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TOXICOLOGY PROTOCOL

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Background

A report by Viola, Bigotti, and Caputo (Cancer Res., 31: 516 (1971) that vinyl chloride (VCM) may cause skin and/or bone cancer in rats chronically exposed to atmospheres containing up to 3 per cent (by volume) of the compound has recently received renewed attention. The totally unexpected finding that this monomer may be retained in extruded polyvinyl chloride resin after its fabrication into bottles has precipitated a major investigation into the prevalence of this situation and the consequent degree of migration of monomer into foods packaged in such plastic articles.

Meanwhile, the question has arisen whether or not the report of Viola, et al, is per se sufficient to invoke Section 409(c)(3)(A) of the Food, Drug and Cosmetic Act (the so-called Delaney clause). On its face, it would appear that the route of administration was inappropriate for the evaluation of a food additive, and that the concentration (in air) employed was excessive, especially in view of the fact that the currently accepted threshhold limit value of VCM is 500 ppm. Torkelson, et al (Am. Ind. Hyg. Assoc. J., 22, 354 (1961)) suggested a 100 ppm limit based on extensive studies with rats exposed for 7 hours per day, 5 days per week for extended periods. Thus Viola's exposure was probably 300 times higher than an acceptable no-effect level for rats. He reported observing partial narcosis in his animals.

It is generally conceded that extrapolation of toxicity data obtained by inhalation to equivalent dietary levels is unsatisfactory. Large areas of uncertainty exist due to lack of exact information with respect to lung retention factors, difference in metabolic fate between direct inhalation and ingestion, effects of preening by the animals on the ratio between inhalation and ingestion of the test compound, and finally, the possibiliti s of various chemical interactions between the compound and exposed surfaces of either the animal itself or the equipment in which it is housed during exposure. If food is exposed with the animal, absorption into the diet may occur with unknown consequences. The tendency of halogenated unsaturated hydrocarbons to react with free sulfhydril groups on protein molecule- is well known.

On balance, therefore, it is unjustified to conclude that the Viola report constitutes proof that VCM is a carcinogen for rats (or man) when the sole exposure is restricted to trace amounts present in the few foods likely to have been packed, stored, or otherwise held in contact with polyvinyl chloride resin containers. At the same time, direct evidence in the form of animal feeding studies with VCM incorporated in the diet are not known to have been conducted. The technical difficulties inherent in such a study are formidable since VCM is a gas at room temperature. Nevertheless, practical solutions to this problem are not impossib and these will be discussed in succeeding sections of this proposal.

Consideration of dosage levels

A major question in designing a feeding study with VCM is that of choosing a suitable dietary concentration consistent with both the expected toxic effect level and the feasibility of maintaining a known compound intake by the animals. Torkelson (see above) reported that rats maintained in atmospheres having VCM levels of 500 ppm (1.3 mcg/ml) and higher exhibited increased liver weights with some histopathological changes. If one mak s the usual assumptions, (e.g., 50% retention by rats weighing 400 g., with tidal volumes of 2.0 ml and a respiratory rate of 100 per minute), it appears that exposure for 7 hours per day would be roughly equivalent to an ingestion of about 100 mg VCM per kilo of body weight per day or a diet concentration for adult rats of 2000 ppm.

While there can be no assurance that this dietary concentration will be an effect level, in either a sub-acute (90 day) study or in life-time studies, it seems to be the only figure available upon which to base preliminary trials. Thus, if there are to be three test levels employed in the proposed project, they might well be set at 20, 200, and 2000 ppm, at least for the 90-day animals. Should the highest dose suggested above prove too toxic to permit life-time survival, a fourth (lower) level of 2 ppm might be conside

Feasibility studies

Based on the considerations set forth in the report of the Special Task Force of the Ad Hoc Liquor Bottle Committee (SPI) which met on January 24, 1974, the first phase of this project will be an

in vitro evaluation of several alternative methods of VCM administration to rats. An essential first step will be the calibration of an analytical procedure for VCM which is capable of determining its concentration in solutions, pre-mixes, and complete diets. Presumably, either of the gas chromatographic procedures described in "Comments to the Hearing Clerk" under date of October 15, 1973, or modifications thereof, will be found suitable. Initially, two suggestions appear worth careful investigation. These are discussed in turn below:

(1) Adsorption-desorption from activated carbon

The high affinity of various grades of activated carbon for small organic molecules is well known and this property has been made use of in protective devices and in air sampling apparatus to determine atmospheric contamination. VCM is currently determined by this type of adsorption/desorption cycle in standard air-sampling equipment. Thus, an obvious and simple solution to the dietary problem would be to impregnate carbon granules with an appropriate amount of VCM; add the carbon to the diet at pre-determined levels; and finally show that desorption takes place in the gastro-intestinal tract of rats. It would not even be necessary that desorption be complete; only that it be reproducible and proportional to the amount initially adsorbed. Furthermore, some spontaneous loss of VCM during handling and storage would be acceptable provided the amount present when fed is known.

The exploration of this line of attack would be readily carried out at the bench level using small samples and exposing the impregnated carbon to artificial gastric and intestinal fluids under conditions simulating those pertaining in vivo. If the preliminary results appear encouraging, a trial in a live rat using ^{14}C-VCM/carbon complex would be done in an enclosed metabolism chamber with particular attention paid to the route of isotope excretion. If successful, the entire procedure could then be scaled up to provide test materials for feeding studies.

(2) Administration in drinking water

The second approach considered by the Task Force was based on the limited but appreciable solubility of VCM in water and included the development of a closed water delivery system to the individual animal wherein monomer loss would be negligible or at least controlled. It was further suggested that solubility might be increased and vapor pressure of the VCM reduced by dissolving the gas in aqueous solutions of harmless materials, such as a 5% solution of propylene or butylene glycol, both of which are known to be palatable to rats at this concentration in drinking water. Since the adult rat consumes between 30 and 50 ml of water per day, loss from the hanging drop left on the tip of the sipper tube between "drinks" may not be a serious problem and, at any rate, could be allowed for in calculating the daily intake. Should this approach be found feasible, an isotopic tracer trial probably would not be necessary.

While a third approach was included in the Task Force agenda, it has been reported to involve concepts for which no well established model exists. This involved the possible microencapsulation of VCM in edible oil solutions employing shell materials which could be controlled with respect to VCM diffusion rate. While still theoretically possible, this line will be deferred pending the outcome of the first two methods since the cost and time will be considerably greater.

Rat feeding studies

Objective

The obvious purpose of the studies to be described below is to determine whether VCM is in fact a carcinogen for the rat when the route of exposure is via the diet. A secondary objective would be to compare the effects induced by toxic (but sub-lethal) levels between the respiratory and dietary routes, using the data of Torkelson et al (see above) as a guide line. In considering the design of such studies, it is obvious that the primary objective will be served only if the exposure (dosage) levels are within a reasonable range and the duration of the study and the numbers of animals are acceptable within the requirements of current guide line for carcinogenicity trials.

There are three possible protocols which can be considered. The first and most efficient in terms of time and cost combines both the primary and secondary objectives, namely, the determination

of the sub-acute no-effect dosage level after 90-day exposure and the evaluation of carcinogenic potential in a life-time trial. This protocol entails the use of a sufficient number of animals to permit the interim sacrifice of a significant number of rats for 90-day evaluation while the main experiment continues without interruption.

The second plan would contemplate conducting the two studies sequentially. Thus, the sub-acute phase would be finished and reported prior to initiating the carcinogenicity trial. This has some theoretical advantage in that it would provide better data on which to base the selection of the chronic dose levels. However, the total elapsed time is considerably longer, and the cost is higher.

The third alternative would involve only the carcinogenicity study for the life-span of rats and would be based on the assumption that the 90-day phase is unnecessary or would serve no useful purpose.

In presenting the detailed protocols which follow, the assumptic is made that the preliminary feasibility studies will have successful identified a method of dietary incorporation, but allowance is made for frequent and continuous analytical monitoring of diet preparatior to ensure proof of compound intake. It is also assumed that a basic traditional protocol will satisfy the needs for the life-span study and that the observations may be limited essentially to the

recognition and identification of any tumor which may occur in any animal assigned to the project.

Combination protocol

A sufficient number of weanling albino rats (FDRL-Wistar derived stock) will be assigned to individual cages in air-conditioned space and supplied with stock diet (Purina Lab Chow) and fresh tap water, ad libitum, so that after 7 days, and after discarding any unthrifty animals, the following distribution will be made:

Four balanced groups comprised of 125 males and 125 females will be identified in which the average body weight shall not differ by more than 3 grams and in which not more than one male and one female shall have been litter-mates. One group of 250 rats will receive the basal control diet while each of the three remaining groups receive that same basal diet with the incorporation of VCM in a suitable vehicle at three graded levels respectively, designated as the "low", "medium", and "high" test groups. The absolute dietary concentrations will be determined on the basis of the feasibility trials described previously. Each diet shall be made fresh as often as necessary to insure constant VCM intake at the designated levels, confirmed by analysis.

All rats will be observed daily for appearance, behavior, and signs of any toxic reaction to the test substance. Body weights will be recorded weekly on all, and average weekly food consumption

measured for 25 males and 25 females (randomly selected) in each group for the first 10 weeks. During the 11th week, these same 25 animals per sex per group will receive a complete hematological examination, and blood samples drawn for not less than four appropriately selected biochemical determinations. During the 13th week, these 200 animals will be sacrificed for gross pathological examination and a record made of fresh weight of liver, kidneys, heart, spleen, gonads (with adnexa), adrenals and pituitary. Specimens of all organs and tissues (approximately 35 in all) will be preserved in neutral 10% formalin. Liver and kidney (both) from all 200 rats will be paraffin-embedded, cut at 4 microns, and stained with hematoxylin-eosin for microscopic pathologic examination. Since all previous evidence points to the liver as the prime target organ in VCM toxicity, a decision regarding the need for complete histopathology in any of the test groups might well be deferred pending the examination of that organ in all animals. At that point, complete histopathology should be carried out on all organs and tissues from all the rats in the highest dosage group in which no liver pathology was seen. Of course, an equivalent number of control animals would be similarly examined.

Upon submission of a written report covering the foregoing procedures, the 90-day, sub-acute phase of the project will have been completed.

From week 13 forward, all surviving rats will be continued on their respective diets under the same control procedures as before. Every animal will be physically examined at least weekly and palpated for the presence of detectable masses. Body weights will be recorded once monthly, and food consumption will be measured during one week per month on 25 randomly selected rats per sex per group. Weekly weights will be resumed for any animal showing signs of toxicity, debility, or suspected tumors and every effort will be made to sacrifice moribund animals prior to death in order to insure proper preservation of tissues.

The study will continue in this manner until 50% of the initial 800 long-term rats have died or been sacrificed moribund. If high mortality occurs specifically in any one test group, that group may be sacrificed ahead of the remainder upon the death of half of the animals.

Any animal which upon death or sacrifice is found to bear a visible tumor will be subjected to gross dissection and complete histopathological examination of all 35 organs and tissues separate and distinct from specimens of the tumor itself. However, all organs and tissues from every rat will be preserved in neutral formalin and will be available for later processing and examination if needed. Liver and kidneys from all rats will be routinely examined in H & E stained sections, regardless of whether or not a tumor is detected.

Based on experience with control rats of the FDRL colony, the end point in studies of this type can be expected to occur between

24 and 30 months on diet if the test material is essentially non-toxic. Assuming that the terminal procedures will involve the autopsy and tissue processing from approximately 400 rats, followed by microscopic examination of the slides, a final report cannot be anticipated until approximately 3 or 4 months after the last rat is terminated.

Sequential sub-acute and chronic studies

In this plan, the total numbers of animals and the procedur s to be employed would be identical with those described in the combined plan (above). The only difference would be that the first phase would be initiated and completed using 200 rats before th life-time (chronic) study was begun. While the availability of the 90-day data would be the same in either the combined or sequential plans, the completion of the carcinogenicity phase would be delayed by at least 9 to 12 months beyond the time required for the combined study.

Life-time carcinogenicity study

The decision to eliminate the 90-day trial could be made without affecting the protocol or schedule described under the combined study. The total of 800 rats would be used and carried through the same observations, except that 25 animals per sex per group would be followed with respect to body weight and food consumption for the first 10 weeks on the test diets. The option to include hematology and biochemistry at the 90-day point can be left pen for later determination by the Sponsor. All terminal procedure would remain as d scribed above.