</tr>
<tr>
<td>Reconstituted vegetable proteins</td>
<td>2.80</td>
</tr>
<tr>
<td>Gravies, sauces</td>
<td>5.00</td>
</tr>
<tr>
<td>Dairy products analogs</td>
<td>1.48</td>
</tr>
<tr>
<td>Seasoning and flavors</td>
<td>5.00</td>
</tr>
<tr>
<td>Baby formulas</td>
<td>3.48</td>
</tr>
<tr>
<td>Baby food processed fruit</td>
<td>2.10</td>
</tr>
</tbody>
</table>

* Level of addition is the weighted mean of the levels reported by manufacturers as their usual addition to one or more products in a food category. For discussion of weighted mean see text, also Section X and Exhibit 50 of reference 26. Information was not requested on usage of soy protein isolates; however, manufacturers volunteered the level of addition information.
Per unit of body weight, infants may receive larger intakes of soy protein isolates than do individuals of other age groups. During the first few months of life, it has been estimated that approximately 10 percent of U.S. infants are fed commercially prepared milk-free formulas. The great majority of such infants receive formulas in which the protein is provided from soy protein isolates (29). Protein concentration of these formulas ranges from 1.8 to 2.7 g per 100 ml or 2.7 to 3.9 g per 100 kcal. During the second month of life, the 50th percentile for energy intake is estimated to be 565 kcal per day and the 90th percentile is estimated to be 680 kcal per day (29). Assuming that the entire energy intake was provided by a formula supplying 4.0 g of soy protein isolate per 100 kcal, 50th and 90th percentile intakes of soy protein isolate would be 22.6 and 27.2 g per day, respectively (approximately 4.8 and 5.8 g per kg per day).

The report of the NRC subcommittee on the use of GRAS substances in foods (26) contains no data on the possible intake of soy protein as a result of its migration into foods from paper and paperboard packaging. Neither are data available on the extent of use of soy protein isolates as components of paper and paperboard products used in food packaging. However, it has been estimated that about 55 million pounds of soy protein isolate are used for all industrial purposes, including paper and paperboard applications (30). If all were assumed to enter foods, this would amount to 300 mg per capita daily intake. Maximum concentration of soy protein in a coated paperboard in which soy protein isolate has been used as a pigment binder is about 0.6 percent (31). Only a very small fraction of the coating from a food package containing this low concentration of soy protein would be expected to be transferred into the food by attrition or migration. Accordingly, the intake of soy protein isolate from these sources is estimated to be only a very small fraction of the hypothetical 300 mg per capita daily intake estimated from its total industrial use.
IV. BIOLOGICAL STUDIES

As pointed out by Rackis (32) the nutritional and biologic effects of feeding soy protein isolates are somewhat different from those of feeding soy meal. When fed as the sole source of protein in the diet, the isolates increase the requirements for vitamins E, K, D, and B₁₂. Phosphorus is utilized poorly. Deficiency symptoms from decreased availability of calcium, magnesium, manganese, molybdenum, copper, iron and zinc have been reported. Availability of zinc is most affected. It is evident that the formation of complexes during processing involving protein, phytic acid and minerals may be responsible for the relatively poor availability of minerals (33). Soy protein isolates are more deficient in sulfur-containing amino acids than are other soy protein products (34), and deficiency of choline reduces nutritive value further (35). Rachitogenic and perotic factors are concentrated in soy protein isolates, and growth-promoting, antirachitogenic, anti-perotic and antithyrotoxic factors are present in lesser quantities than in soy meal extracts (32). Nitrite is another component that may be present in soy protein isolates that are spray dried. A comprehensive discussion of possible toxic effects of this component and its reaction products with amines is given in an appendix to this report.

Absorption and metabolism

Gastric pH and gastric emptying. The effect of feeding soy protein isolate and casein on rate of passage of food and on gastric pH was studied in baby pigs by Maner et al. (36). After a 12-hour fast, ten 4-week-old pigs fed a diet containing soy protein isolate excreted a ferric oxide marker in 14 to 24 hours whereas five pigs of similar age fed a casein diet excreted the marker in 36 to 48 hours. At 10 weeks of age, four pigs fed each diet excreted the marker in 40 to 50 hours.

Six 10-day-old pigs were fitted with polyethylene gastric fistulas and after a recovery period of 7 to 9 days were allotted at random to liquid diets containing either soy protein isolate or casein (36). At age 4 weeks, after a 12-hour fast, gastric pH ranged from 1.2 to 3.0. Five minutes after consuming either diet, pH ranged from 5.1 to 6.0. Two hours after feeding, gastric pH of pigs that had received the casein diet ranged from 1.4 to 2.5 whereas that of pigs fed the soy protein isolate diet ranged from 4.0 to 4.4. The finding of increased gastric pH in 4-week-old pigs after feeding of soy protein isolate is generally similar to that of Osmon et al. (37) concerning gastric pH of humans after consumption of soy flour. The authors suggested that the buffering effect of soy protein may delay activation of pepsinogen, thus delaying and reducing the digestion of the protein.
Protein quality. Heat treatment improves the nutritional quality of protein in soybean meals. The degree of improvement in nutritive value depends upon the temperature, moisture conditions and duration of heating (32, 38, 39). This improvement is in part due to the destruction of trypsin inhibitor(s) (38, 40, 41) and partly to modification of the protein permitting more complete digestion and utilization of sulfur-containing amino acids (39, 40, 42). Longenecker et al. (43) and Bressani et al. (44) have demonstrated that mild heat treatment may substantially improve the protein efficiency ratios (PER) of various soy protein isolates.

Theuer and Sarett (35) have reported that 100 μg protein from unheated soy protein isolate inhibited 3.9 μg of trypsin. After heat treatment in processing of three commercially available edible soy isolate formulas, amounts of trypsin inhibited were 0.05, 0.3, and 0.8 μg per 100 g of protein. Whether this sixteenfold difference in residual concentration of trypsin inhibitor is of nutritional significance to infants is unknown. However, Churella et al (45) found that residual trypsin inhibitor in soy protein isolate formulas has no nutritional significance in rats.

The PER of four different infant soy-based formulas was determined by Harkins and Sarett (46) using groups of ten weanling rats of the Wistar strain. In one of the formulas (probably diet A) protein was supplied by soy protein isolate. The PER values ranged from 97 percent (diet A) to 62 percent that of the casein control.

Nitrogen balance studies. The ability of soy protein isolates to promote positive nitrogen balance has been studied in experimental animals, infants, children, and adults.

Studies of growth and nitrogen balance were carried out by Bressani et al. (44) with twelve 2-month-old mongrel dogs, three males and three females in each feeding group. Textured soy protein food or dehydrated beef were sources of protein and accounted for 50 percent by weight of diet. Growth to age one year was similar for the two groups and no adverse physiologic effects were noted. At the termination of the growth study, nitrogen balance studies were carried out at various nitrogen intakes from zero to more than 600 mg per kg per day. The nutritive value of the two protein sources appeared to be similar.

Fomon et al. (47) reported results of one 3-day metabolic balance study with each of six infants, 113 to 118 days of age, fed a formula with protein from soy protein isolate fortified with 5 mg of L-methionine per g of protein. The protein concentration of this formula was similar to that of human milk (1.1 g per 100 ml). The relation of retention of nitrogen to intake of nitrogen was similar for infants fed the soy protein isolate formula to that of other infants of similar age fed fresh or processed human milk or an experimental formula with protein from cow milk.
Four children 26 to 32 months of age, were studied by Graham et al. (48) during convalescence from protein-calorie malnutrition. Similar retentions of nitrogen were found with intakes of protein of 2 g per kg per day from milk or from a formula providing protein from soy protein isolate (and presumably fortified with methionine).

Eight children between 22 and 72 months of age and weighing between 9.0 and 16.3 kg were studied by Bressani et al. (44). These children were in good health after recovery from protein-calorie malnutrition. With nitrogen intakes averaging 342 mg per kg per day from milk or 312 mg per kg per day from a textured soy protein product, no significant difference was detected in percentage of nitrogen absorbed or retained.

Six healthy men, 22 to 26 years of age, weighing 62.7 to 84.6 kg at the onset of study, remained in a metabolic ward for 108 days and consumed 11 different formula diets in random sequence (49). After an initial standardization period of 18 days, metabolic periods for each diet lasted nine days. Two sources of protein - pasteurized spray dried egg white and soy protein isolate - were tested. These were fed at different levels of intake and the soy protein isolate diets were fed with or without methionine supplementation. All diets were isonitrogenous, the protein being supplemented when necessary with glycine and alanine. Nitrogen retention increased with increasing intakes of nitrogen from soy protein isolate (3.0, 4.5, 6.0 and 7.5 g per day) or from methionine-fortified soy protein isolate (3.0, 4.5, and 6.0 g per day). When intake of nitrogen was 6 g per day, retention of nitrogen from methionine-fortified soy protein isolate was similar to that from egg white.

Two similar studies were carried out with healthy young adult males to determine whether there was a difference in utilization between spun soy protein and a 5:1 casein-lactalbumin mixture when crystalline amino acids were added to provide identical intakes of essential amino acids (50). Eight men, ages 20 to 29, were subjects for the first study, in which the intake of protein was 0.4 g per kg per day; six men, ages 19 to 23 were subjects for the second study, in which the intake of protein was 0.45 g per kg per day. Approximately 50 percent of dietary amino nitrogen was provided by the casein-lactalbumin mixture or approximately 60 percent of dietary nitrogen was provided by spun soy protein isolate. After a 7-day period of adjustment to the low protein diet, two 15-day dietary periods were employed with a cross-over design. Nitrogen balance was determined during the last 10 days of each period. Spun soy protein and the casein-lactalbumin mixture appeared to be equally well utilized under these conditions.

**Mineral metabolism**

Availability of calcium, phosphorus and magnesium. The effects of soy protein isolates on metabolism of calcium, phosphorus and magnesium and on bone mineralization have been studied in swine and in poultry.
Miller et al. (51) studied the effect of dietary source of protein on growth and skeletal mineralization of Yorkshire-Hampshire crossbred pigs of either sex. Diets providing protein from casein or from soy protein isolate supplemented with DL-methionine were fortified with varying amounts of vitamin D₂. Forty-four pigs were weaned at 3 days of age and were fed the experimental diets for 5 weeks beginning at age one week. The requirement for vitamin D was demonstrated to be greater for pigs fed the diet with soy protein isolate than for those fed the diet with casein, as judged by growth, serum concentrations of calcium, magnesium and inorganic phosphorus, activity of serum alkaline phosphatase, chemical analysis of humerus for ash, calcium, phosphorus and magnesium, and metabolic balance studies of calcium, phosphorus and magnesium.

In subsequent studies (52, 53) of the same soy protein isolate (51) fed to Yorkshire-Hampshire crossbred pigs, it was shown that serum concentrations of inorganic phosphorus decreased and serum activity of alkaline phosphatase increased with increasing concentration of soy protein isolate in the diet. Mineralization of bone was less when soy protein isolate constituted 32 percent of the diet than when it constituted 16 percent of the diet. On the other hand, mineralization of bone was similar with the casein diet whether the content was 32 or 16 percent.

Soy protein isolate has growth-depressing and rachitogenic effects in turkeys (54, 55) and chickens (56, 57) when fed as the protein component of their diets. Supplementation with heated soybean meal, water extracts of heated soybean meal, or with an eightfold increase in vitamin D₃ largely overcame the adverse effects in turkeys (54, 55). Bone ash in chickens fed soy protein isolate diets was increased by supplementation with heated soybean meal, calcium and phosphorus or autoclaving the soy protein isolate (56, 57).

Availability of trace minerals

Seven young rhesus monkeys of both sexes initially weighing 1.5 to 2.5 kg were fed a basal diet with protein from soy protein isolate (58). The diet contained 21 mg of iron per 100 g diet, contributed by the soy protein isolate. The basal diet was fed alone or supplemented with choline, with choline plus vitamin E or with methionine and cystine. After receiving the diets for 2 to 7 months, the monkeys demonstrated hemoglobin concentrations of 5.0 to 9.2 g per 100 ml. With administration of iron the concentrations of hemoglobin increased to a level of 10.6 to 14.0 g per 100 ml. In an anemic monkey absorption of $^{59}$Fe from a soybean protein-$^{59}$Fe mixture was approximately 50 percent as great as from a casein-$^{59}$Fe mixture.

The bioavailability of zinc in milk and in infant formulas with protein supplied from soy protein isolate was studied by Momčilović et al. (59). Weanling male Wistar rats were randomly divided into 12 experimental
groups of eight each and fed various diets ad libitum for 3 weeks. A zinc-
deficient diet containing egg-white protein was supplemented with graded
levels of zinc from zinc sulfate, from milk, or from a formula with soy
protein isolate as the source of protein. A log plot of total femur zinc at
the end of the 3-week experimental period gave a linear relationship over
the range 0 to 12 μg per g of added zinc. By using a slope-ratio bioassay
method, the availability of zinc from formulas with protein from soy protein
isolate or milk was 67 and 86 percent, respectively, of that from the egg-
white protein formula containing added zinc sulfate.

New Hampshire chicks, ten per feeding group, were studied for 4
weeks from time of hatching while receiving a basal diet with protein from
soy protein isolate supplemented with 0.5 percent DL-methionine (60).
Three levels of zinc, manganese, copper and iron were fed with and with-
out ethylenediaminetetraacetic acid (EDTA). Deficiency symptoms consisted
of decreased growth rate and, in the case of deficiency of zinc, osteoporosis.
Deficiency of copper or iron resulted in decreased hematocrit and concen-
tration of hemoglobin. The inclusion of EDTA in the diet appeared to increase
the availability of zinc, manganese and copper but not of iron. The findings
suggested that zinc, manganese and copper were poorly available in the soy
protein isolate diet, and that the availability was increased by the inclusion
of EDTA. Other studies with chicks (61) and turkey poult(s) (62) also have
demonstrated a relatively low availability of zinc from soy protein isolates.

Acute toxicity

With the exception of a few cases of adverse reactions to infant
formulas containing soy protein isolate (see Special studies, p. 19), no
reports of acute toxicity studies have been found.

Short-term studies

Growth of animals. Growth of hysterectomy-obtained specific
pathogen-free Hampshire-Yorkshire crossbred pigs was studied by
Schneider and Sarett (63). Eight pigs were fed an infant formula with
protein (15 percent of calories) supplied from soy protein isolate supple-
mented with 8 mg of DL-methionine per g of protein and seven pigs were
fed a milk-based formula providing the same percentage of calories from
protein. Lactose was the carbohydrate in each formula and both were
supplemented with the same vitamins and minerals. Cumulative weight
gain to age 31 days averaged 5.6 kg for pigs fed the soy protein isolate
formula and 8.0 kg for pigs fed the milk-based formula. This difference
was statistically significant (p < 0.02). However, it may be noted that
caloric density of the milk-based formula was approximately 10 percent
greater than that of the soy protein isolate-based formula. Gain in weight
per unit of caloric intake was not significantly different for the two groups.
At age 31 days, carcass composition of the two feeding groups was sig-
nificantly different (p < 0.01) with respect to protein (greater in pigs fed
milk-based formula) and ash (greater in pigs fed soy protein isolate-based formula).

Thirty-six Yorkshire pigs studied by Pond et al. (64) were weaned at 2 or 3 days of age, assigned to three feeding groups and maintained individually in wire-bottomed cages. Diets were identical except for the protein source, which consisted of soy protein isolate, casein or a fish protein concentrate. Diets were supplemented with vitamins and minerals but not with methionine. Each pig was fed the liquid diet from a porcelain bowl four times daily. Over the 21 days of study, average gains were 44, 59, and 57 g per day, respectively, for pigs receiving soy protein isolate, casein and fish protein concentrate. Corresponding gains per gram of protein consumed were 1.9, 2.4, and 2.4 g. In each case, the value for pigs fed the soy protein isolate was significantly (p < 0.05) less than that of pigs fed casein or the fish protein concentrate. At the end of the experiment serum concentration of total protein was also significantly less (p < 0.01) in the soy protein isolate group than in the other two groups.

Growth of infants and young children. Ten male and nine female infants, all or nearly all with birth weights 2500 g or more, were studied by Bates et al. (65) while receiving a formula providing 1.8 g of protein from soy protein isolate per 100 ml (and per 67 kcal) fortified with 0.026 percent DL-methionine, vitamins and minerals. Gains in weight and length to age 112 days (107 to 137 days) and final serum concentration of albumin were judged to be normal on the basis of comparison with breastfed infants and infants fed milk-based formulas.

Performance of infants fed a formula with protein from soy protein isolate was compared by Cherry et al. (66) with performance of those fed a commercially available milk-based formula. The protein content of the soy protein isolate formula was 2.37 g per 100 ml (15.1 percent of calories) and the formula was fortified with vitamins and minerals but apparently not with methionine. The protein content of the milk-based formula was 2.24 g per 100 ml (14.8 percent of calories). Seventy-three infants were enrolled usually by 3 days of age, and 58 completed the planned 6 months of study. Formulas were assigned alternately within narrow birth weight categories of like sex. Consumption of foods other than formula was discouraged during the first 3 months of life and similar foods were consumed by both groups subsequently. By age 56 days mean body weights and lengths were significantly greater for boys and girls fed the milk-based formula than for those fed the formula with protein from soy isolate. Thereafter, the feeding-related difference in body size persisted but was not statistically significant for boys after 122 days of age for weight and 152 days of age for length. Tibial length and width and serum concentrations of total proteins were slightly greater for infants fed the milk-based formula but these feeding-related differences were not statistically significant.
Dean (67) was able to observe 24 of 26 full-term infants from the time of enrollment in the newborn period until six months of age while they received a formula with protein from soy protein isolate fortified with methionine, minerals and vitamins. This formula provided 2.0 g protein and 67 kcal per 100 ml. Values for weight and length of all infants were reported to remain between the 10th and 90th percentiles of a standard size chart and serum concentrations of albumin were reported to remain within normal limits.

Fomon et al. (47) studied 15 normal full-term female infants fed an experimental formula with protein from soy protein isolate. The infants were enrolled before 10 days of age and thirteen completed the planned period of study to age 112 days. The formula provided 1.1 g protein per 100 ml (per 67 kcal) and was fortified with 5 mg L-methionine per 100 ml and with vitamins and minerals. Daily weighed food intakes for each infant were obtained. Mean gains in weight and length were similar to those of a larger number of infants studied in the same manner while receiving milk-based formulas. Serum concentrations of albumin demonstrated a gradual increase between 28 and 112 days of age in a manner similar to that recorded in studies of normal breastfed infants.

Leake et al. (68) studied 22 infants hospitalized for severe, nonbacterial diarrhea. The infants were assigned at random to a skim milk formula or to a commercial formula with protein from soy protein isolate. Treatment failures were reported to be greater in infants fed the skim milk formula than in those fed the soy protein isolate formula.

Several other studies of growth of infants fed formulas with protein from soy protein isolate have been reported (68-71). Some of these studies are difficult to interpret because of the absence of controls or because details about the subjects or their management are not provided.

Study of human adults. Six healthy male volunteers were studied for 24 weeks in a metabolic ward while receiving their entire protein intakes in the form of soy protein isolate (72). They remained healthy and demonstrated normal leukocyte counts, hemoglobin concentrations, hematocrits, serum concentrations of urea nitrogen, vitamin A, carotene and ascorbic acid, and plasma concentrations of vitamin B₁₂.

Long-term studies

With the exception of two multigeneration studies of rats (see Special studies, p. 19), no long-term studies are available.
Special studies

Adverse reactions to soy protein isolates by infants. Mendoza et al. (73) reported the case of an infant fed from birth a commercially available formula with protein from soy protein isolate. At 4 weeks of age the infant developed diarrhea and at 6 weeks of age was admitted to the hospital for treatment of diarrhea. When formula feeding was re instituted, a disaccharide-free formula with protein from soy protein isolate was given and diarrhea recurred. The infant improved when fed human milk and subsequent challenge with 1 oz of soy formula (not identified) was immediately followed by vomiting, explosive diarrhea and signs of peripheral vasoconstriction. The infant was then fed human milk, lamb and rice and at age 6 months passive transfer tests demonstrated reactions to soy on three occasions.

Ament and Rubin (74) reported a case of an infant who developed diarrhea at 4 weeks of age while being fed a commercially available milk-based formula. At 6 weeks of age he was fed a commercially available formula with protein from soy protein isolate and reacted with diarrhea and vomiting. Two inadvertent feedings of 1 oz of the soy formula were followed by ashen color, listlessness, vomiting and passage of guaiac-positive stools. At 10 months of age the infant was able to tolerate feedings of homogenized whole cow milk. At 10½ months, administration of 6.5 g of soy protein isolate resulted in manifestations similar to those observed with the earlier inadvertent challenges. Proximal jejunal biopsy 24 hours after the soy protein isolate challenge revealed a lesion indistinguishable from that seen in untreated celiac syndrome. The histologic appearance returned to normal within 4 days.

Vitamin deficiency. The influence of feeding soy protein isolates on vitamin D requirements has already been discussed. Increased requirements for vitamins E and K also have been reported when diets with protein from soy protein isolate were fed.

Day-old male chicks of the Vantress breed or from a cross between Columbian females and New Hampshire males, seven to ten birds per treatment, were fed diets providing 1 to 5 percent corn oil and 13 or more mg per kg of α-tocopherol (75). When the dietary protein was soy protein isolate but not when it was casein, growth depression, exudative diathesis, encephalomalia and death were observed. These effects could be prevented by higher concentrations of α-tocopherol in the diet or by including the antioxidant, ethoxyquin.

In 1969 Morgan (76) and Moss (77) described bleeding episodes in two infants with diarrhea who were fed formulas with protein from soy protein isolate. These episodes were terminated by administration of vitamin K. It was subsequently demonstrated by Williams et al. (78) in a prospective
study of infants from birth to 3 months of age that prothrombin time remained normal in individuals without diarrhea or other illnesses who were fed formulas with protein from soy protein isolate.

**Multigeneration studies.** Four generations of rats fed a diet in which a commercial soy protein isolate and DL-methionine were the only sources of amino acids made satisfactory postweaning weight gains. The postweaning mortality was low (less than 1 percent) (79). A more highly purified soybean protein was prepared by two reprecipitations of a commercial soybean protein isolate from aqueous medium, followed by extraction with boiling alcohol. This protein was fed to rats of two generations and also gave satisfactory growth and low postweaning mortality. Prewearning mortality was high (up to 50 percent) and appeared to be related to lactation failure. The mortality may have been due to vitamin B\textsubscript{12} deficiency (80, 81). Mortality could be decreased by addition of liver extract or condensed fish solubles to the diets of the dams or by substitution of expeller processed soybean oil meal as the source of protein (82).

A multigeneration study of rats (strain not specified) fed a highly purified sodium proteinate from soy supplemented with DL-methionine was reported by Richardson and Brock in 1956 (81). The protein was exhaustively extracted with boiling water and with 70 percent isopropyl alcohol. Four generations of females, ten animals per generation, were observed. With the exception of the first generation (82 percent weaned), 90 to 100 percent of the offspring of each generation were weaned.

**Studies of alkali-treated soy protein isolates**

**Renal lesions of rats.** Feeding of soy protein isolate prepared for industrial uses or of alkali-treated edible soy protein isolate in large amounts to rats has been reported to result in cytomegalic lesions of the straight portion (pars recta) of the proximal tubule (83, 84). The investigators have presumed that the lesions were caused by lysinoalanine formed during alkali modification of the protein. Parenteral administration of relatively small quantities of lysinoalanine gave rise to lesions similar to those of rats fed alkali-treated protein for a much longer time. A review of these studies follows.

Woodward and Alvarez (85) studied weanling, female Sprague-Dawley rats, ten per feeding group, fed semipurified diets containing 20 to 30 percent of a commercial alkali-treated soy protein isolate (industrial grade protein isolate intended for nonfood applications) or casein as the sole source of protein. Sucrose was the carbohydrate source. The animals were killed after 8 weeks to 1 year. Cytomegalic changes in the pars recta of the renal tubules were found in animals fed the industrial grade soy protein but not in those fed casein. The lesions were not affected by the levels of choline, methionine or vitmain B\textsubscript{12} in the diet and could not be produced by feeding a methionine-deficient casein diet.
Newberne and associates (86, 87) had observed similar kidney lesions in weanling male Charles River caesarean-derived rats fed diets containing 20 percent of commercial alkali-treated soy protein as the only source of protein and with sucrose as the carbohydrate source. In two different experiments, groups of 10 to 39 animals were fed the diets supplemented with various levels of DL-methionine (0.1 to 0.6 percent), choline (0.1 to 0.6 percent) and vitamin B_{12} (0 to 5.0 μg per 100 g diet). Ninety percent or more of the animals on diets supplemented with 0.1 percent methionine and 0.1 percent choline exhibited kidney lesions when autopsied after 6 months feeding. However, maintenance of the low choline intake together with a moderate methionine (0.3 percent) supplementation markedly reduced the incidence of kidney lesions. A further significant decrease was noted when the diets were supplemented with 0.6 percent methionine and 0.6 percent choline, but normal renal morphology was observed only after the addition of vitamin B_{12} to the diet.

Subsequently, it was demonstrated by Woodard (88) that after 1 week of feeding diets containing 20 percent of an industrial grade alkali-treated soy protein isolate as the protein source, the kidneys of weanling rats demonstrated increase in mitotic activity within the inner stripe of the kidney cortex. This change was followed by an increase in number of polyploid nuclei within the inner stripe and by the fourth week of feeding cytomegalic changes became uniformly obvious. No differences in rate of DNA synthesis were detected between normal and enlarged nuclei. The process eventually led to invagination of the cytoplasm to form intranuclear inclusions. Fatty metamorphosis of the proximal tubule was noted early and progressed throughout the experiment.

In other studies, male weanling caesarean-derived Sprague-Dawley rats, 5 to 15 per feeding group were fed a basal diet containing soy protein isolates listed in Table III as the only source of protein. The commercial alkali-treated protein also was fed after pronase digestion and after extraction with water, methanol, chloroform or hexane (17). The diets, containing 20 percent soy protein product, were fed for 8 weeks and the kidneys were then examined for cytomegalic changes in the pars recta. The nephrotoxic factor was found to be heat-stable and could not be extracted with polar and nonpolar solvents. The toxin was not indigenous to soy protein but resulted from the alkali treatment. It was demonstrated that such treatment resulted in formation of an unusual amino acid with chromatographic properties similar to those of chemically synthesized lysinoalanine. Content of lysinoalanine in the commercial alkali-treated protein was 0.6 percent.

Further investigations by Reyniers et al. (89) of male weanling Sprague-Dawley rats fed diets containing 30 percent of a commercial alkali-treated soy protein isolate as the sole protein source demonstrated altered
nuclear characteristics of nephromegalocytes: increased availability of DNA phosphates for cation binding; reduced chromatin thermal stability; and altered ratio of dye-binding capacity of arginine and lysine residues in histone. The investigators considered that the data were consistent with the view that the megalocytes represented renal tubular cells with an altered functional state.

Female weaning Sprague-Dawley rats, three per feeding group, were fed semipurified diets containing 0, 0.025, 0.05, 0.1, and 0.3 percent lysinoalanine (83, 84). Animals were killed after they had received the diet for 4 weeks. Renal lesions were observed in all groups fed lysinoalanine and the lesions were more severe at the higher dose levels. Two other animals received daily intraperitoneal injections of 30 mg of lysinoalanine and two controls received similar injections of L-lysine monohydrochloride. Still other animals received 30 mg of lysinoalanine or 30 mg of L-lysine monohydrochloride by stomach tube. The animals that received daily doses of amino acids by injection or gavage were killed after the seventh treatment. Renal lesions were demonstrated in those given lysinoalanine but not in those given lysine.

De Groot and coworkers (18, 19, 23) also have investigated the effect of alkali treatment on the biological properties of proteins. They confirmed the findings of Woodard et al. (83) that free lysinoalanine induced nephrocytomegaly in rats but observed no renal changes when alkali-treated soy protein or casein containing bound lysinoalanine was fed to rats. After complete acid hydrolysis the renal activity of alkali-treated proteins was found to be that expected from the lysinoalanine liberated. Some nephrotoxicity was observed with peptide-bound lysinoalanine in the breakdown products (molecular weight <5000) of partially hydrolyzed alkali-treated casein, but the toxicity was considerably less than that of free lysinoalanine (19).

De Groot and Slump (18) fed groups of five female Wistar-derived weanling rats for 6 weeks, diets containing 10 percent casein and 20 percent of untreated or alkali-treated soy protein isolate. The diets were supplemented with DL-methionine; wheat starch was the carbohydrate source. Alkali treatment conditions were pH 12.2 and 40°C for 4 hours; lysinoalanine content of the treated protein was 0.80 g per 16 g N. Kidney and liver weights and gains in body weight of the treated animals did not differ significantly from the controls. Kidneys of the test rats showed distinct changes (nephrocalcinosis) consisting of heavy calcareous deposits in the corticomedullary region attended with distorted tubules. Similar renal changes were present in the controls, but were less severe. Nephrocalcinosis was stated to occur commonly in the strain of rat used and was
aggravated by diets either low in calcium, high in phosphorus, or low in magnesium. To counteract the possible effect of phosphorus contained in soy protein isolate, another feeding test was conducted in which the alkali-treated soy protein diet was supplemented with 1 percent CaCl₂·2H₂O. Renal calcinosis in the test rats was less than in the controls fed untreated soy protein isolate.

In a 90-day study, De Groot and Slump (18) fed groups of five male and five female weanling rats untreated or alkali-treated soy protein isolate in diets similar to those used in the 6-week studies just discussed. Body weights of the test animals were lower than those of controls but not significantly (p>0.05). No significant differences were found in blood or urine parameters of the two groups. Organ weights and gross autopsy findings did not show treatment related differences. Microscopic examination of 30 different organs and tissues did not reveal any distinct abnormalities, except for the kidney of one treated female in which severe nephrocalcinosis was observed.

De Groot et al. (19) treated soy protein isolate in 0.1 N NaOH (100 g per liter) for 8 hours at 60°C as described by Woodard and Short (17). After acidifying to pH 4.5, the precipitate was recovered by centrifugation; it was not washed. Amino acid analyses of the untreated and treated proteins are given in Table IV. Semipurified diets containing 20 percent crude protein from treated or untreated soy protein, as such and after acid hydrolysis, were fed to groups of five male weanling rats for 4 weeks as the sole source of protein. Diets were supplemented with DL-methionine and the dietary levels of tryptophan, calcium and phosphorus were equilibrated by appropriate supplementation. Starch was the carbohydrate source. The diet containing alkali-treated protein provided 2400 ppm lysinoalanine; it supported normal growth and no renal changes were observed microscopically. Rats fed the acid-hydrolyzed alkali-treated soy protein exhibited growth depression, increased kidney weights and nephrocytomegaly. De Groot et al. (18) also fed casein that was alkali treated by autoclaving with calcium hydroxide for 1 hour at 105°C. The treated protein was precipitated at pH 4.5, washed with deionized water and vacuum dried. Lysinoalanine content was 6.2 g per 16 g N (Table IV). Fed to male weanling rats for 4 weeks as a supplement to a stock diet at levels (3.9 and 11.7 percent) providing 2,000 and 6,000 ppm lysinoalanine, the treated casein did not induce any histological renal changes. However, substitution of acid-hydrolyzed alkali-treated casein for the nonhydrolyzed, alkali-treated casein in the stock diet resulted in renal cytomegala even though the dietary level of lysinoalanine was only 200 ppm. The renal lesion was slightly less pronounced than with 200 ppm synthetic lysinoalanine added to the stock diet. Free lysinoalanine levels in serum from blood samples collected at day 28 were comparable for rats fed synthetic lysinoalanine and acid-hydrolyzed alkali-treated casein (0.5 and 0.4 μg per ml, respectively) but no measurable quantity was found in the blood of rats fed 2000 ppm lysinoalanine as the protein-bound compound in nonhydrolyzed alkali-treated casein.
De Groot et al. (19) investigated the cytomegaly inducing properties of lysinoalanine bound in oligopeptides by feeding rats a stock diet supplemented with alkali-treated casein which was subsequently partially acid-hydrolyzed. The hydrolyzed preparation contained 6.2 g lysinoalanine per 16 g N, only 8 percent of which was in the free state. The remaining peptide-bound lysinoalanine showed the following molecular weight distribution: 5000 to 1500, 10 percent; 1500 to 1000, 30 percent; 1000 to 500, 45 percent and <500, 7 percent. Cytomegalic reaction was observed in the kidneys of rats fed diets containing levels of the hydrolyzed casein which provided free and bound lysinoalanine at levels of 30 and 400 ppm, and 45 and 600 ppm, but not at levels of 15 and 200 ppm, respectively. No such renal changes were observed in rats fed synthetic lysinoalanine at levels of 30 and 45 ppm, indicating that peptide-bound lysinoalanine as present in the oligopeptides fed, exerted some activity in inducing nephrocytomegaly.

Feron et al. (90) reported renal cytomegaly in weanling Wistar rats fed an alkali-treated soy protein isolate (20 percent dietary level) as the sole source of protein in the diet for 8 weeks. The treated protein contained 1.3 percent lysinoalanine providing 2650 ppm lysinoalanine in the diet. Another group of rats was fed a diet containing 20 percent of the alkali-treated soy protein and 10 percent untreated casein. Rats fed the former diet showed slight to moderate nephrocytomegaly, whereas only minimal or doubtful nephrocytomegaly was observed in the rats fed the diet supplemented with casein.

Slump et al. (91) found that pepsin-pancreatin-pig intestinal mucosa treatment of alkali-treated caseins containing 1.0 and 5.5 percent lysinoalanine liberated 0.5 and 0.2 percent of the lysinoalanine, respectively, whereas similar treatment of alkali-treated soy protein containing 1.3 percent lysinoalanine released 3 percent of the bound lysinoalanine. Nephrocytomegaly was not observed in rats fed the casein containing 5.5 percent lysinoalanine for 4 weeks at a dietary level providing 10,000 ppm lysinoalanine, but was observed with the other casein and the soy protein fed at levels providing 2700 and 2500 ppm lysinoalanine, respectively.

In 90-day feeding studies on synthetic lysinoalanine, De Groot et al. (19) found cytomegaly was present in the kidneys of all female and male rats (10 each) fed a stock diet containing 100 ppm of the compound but no renal changes were observed at levels of 10 and 30 ppm. The stock diet was composed (in percent) of: fish meal 7, meat and bone meal 4, soybean oil meal 11, yellow corn 29.7, whole wheat 36, grass meal 3, brewer's yeast 3, whey powder 2, soybean oil 3, plus vitamin and mineral supplements. In another experiment, dietary levels of 0, 1000, 3000, and 10,000 ppm lysinoalanine in the stock diet were fed to groups of five male rats for 4 weeks. The organ-to-body weight ratios of heart, kidneys, liver, spleen, brain, gonads, thymus, thyroid and adrenals after feeding lysinoalanine at the relatively low levels for 13 weeks, and of heart, kidneys, liver and spleen determined after feeding lysinoalanine at the high levels for 4 weeks,
did not show any treatment related changes. Microscopic examination of organs and tissues revealed marked damage only in the kidneys, mainly in the inner zone of the renal cortex and consisted of necrosis, regeneration and cytomegaly in the proximal tubules. The lesions occurred at all of the levels tested in the 4-week study. The regenerative and fibrotic changes were most marked at 10,000 ppm, whereas the typical cytomegalic changes were most clearly visible with 1,000 ppm.

Of the lysinoalanine consumed by the rats fed the diet containing 1,000 ppm lysinoalanine during 3 days in week 2, 21 percent was recovered in the urine, all as the free compound. Only 10 percent was found in the feces, 6 percent free and 4 percent in bound form. In rats fed alkali-treated soy protein, 0.2 percent was found in the urine and 50 percent in the feces, whereas these values were 25 percent and 7 percent, respectively, after feeding the alkali-treated and subsequently acid hydrolyzed protein (19).

Effect of feeding synthetic lysinoalanine to six species other than the rat was reported by De Groot et al. (18). Two groups, each consisting of 15 newly hatched Japanese quail, 10 male weanling Swiss mice, 10 male weanling Syrian golden hamsters, four male weanling New Zealand white rabbits, and two 6-month-old male beagles were fed appropriate stock diets supplemented with 0 or 1,000 ppm synthetic lysinoalanine. Male rhesus monkeys, 18 months old, were hand-fed 150 g of three casein based diets and 150 g of a fruit mixture daily. Two monkeys were fed the basal diet supplemented with 1,000 ppm synthetic lysinoalanine, two others received a diet containing 10,000 ppm lysinoalanine provided by 16.5 percent alkali-treated casein which was incorporated at the expense of an equal amount of untreated casein. The fifth monkey was fed the basal diet. Except for the monkeys, one-half of the animals in each species group were killed after 4 weeks and the remaining animals were killed after 8 weeks. Growth rate, food intake and food efficiency of mice, hamsters, Japanese quail and rabbits were unaffected by lysinoalanine supplementation of their diets. Liver weight of hamsters was slightly (10 percent) but significantly increased. Histopathological examination of the kidneys of mice, hamsters, rabbits and quail after feeding lysinoalanine for 4 and 8 weeks did not show cytomegaly or any other sign of renal damage. Histopathological findings were negative for the two beagles, one after feeding for 4 weeks and the other after 9 weeks. The monkeys were killed after 9 weeks. Their body weights remained comparable and no abnormalities in condition or behavior were observed. Gross autopsy findings were essentially negative and microscopy of the liver, kidneys, spleen, pancreas, stomach, intestinal tract and adrenals did not reveal any changes attributable to treatment. In monkeys, 13 percent of the amount of synthetic lysinoalanine was recovered in the urine, 11 percent free and 2 percent in a bound form. About 4 percent was
recovered in the feces, predominantly in a bound form. When protein-bound lysinoalanine was fed, only 1 percent was recovered in the urine and 12 percent in the feces, both mainly in a bound form.

Feron et al. (90) found a slight nephrocystomegaly in weanling Swiss mice, but not in weanling Syrian golden hamsters, after feeding diets containing 10,000 ppm free lysinoalanine for 4 to 8 weeks.

In a long-term feeding study with free lysinoalanine in rats, Feron et al. (90) fed diets containing 0 or 200 ppm synthetic lysinoalanine. After 4, 8, 13 and 26 weeks 5, and after 52 weeks 10 of each group were killed. Nephrocystomegaly became increasingly accentuated with exposure time in the treated animals. However, there were no signs of proliferative or neoplastic changes in any of the kidneys examined at 52 weeks.

Van Beek et al. (23) investigated nutritional, renal and other biological effects of feeding spun soy protein isolate in the diets of rats. The spun soy protein isolate was a commercial product used in the fabrication of meat analogs and contained 0.168 percent lysinoalaine on the dry basis. Groups of Wistar-derived weanling rats, ten males and ten females per group, were fed diets containing soy protein isolate and/or spun soy protein isolate, the latter at levels (dry matter basis) of 0, 5.1, 10.3 and 22.6 percent of the diet weight. Dietary levels of lysinoalanine were 0, 100, 210, and 410 ppm, respectively. The diets were supplemented with DL-methionine, minerals and vitamins; cornstarch was the carbohydrate source. The rats were killed during the fourteenth week of feeding. An increase in relative kidney weight and degree of nephrocalcinosis was noted in females and was attributed to the high level of available phosphorus in the diet containing alkali-treated soy protein isolate. Omission of 0.9 percent potassium dihydrogen phosphate in the mineral supplement, or addition of either 1 percent calcium chloride dihydrate or 1.5 percent magnesium oxide to the diet containing 22.6 percent spun soy protein isolate reduced the degree of nephrocalcinosis. The study failed to reveal adverse effects on general appearance, growth, feed efficiency, hematologic indices, blood enzyme levels, urine composition, kidney function, organ weights or gross microscopic findings.

Gould and McGregor (92) and Karayiannis (93) reported renal karyomegaly in Sprague-Dawley and Wistar rats fed diets containing 20 percent alkali-treated soy protein, providing 1400 to 2600 ppm dietary lysinoalanine, as the sole dietary protein source. Soy protein isolate was treated with 0.1 N NaOH (100 g protein per liter) for 8 hours at 60°C, precipitated at pH 4.5, washed and dried. Lysinoalanine contents of three different preparations were 0.68, 0.98, and 1.27 percent, respectively (93). They did not, however, obtain the lesion with 12 percent alkali-treated soy protein and 8 percent untreated lactalbumin in a diet providing 1600 ppm lysinoalanine, nor with a diet containing 10 percent alkali-treated lactalbumin and 10 per-
cent untreated lactalbumin that provided 2500 ppm dietary lysinoalanine. Further, diets containing 20 percent alkali-treated lactalbumin treated under more moderate alkaline conditions produced only mild nephrocytomegaly despite a dietary lysinoalanine level of 500 ppm.

A difference in sensitivity of Sprague-Dawley and Wistar rats to alkali-treated soy protein was observed by Struthers et al. (94) in dose response studies of dietary lysinoalanine. Marked renal cell cytomegaly was seen in Sprague-Dawley rats fed a diet containing 30 percent alkali-treated soy protein isolate (1 percent lysinoalanine) but no changes occurred in Wistar rats fed the same diet. No significant differences from control animals were seen in either strain at lower dietary levels of alkali-treated protein.

In another study, Struthers et al. (95) fed weanling Sprague-Dawley rats a diet containing 30 percent alkali-treated soy protein isolate providing 3000 ppm lysinoalanine in the diet. After 8 weeks the treated animals were fed the control diet for a second 8-week period. Pair fed controls were maintained on a diet containing 30 percent lysinoalanine-free soy protein isolate. Incidence of cytomegaly in the treated animals dropped from 100 percent at 8 weeks to 37.5 percent at 16 weeks; severity dropped 60 percent from the 8-week value.

Finot et al. (96) investigated the distribution and metabolism of $^{14}$C-labeled free lysinoalanine in the rat, mouse, hamster and quail. About 15 percent of the radioactivity from an oral dose of 6.8 mg per kg $^{14}$C-labeled lysinoalanine administered to rats was excreted in expired CO$_2$, 40 percent in the urine and 25 percent in the feces within 24 hours. In addition to free lysinoalanine, more than ten lysinoalanine metabolites were detected in rat urine. At least five of these metabolites appeared to be acetylated lysinoalanine derivatives. In the 24-hour urine sample collected after the oral dose, 60 to 65 percent of the urinary radioactivity was associated with free lysinoalanine, 13 to 19 percent with a major, acid-stable metabolite and the rest with acetylated and other metabolites. The urinary metabolites found in the mouse and hamster were similar to those found in the rat, both qualitatively and quantitatively, with the notable exception that the major, acid-stable, metabolite found in rat urine was absent. This metabolite also was absent in quail urine.

Twenty-four hours after the oral administration of $^{14}$C-labeled lysinoalanine, the rat kidneys contained a higher fraction of the administered dose than the mouse, hamster or quail kidney (96). The total label in the rat kidney, expressed as a fraction of the administered dose, was more than ten times higher than that in hamster kidney and two to ten times higher than in the mouse kidney.
It has been suggested (92) that the biological properties of protein-bound lysinoalanine may depend on the specific protein and on the conditions of alkali treatment. The biological activity of lysinoalanine may depend on its optical configuration which, in turn, may be determined by the conformation of the protein and treatment conditions. Since the mechanism of formation of lysinoalanine is via dehydroalanine in which the \( \alpha \)- and \( \beta \)-carbons are joined by a double bond, it is to be expected that the alanyl part of the lysinoalanine, formed by the addition of the \( \epsilon \)-amino group of lysine to dehydroalanine would be racemic. Because the lysine moiety is present in the native protein in the L-form, the L-lysyl, D,L-alanyl forms observed by Bohak (16) are most likely to be formed. It is possible, however, that the tendency of certain proteins to assume a particular conformation could force the \( \alpha \)-carbon of dehydroalanine to assume one or the other optical configuration during the addition reaction to lysine. Severe treatments of sunflower protein with sodium hydroxide (>0.2 M, 80°C, 1 hour) resulted in a marked degree of racemization of lysine (97) and it has been suggested that milder treatments also could result in racemization in different proteins.

In 4-week feeding studies conducted by Feron et al. (90) with synthetic lysinoalanine stereoisomers, the lowest level at which nephrocytomegaly occurred in Wistar rats was 30, 100, 300, and 1000 ppm for LD-, DL-, LL- and DD-isomers, respectively.

The second chemical characteristic which may influence the biological activity of lysinoalanine in proteins is the nature of the lysinoalanine cross-link which results when the lysyl and dehydroalanyl residues link to form lysinoalanine. Whether these two residues are located at adjacent or remote sites in the same protein molecule, or whether they are located on separate molecules and, therefore, result in intermolecular cross-links, may determine the extent of lysinoalanine release from the protein and perhaps the form in which it is released (92).

Teratogenicity

Struthers et al. (98) observed no teratological effects in Sprague-Dawley rats fed alkali-treated soy protein isolate (1 percent lysinoalanine) during pregnancy at 0, 5, 10, 20 or 30 percent dietary levels providing 1,000, 2,000 and 3,000 ppm lysinoalanine. All diets contained 30 percent protein consisting of untreated and/or alkali-treated soy protein. No significant differences were found in birth weight, mortality, live births or number of births per litter as compared to the control group. Decreased weight gain in pups from dams fed either 2,000 or 3,000 ppm lysinoalanine during lactation was attributed to reduced milk production in dams resulting from decreased digestibility of the alkali-treated protein. No lysinoalanine was found in the milk.
Nitrites in soy protein isolates

An analysis of the health aspects of nitrites contained in soy protein isolates is presented in the Appendix; a summary follows:

1. Soy protein isolates may contain up to 50 ppm nitrite. This leads to an estimated maximum daily nitrite consumption of about 0.04 mg per kg body weight for vegetarians eating meat analogs prepared from spun soy protein isolates (assuming nitrite is not removed in the spinning process) and about 0.25 mg per kg body weight for infants subsisting on formulas based on soy protein isolates if they contain 50 ppm nitrite. However, soy protein isolates currently used in infant formulas are reported to contain no more than 6 ppm nitrite and ingestion of nitrite from this source would be correspondingly lower. Other consumers of soy protein isolates probably ingest much less than 0.04 mg per kg nitrite from this source.

2. Nitrite also occurs in other foods of plant origin and as an ingredient in cured meats. Daily per capita intake from these sources has been estimated to be about 2.4 mg. This intake will be reduced by recent USDA regulations which lowered the amount of nitrite permitted to be added to bacon. Saliva is an important additional source of nitrite entering the stomach being formed by bacterial reduction of nitrate secreted in the saliva. Dietary nitrate, principally from vegetables but also produced in the intestines from ammonia or organic nitrogen compounds, is absorbed from the gastrointestinal tract and concentrated from the plasma into the saliva by the salivary glands. Total daily exposure to nitrite from saliva has been estimated to be about 15 mg. Nitrite also is formed in the intestine from ammonia or organic nitrogen compounds and has been estimated to contribute about 90 mg daily.

3. The LD_{50} for sodium nitrite for rodents lies in the range of 80 to 300 mg per kg. The mean lethal dose for human beings is estimated at 1 to 2 g. Long-term feeding studies for rats suggest a threshold of adverse effects for daily consumption somewhere between 10 and 100 mg per kg body weight. A recent unpublished rat-feeding study indicates that nitrite intake in this range enhances the frequency of lymphoreticular tumors from 8.4 percent in controls to 12.5 percent in the treated animals. Newberne, the investigator, suggested that the effect was not mediated through the formation of nitrosamines, and that nitrite acted as a promoter of the neoplastic process. The significance of the latter findings to humans is conjectural.

4. Nitrite can react with many nitrogen-containing compounds in foods, drugs, and other substances, in vitro and in vivo, to form nitrosamines, many of which have been shown to be carcinogenic in experimental animals.
Many studies of soy protein isolates prepared for food use have been carried out with experimental animals and with human subjects. In most studies of experimental animals, fortification of soy protein isolate with methionine increased nutritional quality. Under certain experimental conditions, feeding of soy protein isolates as the sole or major source of protein to animals has impaired the utilization of fat-soluble vitamins or of minerals, especially calcium, phosphorus, magnesium, zinc, and copper. In a number of studies of experimental animals and in a few studies of human infants, growth was reported to be more rapid when proteins of animal origin were fed than when equal amounts of soy protein isolate were fed. However, in other studies of human infants, rates of gain in weight in infants fed formulas with protein from cow milk were similar to those fed formulas with protein from soy protein isolate. In most studies of human subjects, nitrogen balance has been similar with isonitrogenous diets supplying protein of animal origin or methionine-fortified soy protein isolate.

A 24-week study of six men in a metabolic ward failed to demonstrate adverse effects from consumption of a diet in which all of the protein was provided from soy protein isolate. It is estimated that 10 percent of infants in the United States receive formulas with protein from soy protein isolates during the early months of life, some consuming more than 4 g of soy protein per kg per day, without evidence of adverse effects. Several multigeneration studies of rats fed soy protein isolates have failed to indicate evidence of long-term toxicity. Thus, it seems likely that well-processed soy protein isolate adequately supplemented with methionine, vitamins and minerals, either by addition to the soy isolate product or as provided by other components of the diet, is without hazard.

Soy protein isolates subjected to relatively severe alkali treatments that modify their viscosity and adhesive properties are used as sizing and coating adhesives in the production of certain paper and paperboard products used in food packaging. These alkali treatments also result in marked loss of certain nutritionally essential amino acids and formation of lysinoalanine as a component of the protein molecule. Renal cytomegalic changes have been demonstrated in rats fed diets containing high levels (20 to 30 percent) of alkali-modified soy protein isolate as the sole source of protein. Alkali-treated casein and lactoalbumin at high levels in the diets of rats, and free lysinoalanine above certain levels, also produce cytomegalic changes. Removal of alkali-treated protein from the diet appears to reverse the changes. Cytomegaly was reduced or did not occur in rats in which alkali-treated protein was supplemented with an untreated protein suggesting that the renal syndrome was caused by protein bound lysinoalanine in diets deficient or
It is recognized that soy protein isolates constitute a currently minor source of nitrite as far as human exposure is concerned. Preliminary estimates of per capita exposure indicate that they account for less than 0.5 percent of the amount ingested as food ingredients, less than 0.05 percent of that taken in as food ingredients and present in the saliva; and less than 0.0075 percent of the total taken in as food ingredients, present in the saliva, and generated in the intestinal tract. From the standpoint of relative contributions to the controllable nitrite load and/or total body burden, soy protein isolates do not appear to be cause for concern at this time. Nevertheless, the possible adverse effects of nitrite call for more explicit knowledge and actions for maintaining a low level of the compound in the commercial product and for continued monitoring of its relative contributions, with adjustments as necessary, as the major sources of commercially added nitrites are progressively decreased through regulatory procedures underway.

The Select Committee has weighed the available information and concludes:

1. It is essential that specifications for food-grade soy protein isolates be established including provisions for acceptable levels of lysinoalanine, nitrite and nitrosamines.

2. Assuming that acceptable levels of lysinoalanine, nitrite and nitrosamines are established, there is no evidence in the available information on soy protein isolates that demonstrates or suggests reasonable grounds to suspect a hazard when they are used at levels that are now current or that might reasonably be expected in the future.

3. There is no evidence in the available information on soy protein isolates that demonstrates or suggests reasonable grounds to suspect a hazard when used in paper and paperboard products for food packaging at levels that are now current or that may reasonably be expected in the future.
VI. REFERENCES CITED


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Miles Laboratories, Inc., Elkhart, Ind. 46514.

Infant Formula Council, 64 Perimeter Center East, Atlanta, Ga. 30346.

Report submitted by:

July 30, 1979

George W. Irving, Jr., Chairman
Select Committee on GRAS Substances

- 43 -
PUBLIC HEARING ON SOY PROTEIN ISOLATES HELD ON SEPTEMBER 26, 1978*

Requests for a hearing were received from:

1. Food Protein Council, 1800 M Street, N.W., Washington, D.C. 20036
2. Ralston-Purina Company, Checkerboard Square, St. Louis, Mo. 63188
3. Central Soya Company, Inc., Ft. Wayne, Ind. 46802
4. Mead Johnson Nutritional Division, Evansville, Ind. 47721
5. Grain Processing Corporation, 1600 Oregon, Muscatine, Iowa 52761

All requests were withdrawn after submission of the announcement of the hearing for publication in the Federal Register. Ralston-Purina Company proposed to submit a written statement in lieu of an oral hearing presentation. The hearing was convened and the public was invited to make oral presentations of data, information and views concerning the safety of soy protein isolates as food ingredients that would supplement that available to the Select Committee as reflected in its tentative report. None requested opportunity to be heard.

VIII. APPENDIX

HEALTH ASPECTS OF NITRITES IN SOY PROTEIN ISOLATES

In evaluating the health aspects of nitrates contained in soy protein isolates there are three aspects to be considered: (1) the intrinsic biological properties of nitrite ion in relation to consumer exposure, (2) the possibilities of in vitro formation of N-nitrosamines by reaction of nitrite with components of soy protein isolate or ingredients of formulated foods that contain soy protein isolate and (3) the possibilities of in vivo formation of N-nitrosamines by reaction of nitrite, contributed by soy protein isolate, with substances present in foods ingested with the isolate or formed during digestion. The N-nitrosamines are of concern because a large percentage of those known has been found to be carcinogenic in experimental animals.

In this Appendix, information is presented on consumer exposure to nitrite contributed by soy protein isolate in relation to nitrite from other dietary sources and that formed in the gastrointestinal tract by reduction of ingested nitrate. Background information relating to possible nitrosamine from formation is also presented, followed by a review of the literature on the biological properties of nitrite and some of the nitrosamines.

Consumer exposure to nitrates

As noted in Section II of the report, soy protein isolate as currently produced may contain up to 50 ppm nitrite calculated as nitrite ion (A1-A4). Assuming a per capita daily consumption of 150 mg soy protein isolate based on estimated production (see Section III) the soy protein isolate would contribute up to about 7.5 μg nitrite to the average daily diet. Certain population groups, however, may consume considerably larger amounts of soy protein isolate. Two such groups are vegetarians who eat meat analogs fabricated from spun soy protein isolate, and infants fed formulas based on soy protein isolate. If the vegetarian received 48 g protein daily in the form of meat analog (about 240 g of analog containing 20 percent soy protein isolate and up to 10 ppm nitrite) a maximum of 2.4 mg (about 0.04 mg per kg body weight) nitrite daily would be contributed to the diet. It should be noted, however, that nitrite may be lost to the precipitating bath in the fiber spinning process and the nitrite content of the meat analog would be correspondingly lower.

As discussed in Section II, about 10 percent of U.S. infants during their first few months of life are fed commercially prepared formulas with protein from soy protein isolate containing 1.8 to 2.6 g protein per 100 ml. These formulas might contain up to 1.3 ppm nitrite contributed by soy protein isolate. Also, as discussed earlier, the 50th and 90th percentile intakes of
soy protein isolates by 1 to 2 month old infants, whose total energy intake was provided by a soy-based formula, are estimated to be 4.8 and 5.8 g per kg, respectively. Intake of nitrite (assuming 50 ppm nitrite) would be about 0.25 mg per kg body weight at the 50th percentile intake and 0.30 mg per kg body weight at the 90th percentile intake. However, the Infant Formula Council (A5) has conducted a survey of infant formula manufacturers who reported results of testing infant formulas and a number of the soy protein isolates used in these formulas. The soy protein isolates analyzed contained no more than 6 ppm nitrite. Nitrite contents of soy isolate formulas, ready to feed basis, were less than 0.12 ppm and were comparable to milk-based formulas in this respect. On basis of these concentrations, nitrite intake of 1 to 2 month old infants consuming such formulas would be tenfold or more less than those calculated above. Human milk was reported to contain less than 0.1 ppm nitrite.

The nitrite level in a consumer food product contributed by soy protein isolate will depend on the level of incorporation and nitrite content of the isolate. The U.S. Food Protein Council (A6) has listed maximum practical use levels of soy protein isolate in a number of food product categories (Table IA). On the basis of 50 ppm as the maximum nitrite content in soy protein isolate, food products in five categories might contain 10 ppm and those in one category, egg product analogs, 25 ppm.

White (A7) has discussed the relative significance of dietary sources of nitrates and nitrites. The major sources of nitrites in the average diet are vegetables, cured meats and breads. These, and drinking water, also are the principal dietary sources of nitrates. Of the vegetables, beets contain the highest concentration, 6.0 ppm of nitrite; spinach contains 2.7 ppm, corn 2.0 ppm, tomatoes 1.3 ppm, lettuce, onions, green beans, carrots, peas, celery, cucumbers, sweet peppers, pumpkin, lima beans, egg plant, broccoli, cauliflower, asparagus and squash contain 0.4 to 1.1 ppm. Based on compositional and per capita disappearance data, White calculated a daily per capita intake of 0.2 mg from vegetables. From literature values of the nitrite content of cured meats, an average value of 52.5 ppm was calculated for nitrite in these products. Based on this value and disappearance data, a per capita daily contribution of 2.4 mg nitrite was calculated for cured meats. Breads were reported to have an average nitrite content of 0.17 ppm and to contribute 0.02 mg nitrite to the daily per capita intake. White estimated total direct intake of nitrite from ingested foods to be 2.6 mg daily. A similar value, 3.3 mg, for the daily nitrite intake was reported by Selenka and Brand-Grimm (A8) for German Federal Republic residents. Recent action of USDA (A9) reducing the amount of nitrite permitted to be added to bacon will reduce White's estimate of the intake of nitrite from cured meat products.

Per capita daily nitrate intake was estimated to be 99.8 mg. Vegetables are the major dietary source of nitrates, accounting for about 86 percent of the nitrate intake in the United States (A7).
<table>
<thead>
<tr>
<th>Food Products*</th>
<th>Maximum Use Levels (%)**</th>
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<tr>
<td></td>
<td>(on an as is basis before hydration)</td>
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<tr>
<td>Baked Goods</td>
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<tr>
<td>Beverages, alcoholic</td>
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<td>Soups and Soup Mixes</td>
<td>5</td>
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<tr>
<td>Sweet Sauces, Toppings and Syrups</td>
<td>5</td>
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*See Code of Federal Regulations (2), 21 CFR 170.3(n).
**Different foods within a given category may vary widely as to moisture content.
Saliva is an important additional source of nitrite entering the stomach. Tannenbaum et al. (A10) estimated the salivary contribution to be 6 to 10 mg daily. This value may be low since it was based on the nitrite content of morning saliva (A11) and it has been shown (A12-A15) that nitrite in saliva significantly increased for 4 to 6 hours or longer after ingestion of meals containing high nitrate vegetables. Tannenbaum (A16) recently estimated formation of salivary nitrite to range from 10 to 20 mg daily with an average of 15 mg. If saliva is assumed to provide 15 mg nitrite per day, the total daily intake of nitrite by the stomach from dietary (2.6 mg) and salivary sources would be about 18 mg (about 0.3 mg per kg in adults).

The FAO/WHO Expert Committee on Food Additives estimate of acceptable daily intakes for sodium or potassium nitrites is up to 0.4 mg per kg body weight, unconditional acceptance, and 0.4 to 0.8 mg per kg, conditional acceptance (A17).

**Nitrite levels in saliva**

Although parotid ductal saliva is free of nitrite, nitrite is present in saliva in the mouth and has been shown to be formed by microbial reduction of nitrate naturally present (A10,A18). Tannenbaum et al. (A10) reported that nitrite concentration in the saliva of about 100 people of various age, sex and race was 6 to 10 ppm. Nitrite content did not appear to be strongly influenced by the composition of the meal. Ishiwata et al. (A18) found levels of 2.3 to 18.2 ppm (average 8.3 ppm) nitrite and 24.9 to 148.6 ppm (average 68.9) nitrate in saliva of eight adults. The saliva of one male subject contained 10 ppm nitrite and 119.5 ppm nitrate before ingestion of 200 mg sodium nitrate in 200 ml water and 40.9 and 227.6 ppm, respectively, 2 hours after ingestion. After 6 hours both had decreased to pre-ingestion levels.

In other investigations, Ishiwata and coworkers (A12,A13) found a marked increase in the nitrite and nitrate contents of whole saliva after the ingestion of vegetables containing relatively high levels of nitrate such as salted cabbage, lettuce, spinach or dried cut radish. They reported, for example, that addition of 152 mg nitrate and 0.2 mg nitrite in the form of salted cabbage to one meal of a basal daily diet containing 44 mg nitrate and 3.2 mg nitrite increased the total daily salivary content of nitrate from about 26 (basal diet) to 96 mg and nitrite from 3.7 (basal diet) to 15.8 (assuming 1 liter of saliva secreted per day). Determinations of nitrite and nitrate were made on saliva collected at intervals of 1 to 2 hours except at night. Maximum level of nitrite (25 ppm on the first day and 50 ppm the second day) had been reached 1 to 2 hours after ingestion of the salted cabbage.

In studies of Tannenbaum et al. (A14) average salivary nitrite concentration for 14 individuals reached a maximum of 370 ppm (standard deviation, 115 ppm) 2 hours after ingestion of 200 ml celery juice (240 mg sodium nitrate), then decreased in approximately exponential fashion over 24 hours to the initial base level. Response of two individuals indicated the
maximum concentration of nitrite in saliva was strongly dependent on nitrate intake up to approximately 100 mg sodium nitrate, and relatively independent at higher levels of intake. Salivary nitrite concentration also was dependent on concentration of nitrate in the solution ingested, increasing with concentration at a fixed nitrate intake.

Spiegelhalder and coworkers (A15) reported an average nitrite concentration of 9 ppm (standard deviation, 5 ppm) for 11 adults 1 to 2 hours after a normal continental-type breakfast. A maximum average value of about 90 ppm was reached 1 to 1.5 hours after ingestion of 250 ml beet juice containing 450 mg nitrate. From measurements of salivary nitrite concentrations after ingestion of 30 to 550 mg nitrate in 22 vegetables or vegetable juices, it was concluded that salivary nitrite increased 0.2 ppm per mg of nitrate ingested. The data indicated a threshold dose of about 50 mg nitrate, below which salivary concentrations of nitrate and nitrite remained unchanged.

In study of a group of volunteers (number not stated), Waters et al. (A19) also observed that the salivary nitrite concentration was affected by the quantity of dietary nitrate ingested. Consumption of 70 g lettuce containing 56 to 91 mg potassium nitrate resulted in an increase in salivary nitrite concentration from 4.4 ppm prior to the ingestion to 6.8 ppm immediately after the meal. Subsequent mean values at 2, 4, and 6 hours were 5.3, 3.7, and 3.1 ppm, respectively.

Ruddell et al. (A20) found a mean nitrite concentration of about 4.6 ppm in the fasting saliva of 19 human subjects consisting of nine patients undergoing gastric examination and ten normal volunteers. The salivary nitrite concentration of ten smokers was not significantly different from that of nine non-smokers. Thiocyanate, a catalyst for the reaction of nitrite with amines to produce nitrosamines, was detected in 18 specimens of saliva examined at a mean concentration of 1.6 ± 0.3 mM. The salivary thiocyanate of nine smokers, 2.3 ± 0.4 mM, was significantly greater than that of nine non-smokers, 0.9 ± 0.1 mM.

Nitrite levels and pH of gastric contents

Ruddell et al. (A20) analyzed fasting gastric juice of 17 patients for nitrite and thiocyanate before and after pentagastrin stimulation. Ten patients had chronic duodenal ulcers, three had chronic gastric ulcers including one with additional duodenal ulcer, and in five patients no evidence of gastro-duodenal ulcer was found. Nitrite was present in all 17 samples of fasting gastric juice at a mean concentration of 4.9 ± 1.1 μM (0.2 ppm). The mean concentration in 12 smokers did not differ significantly from five non-smokers. Pentagastrin stimulation of gastric secretion increased the volume of gastric juice from a mean basal level of 21.2 ml to a maximum of 88.8 ml per 15 minutes but did not cause a significant change in mean nitrite concentration.
Since others had shown that pentagastrin did not cause increased salivary flow, the authors proposed that active gastric secretion of nitrite could explain their results. In contrast, the decreased concentration of thiocyanate in gastric juice following pentagastrin stimulation suggested that it was of salivary origin.

In another study, Ruddell et al. (A21) determined nitrite, pH, and total and nitrate reductase positive organisms per ml of the fasting gastric juices collected from 69 patients. Of 30 patients who had no demonstrable gastroduodenal lesion, the gastric juice of 18 had a pH <2.5 whereas gastric juice of 12 had a pH >5. The remaining 39 patients had duodenal or gastric ulcer or gastric cancer. Of the 69 specimens of fasting gastric juice, 20 had a pH >5 and a mean nitrite concentration of 30.4 ± 4.9 μmol per liter (1.4 ppm). The remaining 49 specimens had a pH <2.5 and a mean nitrite concentration of 1.7 ± 0.3 μmol (0.08 ppm). The log₁₀ count of nitrate reductase positive organisms per ml in the 20 specimens having a pH >5 was 5.3 ± 0.5 and was significantly higher than in the remaining 49 specimens, 3.4 ± 0.4. High nitrite concentrations were related to the presence of fasting hypochlorhydria, irrespective of gastric lesion. It was noted that the bacterial flora of the stomach with nearly neutral pH would be metabolically active and capable of nitrate reduction. Nitrate derived from saliva was considered to be the most likely source of gastric nitrite and, in near neutral gastric juice, the nitrite entering the stomach in swallowed saliva would be supplemented by additional nitrite formed by intragastric bacterial reduction of salivary nitrate.

Methemoglobinemia in infants that had ingested water containing high nitrate concentrations has been attributed to the bacterial reduction of nitrate to nitrite. Only infants, and generally those less than 3 months old, whose gastric juice has a pH greater than 4 and who have nitrate-reducing bacteria in the upper gastrointestinal tract are considered susceptible (A22). There is no clear evidence that nitrates in foods have been responsible for methemoglobinemia. However, illness and deaths of infants have been attributed to nitrite formed by bacterial reduction of nitrates in improperly stored home-prepared spinach purees (A23).

Walters et al. (19) determined the pH and nitrite concentration of the gastric contents of a human volunteer before and after intubation of a homogenized meal containing 38 ppm nitrite. The meal consisted of luncheon meat (80 g), one fried egg (40 g), bread (32 g), butter (16 g), cheese (22 g), biscuits (17 g), and milk (200 ml). Samples were withdrawn at intervals up to 88 minutes. Both pH and gastric nitrite level rose rapidly with maximum values (pH about 5 and nitrite concentration about 14 ppm) occurring approximately 40 minutes after intubation of the meal. Thereafter, both fell rapidly and at 85 minutes the pH was 3.2 and the nitrite concentration about 4 ppm. Through use of phenol red, which is not absorbed from the stomach, as a marker, it was found that an average of 85 percent of food slurries could be
recovered after residence times up to 30 minutes in the stomach.

In a similar study Klein et al. (A24) gave seven human subjects, 25 years of age, meals consisting of 45 g milk powder, 45 g glucose, 22.5 g corn oil, 9.9 g polyethylene glycol (PEG) and 112.5 g sodium nitrate. The PEG served as an inert marker for determination of dilution effects. Two individuals swallowed their saliva after intubation of the meal whereas the other five individuals did not. The mean pH of samples of gastric contents of all seven individuals taken 30 minutes after intubation was 5 ± 1. It then gradually decreased to pH 3 at 2 hours. Gastric nitrite level of the individuals who did not swallow their saliva was similar to that contained in the meal (about 1 ppm), decreasing slightly during 2 hours after intubation of the meal. In contrast, the gastric nitrite level in the individuals who swallowed their saliva was 5 ppm at 30 minutes and increased to 7 ppm (standard deviation, 3 ppm) at 90 minutes.

Nitrite and nitrate formation in the intestine

A recent study of nitrate balance in humans and analyses of fecal and ileostomy samples indicate that nitrate and nitrite are formed in the intestine, possibly by heterotrophic nitrification of ammonia or organic nitrogen compounds (A25). Samples of ileostomy effluent from six adult males collected approximately 3 hours after breakfast contained 350 to 1540 μmole NO₂ per kg (16 to 71 ppm NO₂). Fecal samples from eight adult males eating free choice, Western-style diets contained 48 to 425 μmole NO₂ per kg (2.2 to 20 ppm NO₂). Urinary nitrate output of six adult males receiving a protein-free diet or a diet containing 0.8 g egg protein per kg body weight was much greater than intake. On the former diet average daily urinary output expressed as NaNO₃ was 1265 μmoles (107 mg) compared to an average intake of 67 μmoles (5.7 mg); on the latter diet the values were 1179 and 126 μmoles (100 and 10.7 mg), respectively, indicating endogenous synthesis of nitrate. Daily production of nitrite in the intestine has been estimated to range from 60 to 100 mg with an average 90 mg (A16).

Nitrosatable compounds found in foods, drugs, and pesticides

Nitrite can react with many types of nitrogen-containing compounds to form N-nitroso compounds; included are secondary and tertiary amines, quaternary ammonium compounds, amides, ureas, carbamates and guanidines (A26). In a recent review, Magee et al. (A27) listed more than 100 such nitrosated derivatives of which more than 80 percent are carcinogenic in experimental animals.

Nitrosatable compounds may be present in a number of food products either as natural constituents or as inadvertent contaminants. Secondary amines have been identified in fish, vegetables, beer, wine, coffee, tea, and other products (A28, A29). Dimethylamine (45 ppm in herring in oil) and
piperidine, for example, occur in herring; pyrrolidine in Tilsiter (19.9 ppm) and Camembert (1 ppm) cheese; dimethylamine (4 ppm), di-n-propylamine (0.4 ppm) and pyrrolidine (0.3 ppm) in brown bread; and dimethylamine (4 ppm), piperidine (2 ppm) and pyrrolidine (10 ppm) in coffee extract. N-methylphenethylamine was found in samples of cabbage, cauliflower, kale, radish, celery, spinach, beets and carrots. Dimethylamine also was found in the five first vegetables named and pyrrolidine in spinach, radish and celery. Concentrations of these amines in vegetables were below 10 ppm except in case of dimethylamine in cauliflower (14 ppm) (A28, A29). The aliphatic polyamines spermidine and spermine are widely distributed in plant and animal products, e.g., spinach (34.5 and 7.7 ppm), apples (9.8 and 2.2 ppm), wheat germ (254 and 41 ppm), barley germ (291 and 128 ppm), soybean flour (16.4 and 5.7 ppm), fresh pork (up to 1250 and 560 ppm) and processed ham (up to 1270 and 800 ppm, respectively) (A30). All the amines named in this paragraph yield carcinogenic N-nitrosamines (A27). Spermidine and spermine yield several nitrosamines on reaction with nitrite, including nitrosopyrrolidine, a carcinogen (A31).

L-citrulline, a urea derivative, occurs in watermelon (50 ppm), green peppers (350 ppm) and soy sauce (1300 ppm). Naturally-occurring guanidines include methylguanidine (fresh beef, cod, sardines and sharks at concentrations ranging 60 to 1900 mg per kg fresh weight), arginine, creatine, creatinine, phosphocreatine and canavanine (A32).

Naturally occurring quaternary ammonium compounds including neurine, choline, acetylcholine, betaine, and carnitine have been demonstrated to yield N-nitrosodimethylamine on reaction with nitrous acid (A33).

N-nitrosation at the ring nitrogen atom has been reported for proline (A27, A34-A36), N-acetyltryptophan, prolylglycine, and N-acetyltryptophylglycine ethyl ester (A34, A36). N-nitrosopropylamine was not carcinogenic in animal feeding studies (A37, A38). However, heating nitrosopropylamine at 170°C for 10 minutes produced the carcinogen nitrosopyrrolidine (A39, A40). No feeding studies appear to have been conducted on the nitrosated tryptophan derivatives or nitrosated prolylglycine. Arginine, a guanidine derivative, reacts with nitrous acids but no stable products have been isolated and, in particular, no nitroso derivative has been identified (A41). After reaction under strongly acid conditions, butanol extracts showed ultraviolet absorption maxima indicating the formation of N-nitrosoureas (A42). Concurrent administration of arginine and nitrite to mice have not demonstrated carcinogenicity (A37).

Many orally administered drugs contain secondary or tertiary amino groups and N-nitroso compound formation has been demonstrated for more than 30 drugs by reaction with nitrite in vitro (A26, A43-A45). Mirvish (A26) lists ten pesticides (ureas, carbamates or N, N-disubstituted amides) that...
have yielded N-nitroso derivatives. About two-thirds of the nitrosated drugs and one-half of the nitrosated pesticides were carcinogenic in animal feeding tests (A26,A43-A45).

**Kinetics of nitrosation**

The quantity of a nitrosamine or other nitroso-compound formed in a food or in the stomach will depend, in part, on the rate equation for the nitrosation reaction. Reaction of most secondary amines with nitrite follows the equations:

\[ 2\text{HNO}_2 + \text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow \text{N}_2 + \text{H}_2\text{O} \]

\[ \text{R}_2\text{NH} + \text{N}_2\text{O}_3 \rightarrow \text{R}_2\text{N}^+\text{NO}^- + \text{HNO}_2 \]

rate = \[ k_1 [\text{R}_2\text{NH}] [\text{HNO}_2]^2 \] (equation 1)

where \([\text{R}_2\text{NH}]\) is the concentration of nonionized amine, and \([\text{HNO}_2]\) the concentration of undissociated nitrous acid. The rate constant, \(k_1\), is independent of pH. In terms of total concentrations of amine and nitrite, the rate equation becomes:

rate = \[ k_2 [\text{amine}] [\text{nitrile}]^2 \]

where the rate constant \(k_2\) is dependent on pH because the concentrations of the reactive species depend on pH. The basicity of the amine influences the concentration of nonionized amine present at a given pH; at optimum pH and 25°C the rate constants for 14 secondary amines varied from 0.027 moles\(^{-2}\) \(1^2\) min\(^{-1}\) for piperidine to 5000 moles\(^{-2}\) \(1^2\) min\(^{-1}\) for piperazine, increasing as the basicity of the amine decreased. Optimum pH was in the range 2.5 to 3.4 (A26).

Rate constants, \(k_2\), for two secondary amines, dimethylamine and pyrrolidine, that have fairly widespread occurrence in foods are 0.10 and 0.32 mole\(^{-2}\) \(1^2\) min\(^{-1}\), respectively. Using these rate constants and assuming concentrations of 50 ppm for nitrite, 10 ppm for dimethylamine and piperidine and a pH of about 3 as might occur in the stomach, the calculated yields of N-nitrosodimethylamine and N-nitrosopiperidine per 1000 ml are 0.16 and 0.5 µg, respectively, assuming a residence time of 3 hours. If the nitrite concentration were 10 ppm, yields would be reduced 25-fold to 0.006 and 0.02 µg, respectively. An increase of x percent in the nitrite concentration in the gastric contents contributed by a component of the food would result in x² percent increase yield of nitrosamine.

Reaction kinetics of few tertiary amines appear to have been investigated. For the nitrosation of trimethylamine at 25°C Mirvish (A26) reported
\( k_3 \) as \( 1.2 - 2.4 \times 10^{-5} \) mole\(^{-2}\)1\(^2\) min\(^{-1}\) (assuming the reaction obeys equation 1), and hence is about 1000-fold lower than \( k_3 \) for the less reactive secondary amines.

Nitrosation of 21 amides including several ureas, carbamates and guanidines have been shown to obey the rate equation:

\[
\text{rate} = k_3 [\text{amide}] [\text{nitrite}] [H^+] 
\]

where \( k_3 \) varies from 0.084 mole\(^{-2}\)1\(^2\) min\(^{-1}\) for N-methylbenzamide to \( 2.4 \times 10^2 \) for ethylene urea at pH 2 and 25°C. Rate constants, \( k_3 \), for the naturally occurring amides, methyl guanidine and DL-citrulline, are 0.24 and 43.2, respectively. Calculated yields of the nitroso derivatives in solutions containing 10 ppm of the amides and 50 ppm nitrite after 3 hours at pH 2 and 25°C are 4.2 μg/1 of methylguanidine and 780 μg/1 of DL-citrulline.

Quaternary ammonium compounds yield nitrosamines on reaction with nitrite at elevated temperatures although in relatively low yields. Formation of dimethylnitrosamine from several quaternary ammonium compounds after reaction at pH 5.6 and 78°C for 4 hr was demonstrated by Fiddler, et al. (A33). Naturally occurring compounds investigated included neurine, carnitine, betaine, choline and acetylcholine. The greatest yield of dimethylnitrosamine, formed by tetramethylammonium chloride, was about 6 percent as much as was formed from an equal quantity of dimethyamine when treated under similar conditions. Lecithin contains choline as a component and yields dimethylnitrosamine on reaction with nitrite in aqueous solution (A46, A47). Pensabene, et al. (A47) treated 4.56 mmol bovine, egg, edible soy, vegetable or synthetic lecithin with 22.8 mmol sodium nitrite in 65% aqueous solution at pH 5.6 and 78°C for 4 hr; yields of dimethyamine were 30.76, 5.4, 2.05, 1.02 and 319.7 mg per kg of compound for the respective lecithins. For comparison, treatment of dimethyamine under similar conditions yielded 157.6 g per kg. Free dimethyl- and trimethylamines were found in all lecithin samples and probably contributed to the yield of dimethylnitrosamine.

Thiocyanate, bromide and chloride increase the rate of nitrosation of morpholine, N-methylaniline, aminopyrine and sarcosine (A26, A48, A49). The order of catalytic activity is NCS\(^-\)>>Br\(^-\)>>Cl\(^-\). The reaction rate for sarcosine was increased by a factor of 400 at pH 0.5, 100 at pH 1.5 and 10 at pH 2.5 by addition of thiocyanate under certain reaction conditions (A49). Formaldehyde and chloral, but not other carbonyl compounds, catalyzed the nitrosation of various secondary amines in neutral and alkaline media (pH 6 to 11) (A50).
Ascorbic acid, gallic acid, tannic acid, sodium sulfite, glutathione, cysteine, urea and ammonium sulfamate have been reported to inhibit the nitrosation reaction in aqueous solution (A26, A51-A54). For example, 20 mM concentrations of sodium ascorbate, gallic acid, sodium sulfite or cysteine, or 4 g per liter tannic acid, reduced the yield of nitrosomorpholine in aqueous solutions, 10 mM in morpholine and 20 mM nitrite, by 87, 97, 86, 95, and 66 percent, respectively, after 90 minutes reaction at pH 3 and 25°C. At the same concentration of the blocking agents and amine, but 10 mM in nitrite concentration, nitrosation of piperazine was reduced 100, 100, 100, 58, and 37 percent, respectively, after reaction at 25°C for 10 min (A26).

Ascorbic acid (500 mg) or glutathione (349 mg) reduced the yield of mononitrosopiperazine in an aqueous solution containing 1 g piperazine adipate and 1 mg sodium nitrite from 1200 μg in the control to 158 and 100 μg, respectively, after reaction for 2 hr at 37°C and pH 2 (A53).

Formation of N-nitrosamines in vivo

Sen et al. (A55) detected diethylnitrosamine (DEN) in the stomach contents of rabbits (100 to 200 μg) and cats (68 μg) that had been administered 450 mg diethylamine hydrochloride and 300 mg sodium nitrite by stomach tube (rabbits) or in gelatin capsules (cats). The animals had been fed 20 to 25 g of food and were killed and the stomach removed 20 to 30 minutes after administration of the amine and nitrite. The pH of the stomach contents extracted with 100 ml water was 3.8 and 4.8 in two experiments with the rabbits and 5.9 in the one experiment with a cat.

Sander et al. (A56) reported 17 to 30 percent of theoretical yield of diphenylnitrosamine in the stomach contents of rats that had been fed diets containing 0.01 percent diphenylamine and 0.15 percent sodium nitrite. Stomach contents were removed 0.5 to 3 hr after feeding. A yield of 17 μg N-nitroso-N-methylaniline per g stomach contents (about 4 percent of theoretical) was obtained when rats were fed diets containing 0.03 percent N-methylaniline and 0.15 percent sodium nitrite. Stomach contents were removed 2 hr after feeding. Diethylnitrosamine was not detected in the stomach contents of rats 2 hr after feeding diets containing 0.3 percent diethylamine and 0.3 percent sodium nitrite.

After injection of piperidine and nitrite, Alam et al. (A57) detected nitrosopiperidine in rat stomachs and small intestines isolated by ligatures but with vasculature intact. Solutions (total volume 10 ml) of sodium nitrite and piperidine hydrochloride were injected into the intestines, incubated 40 minutes and contents withdrawn. No nitrosopiperidine was detected when 125 mg piperidine and 25 mg sodium nitrite were injected but yields of 5 to 25 μg were found when 625 mg of piperidine and 25 mg sodium nitrite were administered. The pH of intestinal contents was 6.5. Employing similar methodology and concentrations of reactants, yields of 12 to 24 μg nitrosopiperidine were found in stomach contents which had pH values of 3.0 to 4.0.
Sander et al. (A58) detected no dinitrosopiperazine (DNPiZ) in the stomachs of five rats 30 minutes after intragastric administration of 9.7 mg piperazine hexahydrate (neutralized with HCl). DNPiZ (13 μg) was found when 0.86 mg of sodium nitrite was given simultaneously with the amine. Yield of nitrosamine increased rapidly with nitrite concentration; about 750 μg DNPiZ was isolated when 3.44 mg sodium nitrite (an equimolar quantity) was administered with the amine. Recovery experiments with DNPiZ indicated that 16 percent of the compound remained in the stomach 30 minutes after gastric intubation.

Sander et al. (A58) also investigated the formation of DNPiZ in rats when piperazine and nitrite were fed as components of the diet by measuring DNPiZ excreted in the urine. Nitrosamine yield, as percentage of theoretical, was based on recovery experiments in which a linear relation was found between the dose of DNPiZ ingested as a component of the feed and the amount excreted in the urine (about 0.2 percent of the dose) over an 8 hr period. Less than 1 percent of the amine was nitrosated when doses of 5 mg sodium nitrite (about 1000 ppm in the feed) and 6.7 mg piperazine (about 1300 ppm in the feed) per rat were ingested by 20 rats as components of 5 g of feed. Doubling the level of nitrite in the feed increased the yield of DNPiZ to 4 percent and quadrupling it increased the yield to 20 percent.

Mysliwy et al. (A59) demonstrated the formation of N-nitrosopyrrolidine in the stomachs of two dogs after administration via indwelling gastric fistula of 50 ml of an aqueous solution containing 1000 ppm sodium nitrite and 200 ppm pyrrolidine at 37°C and pH 11.2 (unbuffered). Nitrosopyrrolidine was positively identified (combined gas chromatography-mass spectrometry) after one minute and rose to a maximum concentration of 0.96 ppm after 2.5 minutes in dog A and 0.12 ppm after 7 minutes in dog B. After 30 minutes the concentration in dog B had dropped to 0.01 ppm. Over a period of 30 minutes the pH fell from an initial value of about 4 to a final value of about 2. The nitrite concentration decreased to about 10 percent of its initial value in the same time period. In a control experiment, less than 0.001 ppm nitrosopyrrolidine was formed when nitrite and pyrrolidine were allowed to react for 30 minutes in water under conditions similar to those found in the stomach of dog B (364 ppm nitrite, 72 ppm pyrrolidine, and pH 3 — averages over 30 minutes). The higher yields in the dog's stomach indicated pronounced catalytic effects on the rate of nitrosation.

Telling et al. (A60) found 0.006 μg nitrosodimethylamine (NDMA) in the stomach contents of Wistar rats (approximately 100 g body weight) that had been fed 5 g of food and killed 1 hr later. Analysis of the drinking water showed 30 ppm nitrate and 0-0.5 ppm nitrite; the diet contained 70 ppm nitrate and 2 ppm nitrite (nitrate as KNO₃ and nitrite as NaNO₂). Addition of 1000 ppm sodium nitrite to the drinking water resulted in no increase in NDMA or nitrosopyrrolidine (NPY) in the stomach contents. However, concurrent addi-
tion of 500 ppm dimethylamine to the diet resulted in 0.005 µg increase in NDMA in the stomach contents. Increase in NPy content resulted when 1000 ppm pyrrolidine was added to the diet and 1000 ppm sodium nitrite added concurrently to the drinking water. Variation of nitrite concentration in the drinking water (up to 1000 ppm) and dietary amine concentrations showed that the latter had greater effect on nitrosamine formation in contradiction to the theoretical rate equation which predicts nitrosamine formation to be proportional to the square of the nitrite concentration.

The in vivo formation of N-nitrosopiperidine in the urinary bladder was demonstrated in rats with experimental Escherichia coli bladder infections (A61). Sprague-Dawley rats were given water containing 5 mg per ml sodium nitrate to drink ad libitum. Approximately 90 percent of the nitrate consumed was recovered in the urine. After 4 days of nitrate consumption, 500 µg of piperidine hydrochloride was administered by stomach tube to 6 rats with bladder infection on 2 successive days and urine was collected for 24 hr after each dose. Urine also was collected from 2 control, uninfected rats treated as above and from 2 infected rats given distilled water to drink instead of nitrate solution. Urine from 3 of the 6 test animals contained 0.2 µg of nitrosopiperidine by gas chromatography-mass spectrometry analysis, whereas the urine of all 4 control animals was negative.

Lakritz et al. (A62) found nitrosamines in the gastric contents of 6 of 35 patients who had fasted at least 8 hr prior to sample collection. Clinical diagnosis of the patients involved disorders of the gastrointestinal tract including duodenal ulcer - 8; gastric ulcer - 1; peptic ulcers - 2; ulcers - 5; esophagitis, duodenitis, and gastritis - 4. Information on medication or other treatments was not reported. Diethylnitrosamine was found in samples from 3 patients at concentrations of 5-30 µg per kg, dimethylnitrosamine in two patients (in one after pentagastrin or histamine stimulation) at 2 µg per kg and nitrosopyrrolidine in one patient after gastric secretion stimulation at 6 µg per kg concentration. Nitrosamines were detected in the gastric contents of an additional four patients at concentrations too low to confirm by mass spectrometry. The pH of the gastric contents containing nitrosamines ranged from 1.5-2.9 with one sample at 6.4-7.5. Nitrite concentrations were determined in three samples of gastric contents that contained nitrosamines. Two of the samples from the same patient (before and after gastric secretion stimulation) contained approximately 70 mg per kg whereas nitrite was not detected in the third sample. Volatile amines found in pooled gastric contents included dimethylamine, trimethylamine and histamine; nonvolatile amines were cadaverine, putrescine, ethanolamine and tryptamine.

Tumor induction by feeding nitrite and amines, amides and ureas

In the rat, malignant tumors developed following chronic feeding of nitrite and several amines or amides: morpholine, N-methylbenzylamine,
methyl urea, N,N'-dimethylurea, ethyl urea, 2-imidazolidinone, heptamethyleneimine, methylcyclohexylamine, N-methylalaniline, aminopyrine, oxytetracycline, chlordiazepoxide, dimethyldodecylamine, and methapyrilene (A63-A73). In mice, an increased incidence of lung tumors resulted from oral administration of sodium nitrite and various amines and amides including piperazine, morpholine, N-methylaniline, methylurea and ethylurea (A74-A76).

Levels of nitrite and amine or amide ingested in the feeding studies were generally many times greater than would be expected in human experience. Sander and Bürkle (A64), for example, fed Sprague-Dawley rats (120 g body weight) diets containing 0.5 percent sodium nitrite and 0.5 percent (about 500 mg per kg) morpholine or N-methylbenzylamine for 8 weeks. Thereafter, the basal diet was fed until the animals died at 150-234 days. In studies with heptamethyleneimine, Taylor and Lijinsky (A66) gave 8-10-week old Sprague-Dawley rats 20 ml of drinking water containing either 2000 ppm heptamethyleneimine hydrochloride, or this salt together with 2000 ppm sodium nitrite, 5 days a week for 28 weeks. Most females were dead at 50 weeks and most males were dead at 80 weeks, 27 of 30 having tumors not seen in the control groups. A similar protocol was followed in rat feeding tests on 13 secondary and tertiary amines with the exception that the rats received the experimental compounds for 50 weeks (A72). However, feeding tests at low dose levels were conducted by Shank and Newberne (A77) in a dose-response study of the carcinogenicity of morpholine. Dietary levels of nitrite and morpholine ranging from 5 ppm to 1000 ppm of each were fed to rats and hamsters. Hepato-cellular carcinoma and angiosarcoma in some rats resulted at the lowest dose level.

Carcinogenic effects were not observed in rats when mixtures of sodium nitrite at 0.05 percent concentration and the secondary amines pyrrolidine, piperidine, piperazine, morpholine or heptamethyleneimine at 0.025 percent concentration in the diet were fed to rats for 75 weeks (A78). Presumably, insufficient nitrosamine was formed under these conditions.

The inhibition of adenoma induction in mice fed amines or ureas in their diet and sodium nitrite (1-2 g per 1) in drinking water by incorporation of sodium ascorbate, gallic acid or caffeine in the diet was demonstrated by Mirvish et al. (A79). At a level of 23 g per kg in the diet, sodium ascorbate reduced adenoma yield induced by piperazine (6.25 g per kg in diet) 91 percent and by morpholine (6.33 g per kg in diet) 89 percent; 11.5 g per kg of sodium ascorbate reduced adenoma yield induced by methylurea (2.68 g per kg in diet) 98 percent. Gallic acid (21.8 g per kg of diet) reduced the yield of adenomas induced by morpholine (6.35 g per kg of diet) 86 percent. A 65 percent reduction in adenoma yield resulted when 1 g per kg caffeine was incorporated in the morpholine diet.
Sodium ascorbate, propyl gallate, and tert-butylhydroquinone (TBHQ) at doses of 200-225 mg per kg body weight inhibited nitrosamine formation in rats intubated with sodium nitrite (125 mg per kg body weight) and dimethylamine (1000 mg per kg) as measured by activities of serum enzymes and the extent of hepatic necrosis. Butylated hydroxyanisole and butylated hydroxytoluene were ineffective at all dose levels tested (A80).

Tumor induction by feeding nitroso-compounds: levels fed

Druckery et al. (A81) investigated the tumor induction of 65 nitroso-compounds in feeding studies with BD rats. The experimental compounds were generally fed at daily dose levels of 2.5 and 5 percent of the LD50 either in the drinking water or mixed into the feed. Lowest oral dosage of any of the compounds fed which resulted in malignant tumors was 0.075 mg per kg per day (about 1.5 ppm in the diet) of diethylnitrosamine. The mean induction time for the appearance of tumors was 840 days. Dosages in mg per kg of some other N-nitroso-compounds which induced carcinomas were: -dimethylamine, 4; -di-n-propylamine, 4; -di-n-butylamine, 10; -pyrrolidine, 5; -piperidine, 5; -morpholine, 5; and n-dinitroso-piperazine, 4.

Biological properties of nitrite

Acute toxicity. Riemann (A82) reported an LD50 of 214 mg per kg body weight for sodium nitrite administered orally to white mice. Druckery et al. (A83) found the oral LD50 for sodium nitrite was 77 mg per kg for 1 year old rats and 110 mg per kg for rats 3 months of age. LD50 values of approximately 250 and 300 mg per kg for sodium and potassium nitrite, respectively, in mice and 120 and 170 mg per kg, respectively, in rats were reported by Ichikawa et al. (A84). The mean lethal oral dose of sodium nitrite for adult humans has been estimated as 1-2 g. Signs and symptoms include intense cyanosis, nausea, vertigo, vomiting, collapse, tachypnea, coma, convulsions and death. Basic actions of sodium nitrite in vivo are the relaxation of smooth muscle, especially of small blood vessels, and in toxic doses, the conversion of hemoglobin to methemoglobin. Sodium nitrite, 0.064 g, delays the emptying time of the stomach by an average of 24 percent, but with marked individual variation (A85, A86).

Long-term studies. Sodium nitrite (100 mg per kg body weight) was administered daily in drinking water to BD rats over the life span of three successive generations (A83). Treatment of 30 rats (15 males and 15 females) comprising the P generation was begun when the rats were about 70 days old. Rats of the P and F generations were mated at 6 months of age; 30 and 34 weanlings were saved from the F1 and F2 generations, respectively, for life-span treatment with sodium nitrite. Growth rate of the treated rats was about 10 percent less than that of controls. Mean life span for the P, F1 and F2
generations was 630, 625 and 610 days, respectively, as compared to 730 days for untreated rats. Reproduction was normal as judged by litter size and no teratogenic effects were observed. Erythrocyte count and hemoglobin concentration were within normal limits for BD rats. Organ weights and gross examination of the liver, kidney and spleen revealed no treatment related changes. The number of tumors observed was not greater than in untreated animals.

Shuval (A87) administered sodium nitrite for 2 years to four groups of eight male 3-month-old rats in their drinking water at levels of 100, 1000, 2000, or 3000 mg per liter (about 10, 100, 200 and 300 mg per kg body weight, respectively). A control group received tap water. There were no significant differences in growth and development or mortality between the treated groups and the control animals nor were there significant differences in hemoglobin levels. The methemoglobin levels of the groups receiving the three higher levels of nitrite were significantly raised and averaged 5, 12 and 22 percent, respectively. Histological examination of pancreas, adrenals and brain of treated animals at 24 months showed no pathological changes. The liver and spleen were frequently congested, while the kidneys sometimes showed focal inflammatory and degenerative changes. Changes in the lungs that appeared to be dose related were dilation of the bronchi, infiltration of their walls with lymphocytes and atrophy of the mucosa and muscles. Interstitial round cells and fibrosis were sometimes found. These changes were found with increasing frequency and severity in the three groups treated with the higher nitrite doses. There were small foci of cells and fibrosis in the hearts of some animals, while a diffuse interstitial cellularity with pronounced degenerative foci was frequent only in the groups receiving the highest levels of nitrite.

Shank and Newberne (A77) fed diets containing 1000 ppm sodium nitrite to 96 Sprague-Dawley rats of the F₁ (52 rats) and F₂ (44 rats) generations as one of the control groups in long-term studies of the carcinogenic effects of feeding diets containing nitrite and morpholine. All dams received nitrite during pregnancy and lactation. The animals showed a high incidence of tumors in the lymphoreticular system, 27 percent compared with 6 percent in the controls that had no additive in their diets. These lymphatic tumors were often accompanied by infiltrating leukemic cells and were responsible for widespread damage throughout several organs. There also was a high incidence (61 percent) of tumors other than hepatomas and angiosarcomas in the sodium nitrite treated group compared with incidence (18 percent) in the control group.

Newberne (A88) investigated the effects of sodium nitrite fed to groups of Charles River CD® Sprague-Dawley rats in commercial chow, powdered semi-purified and agar gel-bound semi-purified diets, as well as in the drinking water of a group fed agar-bound semi-purified diets, at several levels varying from 250 to 2000 ppm.
In all, 48 of 573 untreated rats as compared with 172 of 1380 nitrite-treated rats developed reactions diagnosed as lymphoreticular tumors, yielding frequency rates of 8.4 percent and 12.5 percent, respectively, which were significantly different statistically. An unsupported assumption was made that the observation of "immunoblastic cell proliferation" in lymphoid tissues of 40 more of the 573 (7 percent) untreated rats and an additional 155 of the 1380 (11.2 percent) nitrite-treated rats was a step in the lymphomagenic process, thus permitting combination of these lesions with the lymphoma accessions, yielding a total frequency of 15.3 percent for untreated rats and 23.7 percent of the nitrite-treated animals. There was no clearly defined dose response relationship. The investigator concluded that nitrite was not a proximate carcinogen, but a promoter of the neoplastic process. As the investigator notes, the data were only suggestive and the biological significance of nitrite-associated lesions of the lymphoreticular system was unclear. The significance of the data, especially as extrapolated to man, is conjectural.

**Special studies.** Shuval (A87) demonstrated the transfer of nitrites to the fetus in situ by oral administration of 2.5 to 50 mg sodium nitrite per kg to pregnant rats and subsequent removal of fetuses over a 2-hr period and measurement of nitrite and methemoglobin levels in their blood. Rise in nitrite and methemoglobin levels in the fetal blood lagged about 20 minutes behind those in the mother. Threshold for transplacental transfer of nitrites was stated to be 2.5 mg per kg. In mature rats, the time needed for reduction of methemoglobin concentration to one-half of the maximum level reached after oral administration of 30 mg per kg sodium nitrite was about 90 minutes.

Three-month-old rats with electrodes implanted in the brain cortex were chronically exposed to sodium nitrite in their drinking water in concentrations of 100, 300 and 2000 mg per liter (about 10, 30 and 200 mg per kg body weight). All treated groups showed major brain electrical activity changes as shown by EEG recordings after 2 weeks as compared to their own controls and the control group. Two months after cessation of exposure to nitrite, brain electrical activity changes in EEG recordings remained, suggesting some form of irreversible damage even at the level of 10 mg per kg body weight (A87).

**Summary**

1. Soy protein isolates may contain up to 50 ppm nitrite. This leads to an estimated maximum daily nitrite consumption of about 0.04 mg per kg body weight for vegetarians eating meat analogs prepared from spun soy protein isolates (assuming nitrite is not removed in the spinning process) and about 0.25 mg per kg body weight for infants subsisting on formulas based on soy protein isolates if they contain 50 ppm nitrite. However, soy protein
isolates currently used in infant formulas are reported to contain no more than 6 ppm nitrite and ingestion of nitrite from this source would be correspondingly lower. Other consumers of soy protein isolates probably ingest much less than 0.04 mg per kg nitrite from this source.

2. Nitrite also occurs in other foods of plant origin and as an ingredient in cured meats. Daily per capita intake from these sources has been estimated to be about 2.4 mg. This intake will be reduced by recent USDA regulations which lowered the amount of nitrite permitted to be added to bacon. Saliva is an important additional source of nitrite entering the stomach being formed by bacterial reduction of nitrate secreted in the saliva. Dietary nitrate, principally from vegetables but also produced in the intestines from ammonia or organic nitrogen compounds, is absorbed from the gastrointestinal tract and concentrated from the plasma into the saliva by the salivary glands. Total daily exposure to nitrite from saliva has been estimated to be about 15 mg. Nitrite also is formed in the intestine from ammonia or organic nitrogen compounds and has been estimated to contribute about 90 mg daily.

3. The LD₅₀ for sodium nitrite for rodents lies in the range of 80 to 300 mg per kg. The mean lethal dose for human beings is estimated at 1 to 2 g. Long-term feeding studies for rats suggest a threshold of adverse effects for daily consumption somewhere between 10 and 100 mg per kg body weight. A recent unpublished rat-feeding study indicates that nitrite intake in this range enhances the frequency of lymphoreticular tumors from 8.4 percent in controls to 12.5 percent in the treated animals. Newberne, the investigator, suggested that the effect was not mediated through the formation of nitrosamines, and that nitrite acted as a promoter of the neoplastic process. The significance of the latter findings to humans is conjectural.

4. Nitrite can react with many nitrogen-containing compounds in foods, drugs, and other substances, in vitro and in vivo, to form nitrosamines, many of which have been shown to be carcinogenic in experimental animals.
IX. NITRITE REFERENCES


A2. Letter, dated September 8, 1977, from A.H. Hanson, Grain Processing Corporation, Muscatine, Iowa, to F.R. Senti, Federation of American Societies for Experimental Biology, Bethesda, Md.


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