IV. BIOLOGICAL STUDIES

Absorption

Hydrogen peroxide in the absence of a stabilizing agent gradually decomposes to oxygen and water. Decomposition is rapid in the presence of catalase. Ingested hydrogen peroxide presumably undergoes such degradation in the gastrointestinal tract, leaving little of the intact compound available for absorption. Perfusion of dog intestine with dilute hydrogen peroxide raised significantly the oxygen saturation of blood (15). No attempt was made to determine if the peroxide breakdown occurred before or after absorption.

The rapid release of oxygen into the tissues and vascular network can readily be observed after administering hydrogen peroxide by various routes. The characteristic whitening of the skin after topical application has been attributed to an avascularity produced by oxygen bubbles acting as microemboli in the tissues and capillaries. Hauschild et al. (16) have demonstrated this reaction in a variety of tissues including human skin, cat and dog tongues, rat paws, and animal hearts (species not stated). When 3 or 30 percent hydrogen peroxide was applied sublingually in rabbits, cats, and dogs, gas bubbles rapidly appeared in the jugular vein (17). With the higher concentration, these bubbles were observed within a few seconds and in sufficient quantity to cause pulmonary gas embolisms. Using ^{18}O-labeled-hydrogen peroxide, Ludewig (18) confirmed its rapid absorption after sublingual administration and the passage of liberated oxygen to the lungs. Approximately 7 percent of the oxygen theoretically available from the administered hydrogen peroxide was detected in the expired air within 18 minutes and 30 percent within 34 minutes. Traces of the labeled oxygen were also detectable in the arterial blood.

Metabolism

Hydrogen peroxide is a normal product of aerobic metabolism and may result from a number of oxidase-catalyzed reactions (e.g., d-amino acid oxidase, urate oxidase, glycolate oxidase) or by the breakdown of superoxide by superoxide dismutase. The hydrogen peroxide thus formed is rapidly decomposed by tissue catalase or peroxidase (19). Enzymes metabolizing hydrogen peroxide are localized largely in specialized vesicles or organelles, known as peroxisomes. The peroxisomes comprise about 2 percent of the liver volume, and are especially rich in catalase (20), which efficiently and rapidly decomposes endogenous hydrogen peroxide. Sies calculated that 1 g of rat liver produces approximately 50nM (1.7 µg) of hydrogen peroxide per minute. Boveris et al. (21) arrived at a higher figure for the normal production of hydrogen peroxide under physiological conditions: 90nM (3.1 µg) per min per g

wet weight of liver. Extrapolating to man, this would be roughly equivalent to the production of 150 to 270 mg hydrogen peroxide per hour by the human liver under normal conditions and even larger amounts under the stimulus of appropriate substrates. Breakdown by catalase is so efficient, however, that the steady-state concentration in the liver is 10^{-9} molar (30 ng per kg). Even with maximal stimulation of hydrogen peroxide production, the concentration increases only to 10^{-7} molar (3 µg per kg) (20).

Despite its rapid destruction, hydrogen peroxide may have an important role in certain localized metabolic reactions. For example, it has been shown to exert an insulin-like effect in fat, and perhaps in other tissues (22). When rat adipocytes were incubated with hydrogen peroxide in the presence of glucose, glycogen synthase I was activated, which stimulated the incorporation of glucose into glycogen. It has been suggested also, that hydrogen peroxide may be involved in the body's defense against bacterial infection (23). When microorganisms are phagocytized, hydrogen peroxide or activated forms of oxygen are generated, which may account at least in part for the bactericidal effectiveness of the phagocytic cells.

Acute toxicity

No reports were available to the Select Committee on the acute toxicity in animals of orally administered hydrogen peroxide. Several cases of accidental poisoning in man have been described (24,25) including the death by respiratory failure of a 1-year-old infant within 1 hour after ingesting an unknown quantity of concentrated hydrogen peroxide solution (24). Five non-fatal poisonings were reported of persons who had consumed 25 to 100 ml of 30 percent hydrogen peroxide (25). The victims experienced sharp pains in the abdomen and behind the sternum, foaming from the mouth, vomiting, fleeting loss of consciousness, transitory motor and sensory impairment, rise in temperature, micro-hemorrhaging in the skin and conjunctiva, and a moderate leukocytosis. One patient, who had swallowed 100 ml of the hydrogen peroxide solution, displayed for several days marked visual and neurological symptoms which the authors attributed to oxygen microembolisms.

Aoki and Tani (26) referred without elaboration to a "mass poisoning in Japan...caused by hydrogen peroxide treated noodles." The authors did not specifically incriminate the residual peroxide, but pointed out that approximately 20 percent of commercial products tested contained hydrogen peroxide in excess of the Japanese permissible limits of 100 ppm.

Acute toxicity data on animals are available only for percutaneous and intravenous administration (Table II). Death generally has been attributed to embolic phenomena resulting from liberated oxygen. As is evident from Table II, there is a marked

TABLE II

LD_{50} of Hydrogen Peroxide

Animal	Route	Concentration (percent)	mg/kg	Reference number
Rabbit	Percutaneous	90	630	27
Pig	"	90	2500	27
Cat	"	90	>4000	27
Rat (white)	"	90	4800	27
Rat (black)	"	90	>7500	27
Rat (white)	"	N.S.*	700	28
Rabbit	IV	90	19	27
	"	58.6	16	27
	"	36.0	10	27
	"	14.4	5	27
	"	3.6	3	27
Rat	IV	N.S.*	21	28

*N.S. = not stated.

species difference in the susceptibility to percutaneous application. There is also an inverse relationship between the concentration of hydrogen peroxide injected intravenously and its toxicity. Hrubetz et al. (27) explained this paradox by pointing out that intravascular oxygen bubbles appear at the site of injection and mechanically hinder further access of hydrogen peroxide to the general circulation. The more concentrated the solution, the more marked is this effect and the less peroxide actually reaches the systemic circulation.

Intraperitoneal injection of hydrogen peroxide (from 0.5 ml of 5 percent to 1.0 ml of 10 percent) into adult mice had a radiation-like effect (29). It produced pyknotic nuclei in the intestine and thymus within 2 hours which, with the strongest dose, persisted for up to 24 hours. Toxic signs were produced in horses, rabbits, and dogs by intravenous injection of relatively dilute hydrogen peroxide (1 to 2 ml of 0.4 to 3 percent solutions per kg) (30). Shortness of breath was evident in all species after injection. The rabbits, in addition, responded with a characteristic backward tossing of the head while the horses and dogs displayed increased peristalsis and repeated defecation. Rabbits and cats dying after intravenously administered hydrogen peroxide or oxygen had pale, emphysematous lungs with considerable amounts of gas in the great veins and in the right side of the heart (31).

Aerosols of hydrogen peroxide containing 3 to 5 mg per liter produced pulmonary irritation and congestion in mice within 5 minutes (32). Exposure to higher concentrations (10 to 40 mg per l) for 15 minutes caused death of a high percentage of mice, usually within an hour, apparently from pulmonary edema.

Short-term studies

Three-week-old dd mice drinking 0.15 percent hydrogen peroxide ad libitum (about 150 mg per kg per day) grew normally and developed no visible abnormalities during a 35-week test period (26). Upon necropsy, degenerative changes were observed in the liver and kidney. The stomach wall was slightly necrotic, inflamed, and irregular and the lymphatic tissue of the small intestinal wall was hypertrophic. Solutions in excess of 1 percent (more than 1 g per kg per day) caused pronounced weight loss and death of the mice within 2 weeks.

Romanowski et al. (33) replaced the drinking water of rats with solutions of 0.25 to 10 percent hydrogen peroxide. All animals receiving 2.5 percent solutions or higher died within 43 days. Nine of ten rats fed the 0.25 percent solution and eight of ten fed the 0.50 percent survived the test period of 146 days, although the weight gain of each group was less than that of the controls. The daily hydrogen peroxide intakes for these two groups were approximately 250 and 500 mg per kg, respectively.

A group of weanling male Osborne-Mendel rats given a solution of 0.45 percent hydrogen peroxide to drink ad libitum for 3 weeks was compared with a similar group receiving tap water (34). Both fluid intake and weight gain of the peroxide group were significantly less than those of the controls. However, when fluid intake of the control rats was limited to that of the experimental animals, there were no significant differences in body or organ weights. The daily consumption of hydrogen peroxide during the test period was approximately 500 mg per kg body weight. Three weanling female rats were given 0.45 percent hydrogen peroxide for 5 months, then switched to tap water and mated with normal males. Normal litters were produced. Male rats from these litters were then given 0.45 percent hydrogen peroxide for 9 months. The only noticeable difference between these rats and male littermates receiving tap water was a decreased weight gain in the peroxide group.

In a similar experiment, young, male Holtzman rats were provided with solutions of 0, 0.5, 1.0, and 1.5 percent hydrogen peroxide (approximately 0, 500, 1000 and 1500 mg per kg per day) as their source of drinking fluid for 8 weeks (35). Growth was significantly retarded in all groups receiving hydrogen peroxide and the degree of retardation was proportional to the peroxide concentration. Seven of 24 rats receiving 1.5 percent hydrogen peroxide died during the course of the experiment. All surviving animals in this group and 15 of 24 rats receiving 1 percent had extensive carious lesions and pathological changes in the periodontium. Seven of 24 rats on the 0.5 percent solution also had mild caries, but no periodontal changes.

In none of the experiments described above was any mention made of the use of stabilizers or of other measures to prevent the decomposition of hydrogen peroxide in drinking water.

Kawasaki et al. (36) reported no adverse effects in male Wistar rats receiving up to 30 mg hydrogen peroxide per kg per day by gastric intubation. Rats given twice this dose showed significantly decreased growth rates after 20 days and decreased hematocrit, plasma protein, and plasma catalase activities after 100 days. When the same amount of hydrogen peroxide was administered in the feed, no harmful effects could be detected.

Young mice were injected subcutaneously twice daily for 2 weeks with 0.1 ml of 1.5 percent hydrogen peroxide, roughly equivalent to 100 mg per kg body weight per injection (31). Several animals showed brief embolic signs immediately after the injection and 5 of 30 mice died with embolic signs during the experimental period. A few mice exhibited brief generalized convulsions. Local subcutaneous emphysema was invariably present after injection and small ulcers eventually developed at the injection sites in about 25 percent of the animals.

Rats have proved to be highly resistant to hydrogen peroxide vapor (37). Only 1 of 10 rats died after exposure to 93 mg per m^3 (67 ppm) for 6 hours daily, 5 days per week for 6 weeks, whereas more than half of the mice under approximately the same conditions died within 2 weeks. However, this greater sensitivity of the mouse to inhaled hydrogen peroxide may be more apparent than real. Because of its small size and rapid respiration, the mouse would inhale more hydrogen peroxide per unit time and weight than the rat. Dogs and rabbits exposed to 7 and 22 ppm hydrogen peroxide on a similar schedule for 6 and 3 months, respectively, showed only minor respiratory damage.

Long-term studies

Molnár (38) injected female mice subcutaneously with 0.5 ml of 0.01 percent hydrogen peroxide (about 2.5 mg per kg body weight) two or three times weekly from the age of 6 weeks to 14 months for a total of 95 injections. The average life-span decreased from 595 days in mice receiving similar injections of physiological saline to 446 days among those receiving the hydrogen peroxide. A weight loss in the peroxide-treated group was noted after about 6 months.

Special studies

Reproduction. As noted above, female rats receiving 0.45 percent hydrogen peroxide (about 500 mg per kg per day) as the sole drinking fluid for 5 weeks produced normal litters when mated with normal males (37). Because hydrogen peroxide is reportedly toxic to spermatozoa in vitro (39), the effect of its oral administration on fertility is of interest. Three-month-old male albino mice were given 0.33 and 1.0 percent hydrogen peroxide (about 330 and 1000 mg per kg per day) as their sole drinking fluid for 7 to 28 days before placing them with normal females. All females became pregnant within a few days and in each case, healthy offspring were born in litters of normal size.

Carcinogenicity. The recognition of the radiomimetic (40) and mutagenic (41,42) properties of hydrogen peroxide, as well as its detection in tumors (43), prompted the direct testing of this compound for carcinogenic or cocarcinogenic activity. Schmidt (44) gave groups of newborn mice consisting of strains AB, C57BL, C57BL/6, A/JAX and X a single subcutaneous injection of 0.1 ml of 0.6 percent hydrogen peroxide (about 300 mg per kg) and another group (strain AB) three such injections. The intervals between injections were not stated. Only about 10 percent of the mice survived for 6 months. Six of 30 survivors (20 percent) in the single injection groups developed tumors (3 leukemias, 3 breast cancers, 1 lymphosarcoma of the thymus). In the triply injected group, 14 of 42 (33 percent) were affected (6 breast

cancers, 3 lung adenomas, 4 leukemias, 1 ovarian tumor, 1 hemangiosarcoma of the liver). No tumors arose at the sites of injection. Although no statistical analysis was reported, Schmidt claimed the incidence of tumors among the injected mice was significantly higher than that normally observed in his colony (2 to 5 percent).

Shamberger (45) reported no cocarcinogenic action of hydrogen peroxide in mouse skin initiated with 7,12 dimethylbenz(α)-anthracene (DMBA). He applied 0.25 ml of 3 percent hydrogen peroxide in acetone daily for 40 weeks to the skin of 30 ICR Swiss female mice previously treated with DMBA. No tumors were present after 40 weeks. These findings were confirmed by Bock et al. (46) who painted the dorsal skins of 33 female ICR Swiss mice with DMBA followed by treatment five times weekly for 56 weeks with 3 percent hydrogen peroxide. No skin tumors were produced. Nagata and colleagues (47) found that injections of hydrogen peroxide in female ddN mice significantly reduced the incidence and delayed the appearance of benzo[α]pyrene-induced tumors, suggesting a possible antitumor effect.

Mutagenicity. Since hydrogen peroxide is a radiolytic product of ionizing radiation, its possible role in radiation-induced mutagenesis has been studied extensively. Its effects on isolated DNA, microbial cells, and tumor cells have been explored in numerous reports. Hydrogen peroxide has been shown to be mutagenic to various microorganisms (41,42,48,49) and to mouse ascites tumors (50). DNA degradation, cell damage, and increase in mutants were reported under the specific conditions of the experiments, usually involving the addition of hydrogen peroxide to the media. Although there is a similarity of effect between radiation and hydrogen peroxide, the concentration of hydrogen peroxide produced by radiation must be increased tenfold to achieve the same effects resulting from exogenously-added hydrogen peroxide. Treatment of DNA solutions or bacterial cells with hydrogen peroxide or with X-rays has caused DNA strand breakage (41). Both treatments generated hydroxyl radicals, which are thought to be the active agents.

Inactivation and mutation leading to respiratory deficiency were induced in yeast cells by hydrogen peroxide. This effect was probably due to selection of preexisting mutants in log phase populations, although a small increase in forward mutations of nuclear genes also was reported (48).

Schöneich (50) studied the chromatid aberrations in several lines of mouse ascites tumors after hydrogen peroxide injection. The S2 sarcoma, Erlich ascites carcinoma, and sarcoma 180 were grown in the inbred mouse strain ABJena Gat. One ml of 0.1 M hydrogen peroxide (about 170 mg per kg) was injected intraperitoneally 48 hours after implantation of the tumor. Less than 1 percent of the cells of untreated tumors contained spontaneous chromatid aberrations whereas 4 to 44.7 percent of the examined tumor

cells in the hydrogen peroxide treated animals showed chromosomal changes. The frequency of aberrations varied considerably from animal to animal, but there was a consistent increase with increasing hydrogen peroxide concentrations.

Destruction of essential nutrients. In evaluating the health aspects of hydrogen peroxide, one must consider not only its intrinsic toxicity, but also any secondary deleterious effects which may result from its addition to foods; e.g., the possible destruction of essential nutrients or the production of toxic substances.

As indicated earlier, hydrogen peroxide is authorized as a bactericidal agent in the manufacture of certain cheeses [21 CFR 133.113 et seq.]. Clarified raw milk is treated with hydrogen peroxide equivalent to 0.02 to 0.05 percent by weight of the milk, heated to 52°C for 25 seconds and cooled to setting temperature (30° to 34.5°C). Residual hydrogen peroxide is destroyed by the addition of a small amount of catalase. Hydrogen peroxide has a selective action on bacteria in milk, destroying most of the facultative anaerobic types that are associated with common defects in cheese, while the desirable aerobic acid-forming species are more resistant to the peroxide treatment (51). This treatment produces a higher quality cheese than would result with pasteurized milk. Inasmuch as this process is not equivalent to pasteurization, cheese thus prepared must be held for 60 days before sale, just as if raw milk had been used.

Jasewicz and Porges (52) found that a concentration of 0.02 percent hydrogen peroxide exerted a preservative effect on freshly obtained cheese whey for as long as 10 days. With grossly contaminated whey (2.8×10^7 microorganisms per ml) this concentration of hydrogen peroxide effected 97 percent destruction within 1 hour and 99 percent after 24 hours.

The nutritional quality and wholesomeness of peroxide-treated milk have been studied by various investigators. In a commercial fractionation of whey, a concentration of 0.02 to 0.04 percent hydrogen peroxide is employed to control bacterial growth. Demineralization is achieved by electrodialysis and normally requires 8 to 12 hours at 30° to 38°C. An additional 22 hours are required for lactose crystallization at 50° to 70°C. Any residual hydrogen peroxide is removed by catalase treatment upon completion of the fractionation and prior to spray drying (53,54).

Tepley et al. (55) added hydrogen peroxide to milk at 49°C in amounts sufficient to produce levels of 0.1, 0.2, or 0.5 percent. The milk was held at this temperature for 10 minutes and then cooled to 32°C. Any remaining peroxide was destroyed by the addition of catalase. The amount of added hydrogen peroxide was 2 to 25 times that normally employed in the manufacture of cheese or the processing of whey. There was no significant effect in milk

or whey on levels of thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, folic acid, vitamin B_{12}, vitamin A, or β-carotene. Ascorbic acid was not determined. Treatment of milk with the highest concentration of hydrogen peroxide (0.5 percent) employed lowered the cystine and methionine content of the corresponding cheese by 10 to 25 percent although this effect was not noted in the milk and whey samples. The amounts of tryptophan and lysine were not affected by any of the treatments. Little, if any, reduction in protein efficiency ratios was noted when treated milk, whey, or cheese was fed for 6 weeks to weanling Sprague-Dawley rats as the sole source of protein in an otherwise complete ration. All animals remained in good health and no abnormalities were detected at necropsy. These findings were essentially confirmed by Gregory et al. (56) who treated milk for a much longer period (8 hours) but at a lower temperature (24°C) and hydrogen peroxide concentration (0.05 percent). No vitamin destruction was detected but the nutritive value of the milk proteins was slightly reduced, probably resulting from a slight reduction in the methionine content. Lück and Schillinger (57,58) treated milk with 0.3 percent hydrogen peroxide for 24 hours at 30°C or for 30 minutes at 51°C with no effect on the fat-soluble vitamins A, D_3, and β-carotene or on the water-soluble vitamins thiamin, riboflavin, and pyridoxine. Ascorbic acid, however, was almost completely destroyed by this treatment (58).

Methionine appears to be the only essential amino acid sensitive to hydrogen peroxide treatment. As suggested above, concentrations considerably greater than those employed in cheese making or whey processing are necessary for significant destruction (55,56). Thus, no reduction of methionine content in fish protein concentrates was noted upon treatment at 50°C for 20 minutes with 1.25 percent hydrogen peroxide and only a slight reduction (8 percent) after treatment with 5 percent hydrogen peroxide (59). To ensure the complete oxidation of methionine in casein, Slump and Schreuder (60) heated each kg of the protein with approximately 750 ml of 30 percent hydrogen peroxide (about 200 g) at 30°C for 2 hours. Under these conditions about 75 percent of the methionine in casein was oxidized to methionine sulfoxide, a nutritionally available derivative, and the remaining 25 percent to methionine sulfone, which has no nutritional value (60,61).

Milk exposed to high concentrations of hydrogen peroxide or to lengthy treatment produces cheese with a relatively high moisture content and a soft body (51). These textural changes are presumably manifestations of milk protein alterations. Fox and Kosikowski (62) detected changes in the casein induced by hydrogen peroxide treatment. Sufficient 33 percent hydrogen peroxide was added to a casein solution to give a final concentration of 1 percent. The solution was heated to 85°C and the excess hydrogen peroxide destroyed with catalase. The treated casein was more susceptible to proteolysis, especially by rennin, and formed

a more soluble calcium caseinate than the protein from untreated milk. Other changes in milk and whey proteins have also been reported (63,64). Grindrod and Nickerson (63) treated individual proteins for 30 minutes at 49.9°C or 20 minutes at 26°C with 1 percent hydrogen peroxide. The electrophoretic mobilities of β-casein and of bovine serum albumin were increased while those of α_s-casein and β-lactoglobin were decreased. Treatment of skim milk with 1.0 percent hydrogen peroxide at 49.9°C for 2 hours reduced the whey protein nitrogen from 115 to 100.9 mg per 100 ml, with a corresponding increase in the non-protein nitrogen. Cooney and Morr (64) reported that minimal whey protein denaturation and aggregation occurred at room temperatures with 0.5 percent hydrogen peroxide but that extensive whey protein alteration resulted when higher temperatures (55°C) and peroxide concentrations (1 to 2 percent) were used.

Giolitti (65) reported no change in the lactose and butterfat content of milk treated with 400 mg hydrogen peroxide per liter.

Production of toxic compounds

Lipids. In exploring possible adverse consequences of treating foods with hydrogen peroxide, the Select Committee also considered the possible formation of toxic oxidation products. As an oxidizing agent, hydrogen peroxide theoretically can form a number of reaction products with food constituents, whose nature and significance are largely speculative. Of special interest are the unsaturated fatty acids and the sterols that may be present in the treated foods. Both groups of compounds are vulnerable to oxidation and may yield products with putative carcinogenic or other toxic properties.

A number of vegetable oils and animal fats, oxidized by aeration, have proved toxic when fed to rats, with a rough correlation between toxicity and the extent of oxidation (66-69). Since oleic and linoleic acids are the most prevalent unsaturated acids in edible fats and oils, most studies have focused on their oxidation products in search for the toxic principles. Both peroxides and epoxides of these acids have been investigated. Holman and Greenberg (70) reported LD_{50} values of 6 and 12 mg per mouse after intraperitoneal injection of the peroxides of methyl oleate and ethyl linoleate, respectively, (about 300 and 600 mg per kg body weight). Given by mouth, however, as much as 200 mg of either compound (10 g per kg) failed to kill a single mouse during a 48 hour observation period. Similarly, no deaths resulted among rats fed a daily dose of 75 mg of either peroxide (about 300 mg per kg) for 6 weeks. A single intragastric administration of about 1300 mg per kg methyl linoleate hydroperoxide in rats produced no toxic signs beyond a slight loss in weight (71). The rats died when approximately 5 g per kg of the hydroperoxide were given by stomach tube.