EVALUATION OF THE HEALTH ASPECTS OF CASEIN, SODIUM CASEINATE, AND CALCIUM CASEINATE 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 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 of 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.

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 casein, sodium caseinate, and calcium caseinate 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; 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, an announcement was made in the Federal Register of April 6, 1979 (44 FR 20797-20800) 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 casein, sodium caseinate and calcium caseinate as food ingredients. The Select Committee received no request for such a hearing on casein, sodium caseinate and calcium caseinate.

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 Select Committee on GRAS Substances of LSRO is making its evaluation 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

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*The document (PB-234 902/5) is available from the National Technical Information Service, U.S. Department of Commerce, P.O. Box 1553, Springfield, Virginia 22161.
on absence of substantive evidence of, or reasonable grounds to suspect, a significant risk to the public health. While the Committee realizes that 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 casein, sodium caseinate, and calcium caseinate 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

Casein, the principal protein of bovine milk, is synthesized and secreted by the mammary gland of lactating mammals. The composition of casein varies among species. Cow milk contains about 2.8 percent casein and human milk, about 0.4 percent (3).

Casein exists in milk as a stable suspension of calcium caseinate micelles, particles about 100 nm in diameter (4). Solubility of the protein at its isoelectric point, pH 4.6, is 0.11 g per liter at 25°C. In commercial preparation, casein is precipitated from milk by adjusting the pH to about 4.6 with mineral acid or lactic acid; the latter is generally produced in situ by fermentation of the lactose in milk. The resulting curd is drained, washed several times, pressed overnight, shredded, and dried. Alternatively, casein may be precipitated by treatment of milk with the enzyme rennet. The latter method is used for producing edible casein (5) and also casein for the manufacture of plastics since rennet casein has peculiar properties considered essential for these products (6,7). The health aspects of rennet have been evaluated in another report of the Select Committee (8).

The casein of commerce is prepared from skim milk (9). Little or no casein is manufactured in the United States and most domestic supplies are imported from New Zealand, Argentina, Ireland, and Poland (10). As used in the food industry, casein must nearly always be converted to a caseinate. Caseinates are manufactured in the United States as well as imported (7).

Although often referred to as a single protein, casein is a mixture of phosphoproteins. At least 20 casein components have been found by electrophoretic analysis of samples of pooled cow milk. The main fractions, designated α-casein, β-casein, and κ-casein, are known to be mixtures rather than single proteins. Moreover, genetic polymorphs have been found for each major component (4). Casein is comprised of 18 amino acids including all those essential in human nutrition (Table I). The nitrogen content is about 15.6 percent, phosphorus about 0.9 percent, and sulfur about 0.6 percent. Casein may be classified as having intermediate biological value, i.e., 72 percent compared to whole egg protein as a standard (11).

The U.S. Department of Agriculture (9) has established U.S. Standards for food grades of edible dry casein prepared by acid precipitation. U.S. Extra Grade is required to have a bland natural flavor and odor (not cheesy or sour) with a white to cream-colored physical appearance. Bacterial count is limited to 30,000 per g with coliform count negative per 0.1 g. The protein content (Nx6.38) must be 95 percent or more on a dry basis. Moisture should be no more than 10 percent and fat no more than 1.5 percent. The content of scorched particles must be no more than 15 mg per 25 g with no foreign materials present. The free acid content must be less than equivalent to 0.20 ml of 0.1 N NaOH per g.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>g per 100 g protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>2.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.2</td>
</tr>
<tr>
<td>Valine</td>
<td>7.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.1</td>
</tr>
<tr>
<td>Proline</td>
<td>10.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.7</td>
</tr>
<tr>
<td>Serine</td>
<td>6.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.9</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.34</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.1</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.2</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>7.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>22.4</td>
</tr>
<tr>
<td>Total N</td>
<td>15.6</td>
</tr>
<tr>
<td>Total P</td>
<td>0.9</td>
</tr>
<tr>
<td>Total S</td>
<td>0.8</td>
</tr>
</tbody>
</table>
A commercial casein meeting specifications for U.S. Extra Grade is reported to contain the following typical percentage composition: protein (dry basis) 96.5; moisture, 9.0; fat, 1.3; lactose, 0.1; ash, 2.0; and trace amounts of vitamins B₁, B₂, B₆, niacin, biotin, pantothenic acid, and folic acid (12). Southward and Aird (13) reported whey protein comprised 1.7 percent of total protein in an acid casein prepared from pasteurized milk.

U.S. Standard Grade allows slightly higher tolerances than U.S. Extra Grade (9): bacterial count 100,000 per g, including 20 coliform; a protein content not less than 90 percent (dry basis); moisture not more than 12 percent; and fat content not more than 2 percent. Extraneous material and free acid are permitted at a slightly higher level than for Extra Grade. No specifications for food grade casein are given in the Food Chemicals Codex (14).

Calcium, magnesium, potassium, ammonium, and sodium caseinates are prepared by dispersing acid casein with the appropriate hydroxide or carbonate. Solutions having pH about 6.7 are spray-dried or roller-dried and milled (7). Calcium caseinate contains about 1.6 percent calcium and sodium caseinate about 1.3 percent sodium, dry basis (15). No specifications for food grade caseinates appear in the Food Chemicals Codex (14).

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 crosslinks in the protein. The crosslinks were characterized, after hydrolysis of the treated proteins, as lysinoalanine [N⁶-(DL-2-amino-2-carboxyethyl)-L-lysine]. Although other amino acids, e.g., lanthionine and ornithinoalanine, may be formed when proteins are treated with alkali (17), lysinoalanine (LAL) is of particular interest because renal lesions have developed in rats fed diets containing free LAL (18). The biological properties of free lysinoalanine and alkali-treated casein will be discussed in a following section of this report.

Formation of LAL was demonstrated in 5 percent casein solutions heated at 60°C for 1 hour at pH 8.0 to 13.2 (19). Yield was greatest at pH 12, 2.4 g LAL per 100 g casein being formed. Formation of LAL was catalyzed by multivalent metal chlorides, the activity at pH 10 increasing in the order: MgCl₂<CaCl₂<SrCl₂= BaCl₂ LaCl₃ AlCl₃. Casein, alkali treated by autoclaving with calcium hydroxide for 1 hour at 105°C, contained 6.2 g LAL per 16 g N (20) and 1.15 g per 16 g N after treatment for 4 hours at 40°C and pH 12.2 (21). Sternberg et al. (22) found that LAL formation also occurred in several proteins heated at 120°C under nonalkaline conditions. Casein, for example, heated in 1 percent solution, pH 6 for 1 hour at 120°C, contained 1.7 mg LAL per g protein.

Creamer and Matheson (19) found a wide range in the LAL content (μg per g) of sodium caseinate samples available commercially from different countries: the Netherlands, four samples,
0, 0, 0, and 1120; Canada, one sample, 0; Australia, one sample, 550; and Scotland, one sample, 1560. LAL content (µg per g) of calcium caseinate was also variable: two samples from the Netherlands contained 360 and 4200, respectively; and one sample, country of origin unknown, 6800. Thirty-three samples of sodium and calcium caseinates, number of each not stated, manufactured in New Zealand, contained no detectable LAL. Limit of detection in these analyses was 200 µg per g.

Sternberg et al. (22) reported LAL analyses (µg per g protein) of several commercial casein products available in the U.S.: acid casein, two samples, 70 and 190; calcium caseinate, two samples, 370 and 1000; and sodium caseinate, five samples, 430, 600, 800, 1190, and 6900, respectively. LAL also was reported (µg per g) in commercial corn chips, 390; pretzels, 500; hominy, 560; dried egg white solids, 160 to 1820; infant formulas, 150 to 640; home-cooked frankfurters, 50 to 170; chicken, 100 to 200; and egg white, 140 to 1100.

Although limited data are available on the nitrite content of caseinates dried in direct fired spray driers, a report of one company indicates the product may contain 20 ppm nitrite (23). Soy protein isolates dried in similar equipment may contain comparable levels of nitrite (24). Nitrite also has been reported in spray dried eggs and egg white at similar levels (23) and in spray dried nonfat dry milk at considerably lower levels (25).

Casein is listed as generally recognized as safe in the Code of Federal Regulations (2) as a substance that migrates to food from paper and paperboard packaging materials [21 CFR 182.90]. Sodium caseinate is considered a multiple purpose GRAS food substance [21 CFR 182.1748] and is approved for use as a binder and extender in imitation sausages, nonspecific loaves, soups, and stews [9 CFR 318.7] (26). Calcium caseinate has GRAS status for use in dietary supplements (27). Casein and sodium caseinate are recognized as safe as clarifying agents in wine [27 CFR 240.1051] (28). A petition has been filed proposing affirmation of GRAS status of magnesium caseinate for use as an ingredient for making cheese alternate products (29). Caseins prepared by precipitation with gums, ammonium caseinate, calcium caseinate, potassium caseinate, and sodium caseinate are permitted as optional ingredients in the identity standards for frozen desserts except those for nonfruit sherbets [21 CFR 135.65], nonfruit water ices [21 CFR 135.70], and water ices [21 CFR 135.90].

Casein is also used in the manufacture of plastics, paper coatings, adhesives, paints, and leather finishes. The most extensive nonfood use is in paper coating; about 34 million pounds was used for this purpose in 1967 (6).

In this report, casein, sodium caseinate, and calcium caseinate are evaluated for their GRAS uses.
III. CONSUMER EXPOSURE DATA

A report of a subcommittee of the National Research Council (NRC) has provided information on the usual use levels of sodium caseinate in various categories of foods (30). The subcommittee surveyed food processors by questionnaire concerning the level of addition of sodium caseinate to their processed products, grouped by category. In addition to the requested information, a few companies volunteered information on their usage of casein and calcium caseinate. Based on information supplied by those companies that reported adding casein or a caseinate to at least one food product in a food category, weighted means were calculated for the usual percentage addition of these substances to the food category. Usage of casein was reported in only two categories, milk products and dairy product analogs; usual levels of addition were 25 and 4.1 percent, respectively. Calcium caseinate was reported to be used as an ingredient in only one food category, milk products, at a level of addition of 4.2 percent. Levels of addition of sodium caseinate, the most widely used caseinate, are given in Table II. An entry in Table II does not mean that all foods in the category contain added sodium caseinate or that any one product contains the indicated amount of sodium caseinate.

Assuming all foods within a category contained sodium caseinate at the levels given in Table II, the NRC subcommittee calculated a possible average daily intake for added sodium caseinate using data on the mean frequency of eating foods by food category from the Market Research Corporation of America and U.S. Department of Agriculture data on mean portion size. The intake of sodium caseinate estimated in this way averaged 6.9 g daily for individuals more than 2 years of age. Usage data on casein and calcium caseinate were considered insufficient for the calculation of possible intakes of these substances from all food categories.

The NRC subcommittee noted in its report that its method of calculation was likely to overestimate intakes, often by considerable margins. This appears to be the case for sodium caseinate as indicated by the per capita daily intake, 0.13 g, calculated from the total quantity added to processed foods, data that were collected in the NRC survey (Table III). Others (6) have estimated that about one-third of the approximately 100 million pounds of casein and caseinates (31) marketed annually in the United States is used in the food industry. Per capita daily consumption, 0.2 g of casein and caseinates based on this estimate, is in good agreement with the value for sodium caseinate calculated from NRC poundage data. Sodium caseinate is the most widely used casein product and the Select Committee considers that the poundage data provide the most reliable estimate of actual consumption.

These estimates of consumption of casein and caseinates may be compared with the consumption of casein as a component of milk and other dairy products. Per capita utilization of milk in
1970, as fluid milk and as milk equivalent of manufactured prod-
ucts, was 550 pounds (250 kg) (32); per capita daily consumption
of casein from this source was about 20 g as compared to 0.2 g or
less consumption of isolated casein and caseinates as estimated
above. Per capita consumption of protein from all sources in
1970, based on commodity disappearance data, was about 99 g (33).

**TABLE II**

Level of Addition of Sodium Caseinate
to Foods by Food Category (30)

<table>
<thead>
<tr>
<th>Food category</th>
<th>Weighted mean percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baked goods, baking mixes</td>
<td>2.27</td>
</tr>
<tr>
<td>Breakfast cereals</td>
<td>4.46</td>
</tr>
<tr>
<td>Milk products</td>
<td>2.36</td>
</tr>
<tr>
<td>Cheese</td>
<td>3.94</td>
</tr>
<tr>
<td>Frozen dairy desserts, mixes</td>
<td>0.94</td>
</tr>
<tr>
<td>Meat products</td>
<td>0.75</td>
</tr>
<tr>
<td>Poultry products</td>
<td>2.18</td>
</tr>
<tr>
<td>Sugar, confections</td>
<td>0.20</td>
</tr>
<tr>
<td>Sweet sauces, toppings, syrups</td>
<td>1.80</td>
</tr>
<tr>
<td>Gelatins, puddings, fillings</td>
<td>1.03</td>
</tr>
<tr>
<td>Soups, soup mixes</td>
<td>0.10</td>
</tr>
<tr>
<td>Snack foods</td>
<td>0.61</td>
</tr>
<tr>
<td>Beverages, nonalcoholic</td>
<td>0.08</td>
</tr>
<tr>
<td>Gravies, sauces</td>
<td>0.40</td>
</tr>
<tr>
<td>Dairy product analogs</td>
<td>3.37</td>
</tr>
<tr>
<td>Seasonings and flavors</td>
<td>2.00</td>
</tr>
</tbody>
</table>
TABLE III

Consumption of Sodium Caseinate Based on Total Quantity Used Annually in Foods (30)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative amounts used\textsuperscript{a} 1970/1960</th>
<th>Total used\textsuperscript{b} (1970)</th>
<th>Intake calculated from quantity used\textsuperscript{c} mg/person/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium caseinate</td>
<td>2.2</td>
<td>10,000,000\textsuperscript{d}</td>
<td>130</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Based only on reports of those respondents to the National Research Council (NRC) survey who submitted information for both 1960 and 1970.

\textsuperscript{b}Total usage is the sum of the total kilograms used in foods recalculated to 100 percent based on the NRC subcommittee estimate that their survey data represented about 60 percent of actual usage.

\textsuperscript{c}Based on total consumption in 1970 (column 2) and a U.S. population of 205 million.

\textsuperscript{d}A resurvey in 1975 (79) revealed the use of about the same quantity in processed foods.
IV. BIOLOGICAL STUDIES

Absorption and metabolism

Gupta et al. (34) compared the rates of disappearance of nitrogen from the stomach and small intestine of adult rats fed diets containing 15 percent casein, zein, beef proteins, or an amino acid mixture containing the essential amino acids plus glycine and glutamic acid. Rates of stomach emptying were comparable during the first 2 hours after ingestion by which time about 60 percent of the nitrogen had been emptied. Zein nitrogen disappeared less rapidly than nitrogen from the other protein sources. Less than 10 percent of the nitrogen from casein, beef proteins, or the amino acid mixture accumulated in the small intestine at any time, indicating rapid digestion of the proteins and absorption of their hydrolytic products.

Mason and Palmer (35) determined the absorption and retention of casein in studies on the relative nutritional values of casein, zein, and gelatin in the maintenance of adult rats. After feeding a nitrogen-free diet, 0.4 g (1 g per kg body weight) of each of the three proteins was added to the daily ration. Feces and urine were collected and analyzed for excreted nitrogen. Additional test protein was added to the diet in quantities required for nitrogen balance. After several days, the animals were returned to the nitrogen-free diet and the excreted nitrogen was again determined. Digestion of casein was essentially complete as indicated by analysis for fecal nitrogen; percentage retention was 74 as compared with 57 for zein and 23 for gelatin.

In a nitrogen balance study with five human subjects 19 to 23 years of age, Jekat and Kofranyi (11) found casein to have a biological value of 72 compared to 100 for whole egg protein and 80 for whole milk powder.

Acute toxicity

Boyd et al. (36) administered "high-protein" casein and "vitamin-free" casein (defined below) by intragastric cannulae to groups of 20 young male albino rats in doses of 50 ml per kg of 15 percent suspensions at five successive hourly intervals each day (37.5 g per kg per day) for 3 days; the dose was then increased to nine administrations per day until death occurred or for 3 weeks, whichever came first. Forty percent mortality resulted from stomach rupture after administration of 37.5 g per kg on day 1. No further deaths occurred until the eighth day when daily administrations had increased to seven, at which point 50 percent of the rats had died of gastric rupture. The authors estimated the lethal oral dose of casein to be over 1000 g per kg body weight when administered in multiple doses over a period of 2 weeks.
Similar administration of sodium caseinate or calcium caseinate solutions in quantities up to 75 g caseinate per kg per day in multiple doses produced a number of deaths from stomach rupture. Necropsy of animals that died after 3 to 5 days of treatment without gastric rupture showed generalized organ degeneration to which, the authors stated, high sodium or calcium intake was a contributing factor. The LD$_{50}$ of sodium or calcium caseinate was estimated to be 400 to 500 g per kg when administered over a 5-day period (36).

The "high-protein" casein used in the above studies was prepared by lactic acid fermentation of skim milk and contained 85 percent protein, 11 percent moisture, 1.9 percent ash, 1.5 percent fat, and small quantities of vitamins (36). The "vitamin-free" casein was prepared by multiple extraction of casein with hot alcohol and contained 89 percent protein, 8.0 percent moisture, 2.0 percent ash, and 0.5 percent fat. Alcohol extraction was stated to remove some 75 percent of the vitamins contained in the "high-protein" casein. Sodium caseinate contained 92.5 to 94.5 percent protein, 4.0 percent ash, 1.3 percent sodium, and 1.5 percent fat. Composition of calcium caseinate was not reported.

Short-term studies

Pond et al. (37) compared the growth rates of Yorkshire pigs, 12 per group, fed liquid diets containing casein, fish protein concentrate, or soy protein isolates as the protein source but identical in other respects. The pigs were weaned at 2 or 3 days of age and fed the liquid diets for 21 days. Average gains in body weight were 59, 57, and 44 g per day for pigs receiving casein, fish protein concentrate, and soy protein isolate, respectively. Corresponding gains per g of protein consumed were 2.4, 2.4, and 1.9 g. Similar gains per g of protein were reported for a second experiment in which casein and two fish protein concentrate products were fed.

Growth rates, nitrogen efficiency ratios, and retention of calcium and phosphorus were compared in studies with groups of five 5-week-old male Wistar rats fed semipurified diets containing 18 percent casein, soy protein, or a 1:1 mixture of the two for 3 weeks (38). There were no significant differences in body weight or length gains among the three groups. The nitrogen efficiency ratio, 13.3 ± 0.5 for the casein group, was significantly greater than for the other two groups. Calcium consumption and accumulation rates were slightly but not statistically significantly higher in the group fed casein. Rate and amount of accumulation of phosphorus were similar in the three groups.

Copping et al. (39) compared growth rates of rats fed three types of casein in studies on the formulation of a complete purified diet. The percentage composition of the basal diet was: casein, 20; sucrose or maize starch, 60; hardened arachis oil, 12;
lard, 3; and salt mixture, 5. The basal diet was supplemented with either vitamins or vitamins and liver extract. The caseins were identified as an alkaline caseinate, a highly purified casein, and a casein prepared by acid washing but without extraction with organic solvents. The caseins appeared to be equivalent in nutritive value as indicated by similar weight gains in weanling Lister rats fed the three casein diets with either type of supplementation for 14 weeks.

Osborne and Mendel (40) showed that cystine was a limiting amino acid in casein in supporting growth of rats at dietary levels of 12 percent or less. Mueller and Cox (41) found that utilization of the nitrogen in lactalbumin by rats was greater than their utilization of the nitrogen in casein. However, studies with four adult men showed that casein and lactalbumin were equally effective in maintaining nitrogen balance when fed at a level of 2 percent of total calories (80 percent of total protein intake) for 4 days following a 12-day protein depletion.

Kittens fed a semipurified diet containing 35 percent casein developed classical signs of vitamin A deficiency in 6 to 20 months in spite of receiving 5000 IU vitamin A palmitate three times weekly (42). Growth rates were comparable to those of control animals fed a fresh meat diet supplemented with calcium and vitamin A until the kittens were 3 to 4 months old, after which a decline in rate occurred. Both sexes receiving the experimental diet reached a weight 20 to 40 percent below their expected body weight. Conjunctivitis, xerosis with keratitis and vascularization of the cornea, progressive destruction of the visual cells of the retina, and formation of cataracts were noted. In a subsequent series of studies with kittens and adult cats, it was found that casein contains insufficient quantities of taurine (2-aminoethanesulfonic acid) to maintain the function and structure of the retina (43-45). It appears that the cat, and particularly the kitten, is incapable of synthesizing sufficient taurine from the sulfur amino acids in casein to meet its requirements (46).

Nayak and Higginson (47) fed groups of 20 C3H or CFW mice diets containing 20 percent casein for 100 days. Examination at necropsy showed that three of seven CFW mice, but none of 12 C3H mice had generalized amyloidosis involving the liver, spleen, kidneys, adrenals, and small intestine. The authors stated that they had previously observed similar amyloidosis in 33 of 55 Hauschka mice fed a 20 percent casein diet for up to 400 days. Janigan (48) reported that sodium caseinate administered by subcutaneous injection resulted in amyloidosis in male C57BL/10J mice. However, Stora et al. (49) found that casein from the same source was contaminated with Bacillus cereus and that a bacteriologically sterile product did not produce amyloidosis in mice after repeated subcutaneous injections of the product (administered as the caseinate) in Swiss mice, whereas amyloidosis resulted from injection of the unsterilized casein. Irwin and
Smith (50) observed that amyloidosis was induced in 20 of 27 4-month-old male golden hamsters that were inoculated intravenously with Leishmania donovani and fed a diet containing 27 percent casein for 20 or 28 weeks. Amyloidosis was not detected in inoculated animals fed an 8 percent casein diet and sacrificed at 28 weeks; neither was amyloid found in noninoculated control animals. Amyloidosis has been induced in various species by injections of egg albumin, gelatin, zein, blood proteins, turpentine, phenols, or saline, and by dietary factors such as goitrogens, excess cholesterol, and vitamin C deficiency. Although antigenic challenge may be involved in some cases of amyloidosis, the mechanisms involved in others are not apparent (48).

Long-term studies

It has been well established that very high-protein diets induce degeneration of the glomeruli and tubules in the rat kidney. The onset usually does not occur until advanced age but both the quality and the quantity of dietary protein affect the development of renal degeneration (51).

Newburgh and Curtis (52) investigated the effect of high dietary levels of protein on urine composition and kidney histology in long-term rat feeding studies. Weanling rats (breed not stated) were fed diets containing 7 to 61 percent casein, 12 to 72 percent beef muscle protein, or about 70 percent beef liver protein for 450 days. Twenty-four-hour urine specimens taken every second month were examined for albumin content and presence of casts. An abnormally large number of casts appeared in the urine of rats fed 33 percent casein or beef muscle protein for 240 days and the number was greater at 450 days. Renal injury as indicated by number of casts was greater in the groups fed beef muscle for 240 and 450 days. Albumin content of the urine of the animals fed beef was also greater (1.1 percent) than that of the casein-fed group (0.1 percent) at 450 days. Histologic examination of the kidneys of rats fed a diet containing 75 percent casein for more than a year showed moderate degeneration of the epithelium of the convoluted tubules and some tubular dilatation. Beef muscle protein at a comparable dietary level caused more severe tubular injury and injury also was evident in rats fed a 31 percent beef protein diet for 15 months. Kidneys of animals fed the diet containing beef liver protein for 10 months were enlarged and granular in appearance; microscopic examination of kidney tissues of animals sacrificed at 350 days revealed marked cystic dilatation of some tubules and various forms of glomerular injury.

Addis et al. (53) reported histologically normal kidneys and no more casts in the urine of rats fed a diet containing 74 percent casein for 330 days than were present in control animals. However, samples of fresh urine were examined for casts rather than the concentrates obtained by centrifuging all urine collected over a 24-hour period as was done by Newburgh and Curtis (52).
Bras and Ross (54) found that renal damage in rats, described as progressive glomerulonephrosis (PGN), was influenced by both protein and caloric intake. In lifetime feeding studies with male Charles River SD rats, a higher incidence (46/209) of PGN was found in group A fed daily 10 g of a diet containing 30 percent casein than in group B (16/233) that received 10 g of a similar diet containing 8 percent casein. Lowest incidence (1/119) of PGN was in group C, fed 5.9 g of a diet containing 51 percent casein, i.e., the same daily consumption of protein (3 g) as group A but a lower caloric intake.

Lalich et al. (51) found that degeneration in glomeruli and tubules could be accelerated by combining excess dietary protein with uninephrectomy in rats. Glomerular degeneration, tubular dilatation, and hyaline cast formation were observed in uninephrectomized male Sprague-Dawley rats, about 100 g initial weight, when fed for 150 days or more diets containing 40 percent protein of which about one-half was casein, soy protein, or peanut meal. Early development of glomerulosclerosis and tubular degeneration was attributed to the reduced number of functioning nephrons. Hay et al. (55) showed that nephrosclerosis could be produced in partially nephrectomized castrated male rats, 40 to 60 g initial weight, in 21 days if subcutaneously injected with lyophilized anterior pituitary extract and fed diets containing 30 percent protein. Dietary casein, egg albumin, and wheat gluten produced greater kidney damage than lactalbumin, zein, or gelatin.

Special studies

Untoward reactions. Clinical sensitivity to cow milk has been estimated to occur in 0.3 to 7 percent of all children (56). Comparison of the relative allergenicity of guinea pigs to α-casein, the major protein component of casein, and β-lactoglobulin and α-lactalbumin, the principal proteins of whey, showed that β-lactoglobulin was the most allergenic whereas α-casein and α-lactalbumin were of a much lower order of allergenicity (57). However, one study showed 60 percent of subjects allergic to milk gave positive reactions when challenged orally with casein (58). Casein and other milk proteins may yield a new allergen(s) on partial digestion with pepsin (59). Milk allergy usually develops during the first few months of life and may create a serious problem in infant nutrition. However, Clein (60) reported that only 2 percent of his patients remained allergic beyond the age of six years. About 6 percent of the infants in his practice were allergic to milk. Anderson et al. (61) demonstrated close antigenic relationships between cow, goat, and human casein by active and passive sensitization of guinea pigs.

Among subjects demonstrating untoward reaction to milk, Hanson and Johansson (62) reported the following types of manifestations: gastrointestinal, 25 to 40 percent; respiratory, 30 to 45 percent; atopic dermatitis, 42 percent; urticaria, 8 percent;
and anaphylaxis, 5 percent. Hypersensitivity to casein has been reported in steatorrhea, celiac disease (63), and in hypochromic microcytic anemia (56).

Wright et al. (64) found that 91 of 100 newly born infants had antibodies to casein in the cord blood and 88 percent of the mothers were positive also. In 75 infants with eczema who gave positive intracutaneous skin tests to casein or lactalbumin or both, Hill and Pratt (65) found 35 reacted to both casein and lactalbumin, five reacted to casein alone, and 35 reacted to lactalbumin alone.

Alkali-treated proteins. Lysinoalanine (LAL), an amino acid found in alkali-treated proteins (15-19) and in proteins heated under near neutral conditions (22), has been demonstrated to produce renal lesions in rats when fed in the free form (18, 20, 66). Groups of rats fed dietary levels of 10, 30, or 100 ppm free LAL for 13 weeks exhibited nephrocytomegaly only at the 100 ppm treatment level (20). The treated animals exhibited alterations of the pars recta characterized by karyomegaly, tubular dilatation, and loss of some tubular epithelial cells.

De Groot et al. (20) fed casein that was alkali-treated by autoclaving with calcium hydroxide for 1 hour at 105°C. LAL content was 6.2 g per 16 g N. Fed to male weanling Wistar rats for 4 weeks as a supplement to a stock diet at levels (3.9 and 11.7 percent) providing 2000 and 6000 ppm LAL, the treated casein did not induce any histological renal changes. However, substitution of acid-hydrolyzed alkali-treated casein in the stock diet resulted in renal cytomegaly even though the dietary level of lysinoalanine was only 200 ppm. The lesion was slightly less pronounced than with 200 ppm synthetic LAL added to the stock diet.

Feron et al. (67) observed nephrocytomegaly in rats (presumably the CIVO Wistar strain) fed an alkali-treated casein containing 5 percent lysinoalanine at a level providing 10,000 ppm lysinoalanine in the diet after feeding for 52 weeks but not at 4, 8, 13, and 26 weeks. After 52 weeks, a minimal degree of nephrocytomegaly occurred in the kidneys of 6 of 10 rats examined. However, Slump et al. (68) reported nephrocytomegaly in rats (presumably the CIVO Wistar strain) after 4 to 8 weeks feeding of an alkali-treated casein (1 g LAL per 16 g N) at a dietary level of 2700 ppm LAL. Hydrolysis of the alkali-treated caseins with pepsin, pancreatin, and intestinal mucosa sequentially under similar conditions liberated 0.0 to 0.2 percent of the lysinoalanine from the sample containing 5 percent LAL and 0.5 percent of the bound LAL in the casein containing 1 percent LAL.

The cytomegaly-inducing properties of LAL bound in casein oligopeptides were investigated by De Groot et al. (20) by feeding rats a stock diet supplemented with partially acid-hydrolyzed alkali-treated casein. The hydrolyzed preparation contained 6.2 g
LAL per 16 g N, only 8 percent of which was in the free state. The remaining LAL was distributed according to peptide molecular weight as follows: 5000 to 1500, 10 percent; 1500 to 1000, 30 percent; 1000 to 500, 45 percent; and <500, 7 percent. Cytomegalic reaction was observed in rats fed diets containing levels of the hydrolyzed casein which provided free and bound LAL 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 LAL at levels of 30 and 45 ppm, indicating that peptide-bound LAL, as present in the oligopeptides fed, exerted some activity in inducing nephrocytomegaly.

Histopathological examination of the kidneys of weanling Swiss mice, Syrian golden hamsters, New Zealand rabbits, and newly-hatched Japanese quail after feeding 1000 ppm LAL in their diets for 4 or 8 weeks showed no cytomegaly or other sign of renal damage (20). Growth rate, food intake, and food efficiency were not affected by LAL supplementation of their diets. Histopathological findings were negative for two 6-month-old male beagles fed a stock diet supplemented with 1000 ppm LAL, one after feeding for 4 weeks and the other after 9 weeks. Findings were also negative in male rhesus monkeys, 18 months old, hand-fed 150 g of three casein-based diets and 150 g of a fruit mixture daily for 9 weeks. Two monkeys were fed the basal diet supplemented with 1000 ppm synthetic LAL; two others received a diet containing 10,000 ppm LAL 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. 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. Feron et al. (67) fed Swiss mice and golden hamsters diets containing 10,000 ppm free LAL. Slight nephrocytomegaly was observed in the mice but not in the hamsters after feeding for 4 and 8 weeks.

Renal lesions have been observed in rats fed diets in which the sole source of protein was alkali-treated soy protein isolate containing 0.6 percent or more of LAL (18,66,69,70,71,72). Others found renal lesions in rats fed alkali-treated soy protein or alkali-treated lactalbumin as the only source of protein but not when the ratio of untreated to alkali-treated protein was 1:1 (lactalbumin:alkali-treated lactalbumin) or 2:3 (lactalbumin: alkali-treated soy protein), and minimal or doubtful cytomegaly at a 1:2 ratio (casein:alkali-treated soy protein) (68,71,73).

Finot et al. (74) found differences in the metabolism and distribution of ^14C-labeled free LAL in studies with the rat, mouse, hamster, and quail. 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 oral administration of $^{14}$C-labeled LAL the rat kidney contained a higher fraction of the administered dose than the mouse, hamster, or quail kidney. The total label in the rat kidney, expressed as a fraction of the administered dose, was more than 10 times higher than that in the hamster kidney and 2 to 10 times higher than in the mouse kidney.

Carcinogenicity

A high casein diet has been reported to promote the induction of lung tumors in mice with 9,10-dimethyl-1,2-benzanthracene (75) and mammary cancers in rats with 3-methylcholanganthrene (76). However, it protects against liver carcinogenesis in rats fed di-methylaminoazobenzene (77). The effects in the case of polycyclic hydrocarbons may be nonspecific and reflect the levels of protein in the diet.

Mutagenicity and teratogenicity

No reports on tests for mutagenicity or teratogenicity of casein or caseinates were available to the Select Committee. However, Struthers et al. (78) reported that diets containing 5 to 30 percent alkali-treated soybean protein containing 1 percent LAL produced no teratological effects when fed to pregnant Sprague-Dawley rats.

Nitrite in spray-dried caseinates

Although analytical data on the nitrite content of caseinates available to the Select Committee were limited, these data and the occurrence of nitrite in other products (23-25) dried in direct fired spray driers indicated that caseinates dried in this type of equipment may contain nitrates. The Select Committee analyzed the health aspects of nitrates in connection with a previous report on the health aspects of soy protein isolates as food ingredients (24) and came to the following conclusions:

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 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 2.6 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 promotor 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.
V. OPINION

Casein in milk and milk products has been a major component of the diet of man for centuries. Long-term animal feeding studies have shown that extremely high dietary levels of casein, in common with other food proteins, may be injurious to the kidneys. However, per capita daily consumption of casein and caseinates added to foods is less than 0.2 g and represents a minor contribution to the total average daily intake of protein, about 99 g, by the U.S. population.

Heating casein under strongly alkaline conditions at 90°C or autoclaving at higher temperatures under near neutral conditions has been shown to result in the formation of lysinoalanine as a component of the protein molecule. Lysinoalanine has been reported in commercial samples of casein, calcium caseinate, and sodium caseinate. Lysinoalanine also has been detected at relatively low concentrations in a number of commercial food products and in home-cooked frankfurters, chicken, and egg white.

Sensitivity to lysinoalanine differs among animals species and is dependent on the protein nature of the diet; feeding free lysinoalanine at dietary levels of 1000 ppm to five mammalian species other than the rat, including subhuman primates, failed to produce renal cytomegalic changes. Renal cytomegalic changes have been demonstrated in rats fed free lysinoalanine, or alkali-treated soy protein isolate, lactalbumin or casein containing bound lysinoalanine. The alkali-treated proteins were fed as the sole source of protein at dietary levels of 20 to 30 percent; cytomegaly did not occur or was markedly reduced in rats fed diets 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 imbalanced with respect to one or more amino acids. Although the available information indicates that the present consumption levels of casein and caseinates, less than 0.2 g per capita as currently used, pose no hazard to the consumer, a limitation with respect to lysinoalanine content in specifications for food grade products would avoid possible future problems in this regard.

A small proportion of infants and lesser numbers of children and adults experience untoward reactions to casein and other milk proteins. Casein is only mildly antigenic but the presence of whey proteins in commercial casein may increase the frequency of hypersensitivity reactions. However, the relatively low consumption of casein, sodium and calcium caseinates added to processed foods, as compared to consumption of casein in milk, cheese, other dairy products, and foods containing dairy products as an ingredient, does not appear to significantly increase exposure to this antigen.
Although few nitrite analyses of caseinates dried in direct fired spray driers were available to the Select Committee, the available data and the finding of nitrite in samples of other products dried in similar equipment indicates that nitrite can occur at low levels in spray-dried products. According to available information, casein and some caseinates are dried in indirectly heated driers and would not be exposed to nitrite from the drying gases. Nitrite content is of concern because of its toxicity, per se, and because it can react with other nitrogen-containing compounds to produce nitrosamines, many of which have been shown to be carcinogenic in experimental animals. It is important to ensure that preformed nitrosamines are not present in caseinates and are not formed in processed foods containing caseinates.

Consideration must be given to the potential for nitrosamine formation in vivo from ingestion of foods containing nitrite, and the latter reacting with other nitrogen-containing compounds in foods, drugs, and endogenous amines, must be considered. Many natural and processed foods, including spray-dried products, contribute to the total human intake of nitrite even though bacterial reduction of nitrate to nitrite in saliva is the major source of nitrite entering the stomach.

It has been reported from recent unpublished work with rats fed nitrite that the frequency of lymphoreticular tumors was increased. The possibility that nitrite has a direct adverse effect of this type raises questions about the total body burden of nitrite and the relative contributions from food, saliva, and the estimated larger amount produced by bacteria in the intestinal tract. A study of these sources and their relative importance is required for individuals of different age groups and dietary habits to obtain reliable figures. Specifications should be developed for food grade caseinates which limit the content of nitrite and nitrosamines.

Caseinates appear to contribute a minor quantity of nitrite to total human exposure. Preliminary estimates of per capita exposure indicate they account for less than 0.2 percent of the amount ingested as food ingredients, less than 0.02 percent of that taken in as an ingredient of food and present in saliva, and less than 0.005 percent of the total taken in as a food ingredient, 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, caseinates 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 foregoing information and concludes that:

It is essential that food grade specifications for casein, sodium caseinate, and calcium caseinate be established including provisions for acceptable levels of lysinoalanine, nitrite, and nitrosamines.

Assuming that acceptable levels of lysinoalanine, nitrite, and nitrosamine are established, there is no evidence in the available information on casein, sodium caseinate, or calcium caseinate that demonstrates or suggests reasonable grounds to suspect a hazard when they are used at levels that are now current or that may reasonably be expected in the future.

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


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VII. SCIENTISTS CONTRIBUTING TO THIS REPORT

1. Members of the Select Committee on GRAS Substances:

Joseph F. Borzelleca, Ph.D., Professor of Pharmacology, Medical College of Virginia, Health Sciences Division, Virginia Commonwealth University, Richmond, Virginia.

Harry G. Day, Sc.D., Professor Emeritus of Chemistry, Indiana University, Bloomington, Indiana.

Samuel J. Fomon, M.D., Professor of Pediatrics, College of Medicine, University of Iowa, Iowa City, Iowa.

Bert N. La Du, Jr., M.D., Ph.D., Professor and Chairman, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan.

John R. McCoy, V.M.D., Professor of Comparative Pathology, New Jersey College of Medicine and Dentistry, Rutgers Medical School, New Brunswick, New Jersey.

*Sanford A. Miller, Ph.D., Professor of Nutritional Biochemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts.

Gabriel L. Plaa, Ph.D., Professor and Chairman, Department of Pharmacology, University of Montreal Faculty of Medicine, Montreal, Canada.

Michael B. Shimkin, M.D., Professor of Community Medicine and Oncology, School of Medicine, University of California, San Diego, La Jolla, California.

Ralph G.H. Siu, Ph.D., Consultant, Washington, D.C.

John L. Wood, Ph.D., Distinguished Service Professor, Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee.

George W. Irving, Jr., Ph.D., (Chairman), Life Sciences Research Office, Federation of American Societies for Experimental Biology, Bethesda, Maryland.

*Did not participate in the final opinion reached in this report.
2. LSRO staff:

Kenneth D. Fisher, Ph.D., Director
Frederic R. Senti, Ph.D., Associate Director
Richard G. Allison, Ph.D., Staff Scientist
Sue Ann Anderson, Ph.D., Staff Scientist
Herman I. Chinn, Senior Staff Scientist
Andrew F. Freeman, Senior Staff Scientist
John M. Talbot, M.D., Senior Medical Consultant
Michael J. Wade, Ph.D., Staff Scientist

Report submitted by:

September 12, 1979

Date

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