A Contribution to the Knowledge of the Structure of Two Hydropericardium-Producing Factors from a Toxic Fat

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Two compounds, previously isolated from a toxic feed fat and capable of producing hydropericardium in chicks, were further characterized. Mass and other spectral data indicate that they are isomers with the molecular formula $C_{14}H_{10}Cl_6$. A procedure was developed for reductive dehalogenation of 1-mg. quantities of chlorinated hydrocarbons, with the resulting saturated hydrocarbons being identified by gas chromatography. Application of this procedure to the toxic compounds produced from each of them a mixture of isomeric perhydrophenanthrenes. This result provides strong evidence that the toxic compounds are hexachlorohexahydrophenanthrenes.

During the fall of 1957, the poultry industry suffered a heavy economic loss when millions of broilers died in the central and eastern parts of the United States. The birds suffered from hydropericardium, edema, ascites, and gross liver and kidney damage. The cause was found to be the use of specific lots of feed-grade animal fats in the manufacture of the chicken feed. Certain batches of USP oleic acids and food products derived therefrom (*1*) were also found to contain toxic factors causing this condition in chickens. This led to a Food and Drug Administration food additive regulation requiring that oleic and stearic acids be "prepared from edible fats and oils, free from chick edema factor" (*3*). The toxic compounds have an unusually high degree of physiological activity for the chicken. It is estimated that 5 μg. of one of the toxic factors is enough to kill one chick. Available information about the composition of the toxic factors makes it almost certain that they are not naturally occurring, but rather are contaminants introduced during the production or processing of the fat. The source of the contaminants is still unknown.

Two compounds which produce hydropericardium in chickens have been isolated in pure form from a toxic fat. The isolation and partial characterization of these compounds have been previously described (*6*). These compounds are referred to there and in this paper in terms of their α values. The α value of a compound is the gas chromatographic separation factor calculated on the basis of the retention volume of the compound relative to the retention volume of a reference material. When a 10-foot column of $^3/_{16}$ inch o.d. packed with 20% silicone on 60/80 Chromosorb W and operating at 250° C was used, the toxic compounds had α values of 3.02 and 3.17 relative to methyl arachidate. Thus the authors refer to these two compounds as α 3.02 and α 3.17.

In this communication, the molecular formula for these materials is deduced from mass and other spectral evidence. The parent ring system of the molecule is established by reductive dehalogenation of the parent compounds and identification of the resulting hydrocarbon.

Experimental Procedure

Mass Spectra. Mass spectra were obtained with a modified (*5*) Atlas CH4 mass spectrometer (Atlas-Werke AG, Bremen, Germany) using approximately 20 μg. of sample. The sample was continuously evaporated into the ion source of the mass spectrometer during the run. The whole mass spectrum (m/e 0-400) was scanned in approximately 3 seconds. This was necessary when earlier runs using a conventional glass heated inlet system (270° C.) and a 180° mass spectrometer (*4*) indicated that the fragmentation patterns were changing with time.

Reductive Dehalogenation. One milligram of the sample, 5 to 10 mg. of Raney nickel W-5 catalyst (estimated), and 2 ml. of recently distilled methylcyclohexane were placed in a glass liner (Figure 1). The liner was placed in a 300-ml. high pressure hydrogenation bomb, and the bomb was packed with glass wool to prevent movement of the liner. The liner was so positioned that the opening in the liner always pointed upward while the bomb was being rocked during the hydrogenation. Twenty-five milliliters of methylcyclohexane was poured onto the glass wool, and the bomb was closed. The bomb was flushed with hydrogen and filled to a pressure of 1800 to 2000 p.s.i. The temperature was raised to 250° C., and the bomb was rocked under these conditions for 12 hours. After 12 hours, the bomb was allowed to cool to room temperature, and the hydrogen was slowly released.

The liner was removed from the bomb, the narrow neck was cut off, and the contents were poured from this end. The liner was thoroughly rinsed with benzene. The catalyst was filtered off and rinsed with benzene. Most of the solvent was then removed by a slow distillation through a glass helices-packed column, $^3/_8$ inch i.d. and 10 inches in length. Final concentration was carried out in a small centrifuge cone at room temperature under a stream of nitrogen. When the volume was approximately 1 ml., the sample was analyzed by gas chromatography.

Gas Chromatography. The column used for the analysis was 10 feet in length by $^1/_8$ inch o.d. It was packed with 10% Dow-Corning high vacuum silicone grease from which the silica was removed by filtration. The support was 80-100 mesh, Siliclad-treated Chromosorb W. Column temperature was 179° C. with an injection port temperature of 190° C. The gas chromatographic unit was an Aerograph model A-90AC-5 (Wilkens Instrument Co., Walnut Creek, Calif.) equipped with a thermal conductivity detector. A helium flow rate of about 28 ml. per minute, and a filament

Table I. Theoretical and Observed Intensities for Six Chlorine Atoms

Theoretical No. of Chlorines, RCl_6			Hydropericardium Factors		
			Rel. intensity		
^{35}Cl	^{37}Cl	Rel. intensity	α 3.02	α 3.17	m/e
6	0	18	18	18	388
5	1	36	36	35	390
4	2	29	26	28	392
3	3	13	12	13	394
2	4	3	7	6	396
1	5	0.4	0.9	0.3	398
0	6	0.02	...	...	400

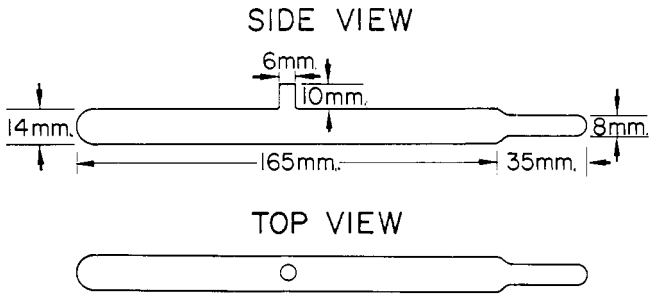


Figure 1. Glass liner used in reductive dehalogenation of 1-mg. quantities of chlorohydrocarbons

current of 300 milliamperes were used. Ten microliters of solution were used, and, if this solution contained approximately 40 μg. of sample, a satisfactory gas chromatogram was obtained.

Results and Discussion

The mass spectra of the α 3.17 and 3.02 materials are shown in Figure 2. Since these mass spectra are essentially identical, the two materials must be isomers. No peaks are observed at larger m/e than 398, so the set of peaks occurring at m/e 388–398 is taken to be the molecular ion, that is, the ion formed by loss of one electron when subjected to electron bombardment in the ion source of the mass spectrometer. Since about 30% of the total ionization occurs in this set of mass peaks and they are the largest peaks in the spectrum, the molecule is probably a relatively stable ring system. This conclusion is based on the fact that in such compounds the molecular ion is the largest peak in the spectrum since the presence of rings has the effect of increasing the parent ion intensity above that in a nonring compound having the same number of carbon atoms. In fused ring systems, this tendency is even more pronounced.

The pattern of peaks at m/e 388-398, differing by 2 units of m/e, suggests a halogenated compound since both Br and Cl have relatively abundant isotopes which differ from the most common one by two mass units. The relative intensities of the peaks at m/e 388, 390, 392, 394, 396, and 398 fit the pattern expected for six chlorine atoms (Table I) and do not fit any other combination of Cl and Br atoms totaling eight (*2*). Thus the authors conclude that both α 3.17 and α 3.02 molecules contain six chlorine atoms. Since m/e 388–398 has been taken as the molecular ion, this means the molecular weight of both of these materials (with six ^{35}Cl) is 388. The chemical molecular weight, taking into account the natural abundance of chlorine isotopes, is therefore 391. The presence of six chlorine atoms was confirmed by chemical analysis as has been discussed previously (*6*).

With a molecular weight of 391 and six chlorine atoms, 178 mass units are left to be accounted for. Since no functional groups other than C—H could be found in the infrared spectra of these materials, the molecule must contain only carbon, hydrogen, and chlorine. This would lead to a molecular formula of $C_{14}H_{10}Cl_6$ or $C_{13}H_{22}Cl_6$ for molecular weight 391. But $C_{13}H_{22}Cl_6$ is the empirical formula of a substituted hydrocarbon containing no ring system and no double bonds. Such a formula is not compatible with either the infrared (*6*), ultraviolet (*6*), or mass spectra of these compounds. Therefore, on the basis of mass and other spectral evidence, the molecular formula for both the α 3.17 and α 3.02 material must be $C_{14}H_{10}Cl_6$.

The fragmentation peaks of interest are those which occur at large m/e. Four sets of high mass peaks are observed —at m/e 353, 325, 262, and 194. The set at m/e 353–361 corresponds to loss of one chlorine atom from the molecule and is to be expected in such a heavily chlorinated compound. The relative intensities of this set of peaks is correct for five chlorine atoms. The set of peaks at m/e 194–199 probably corresponds to doubly charged molecular ion, since the intensities of this set of peaks are correct for six chlorine atoms. This observation lends support to the assignment of m/e 388–398 to the molecular ion. The peaks at m/e 325–333 correspond to loss of 63 mass units from the parent molecule. The relative intensities (in the α = 3.02 spectrum) are correct for five chlorine atoms and correspond to loss of $—C_2H_4Cl$ from the parent molecule to form the ion $C_{12}H_6Cl_5^+$. This set of peaks is also relatively intense, which means there is a large probability that this fragmentation will occur, either because of the ease with which certain bonds in the parent compound are broken or because of the stability of the resulting fragments. Similarly, the set of peaks at m/e 262–270 corresponds, to loss of 126 units from the parent molecule, and the relative intensities are correct for four chlorine atoms. Thus, this set corresponds to loss of two units of $—C_2H_4Cl$ from the parent molecule. The relation between these peaks and the proposed structure of the molecule is pointed out later.

Since the spectral data demonstrated that the two compounds are isomers with the molecular formula $C_{14}H_{10}Cl_6$, the parent hydrocarbon of these compounds is represented by the formula $C_{14}H_{16}$. A number of structural formulas can be drawn to fit this molecular formula, but, if the available data are considered, the most likely structures are a substituted naphthalene or a partially hydrogenated phenanthrene, anthracene, stilbene, or bitolyl. In an effort to demonstrate which, if any, of these structures constituted the hydrocarbon nucleus of the unknown compounds, they were reductively dehalogenated and the resulting perhydro compounds were characterized.

The expected perhydro compounds could be distinguished one from the other by means of gas chromatography. Phenanthrene, anthracene, stilbene, and 3,3′-dimethylbiphenyl were hydrogenated, and the resulting perhydro compounds were analyzed by gas chromatography as described in the experimental section. The gas chromatographic curves obtained are shown in Figures 3 to 6. Although the retention times of 1,2-dicyclohexylethane and 3,3′-dimethylbicyclohexyl were nearly the same, analysis of a mixture of the two gave two distinct peaks demonstrating that these two compounds could be easily distinguished. The gas chromatogram for perhydroanthracene showed three distinct peaks and a shoulder, while that for perhydrophenanthrene had four distinct peaks and a shoulder. Since six stereoisomers of perhydrophenanthrene and five stereoisomers of perhydroanthracene exist, these peaks probably represent stereoisomers. This was demonstrated in the case of perhydrophenanthrene by isolating the four major components from the mixture by collecting fractions from the gas chromatograph and examining each by mass spectrometry. All four components had molecular weights of 192, the correct molecular weight for perhydrophenanthrene, and essentially identical fragmentation patterns.

Next, samples of naphthalene, stilbene, 3,3′-bitolyl, phenanthrene, and anthra-

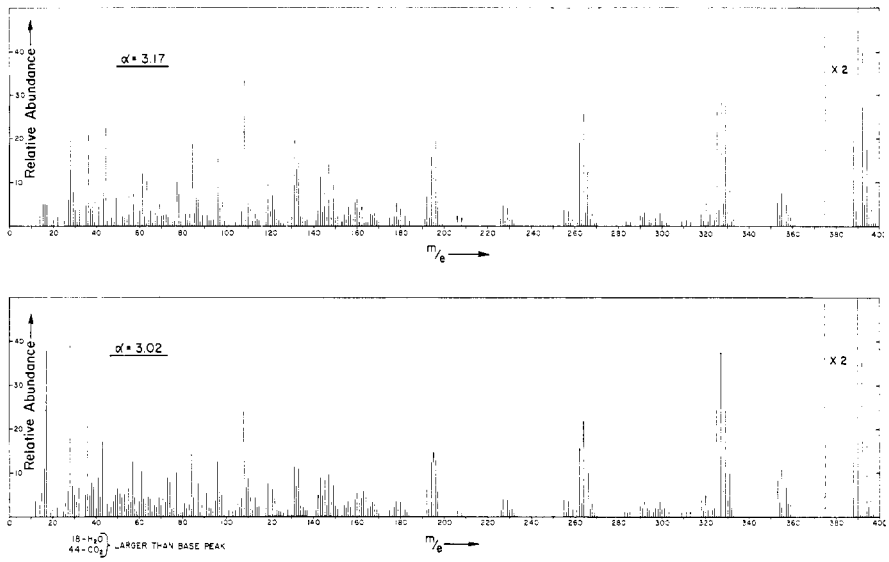


Figure 2. Mass spectra of hydropericardium-producing factors

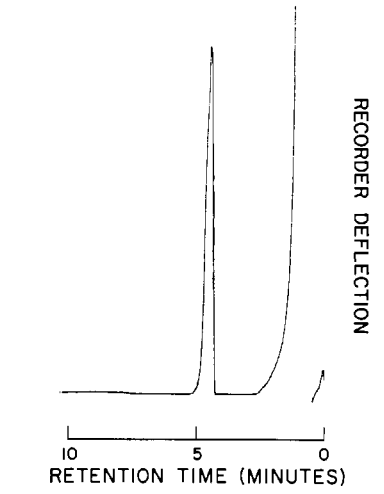


Figure 3. Gas chromatogram of 3,3′-dimethylbicyclohexyl

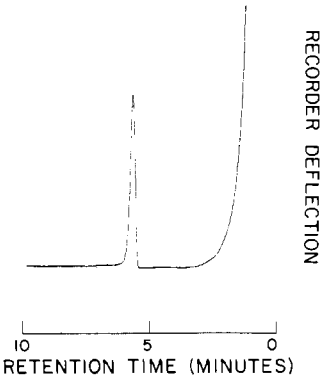


Figure 4. Gas chromatogram of 1,2-dicyclohexylethane

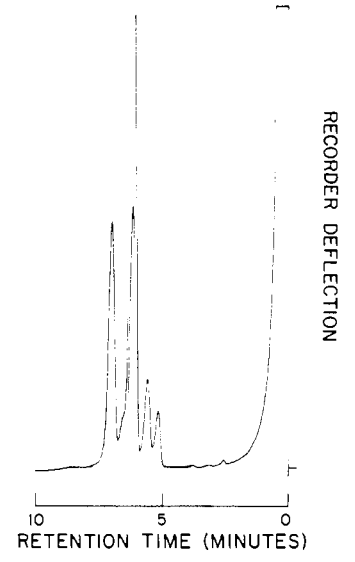


Figure 5. Gas chromatogram of perhydrophenanthrene

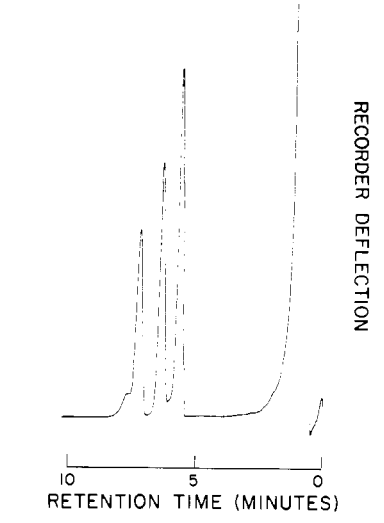


Figure 6. Gas chromatogram of perhydroanthracene.

cene were each chlorinated until the products contained 50 to 60% chlorine. These chlorinations were carried out in CCl_4 in the presence of $FeCl_3$. The complex mixtures thus obtained were hydrogenated as described in the experimental section. The elevated temperature and prolonged reaction time described there were necessary to remove the halogen completely. The halogen apparently tends to poison the catalyst and slow the reaction. In each instance, hydrogenation of the heavily chlorinated compound gave the corresponding perhydro hydrocarbon. With anthracene and phenanthrene, where several isomers were formed in the reaction, the proportions of these isomers tended to vary somewhat from run to run. This variation is thought to be the result of a varying sample-to-catalyst ratio. The quantity of catalyst used was small and necessarily wet with solvent. It could not be accurately weighed and thus represented an uncontrolled variable in the procedure. This does not, however, invalidate or obscure the result of the hydrogenations.

A sample of material recovered from the toxic α 3.17 recrystallization mother liquors was also hydrogenated. This sample consisted primarily of a nontoxic isomer of the toxic α 3.17. A mixture of perhydrophenanthrene isomers resulted from this hydrogenation (Figures 7 and 8).

Similarly, 1-mg. samples of the toxic α 3.02 and the toxic α 3.17 compounds were reductively dehalogenated. In both instances, a mixture of perhydrophenanthrene isomers was obtained. The gas chromatograms of the products obtained from these hydrogenations as well as the chromatograms obtained on

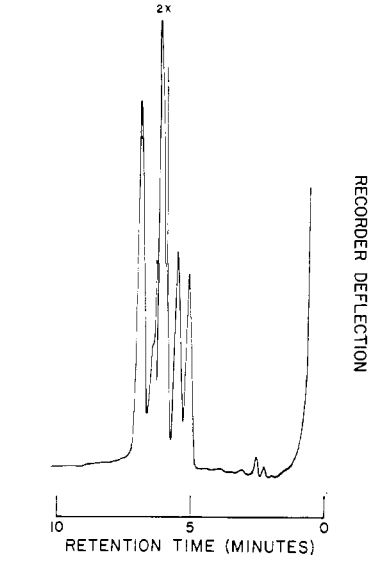


Figure 7. Gas chromatogram of product of hydrogenation of nontoxic α 3.17

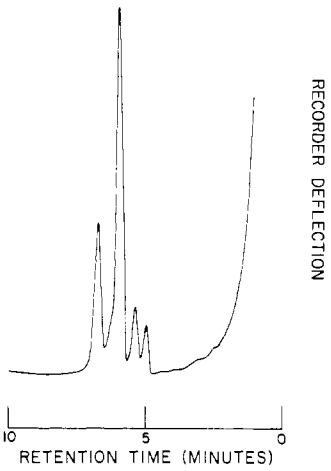


Figure 8. Gas chromatogram of a mixture of perhydrophenanthrene and the product of hydrogenation of non-toxic α 3.17

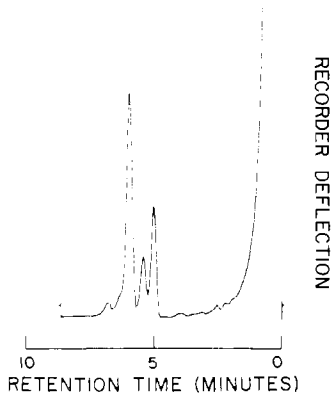


Figure 9. Gas chromatogram of product of hydrogenation of toxic α 3.02

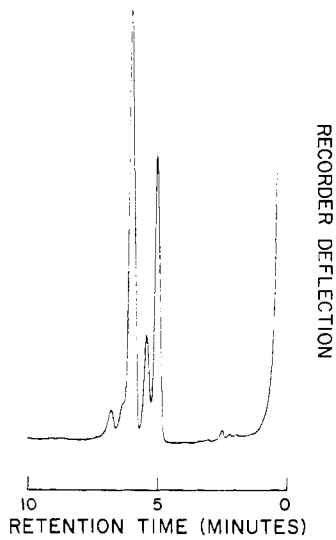


Figure 10. Gas chromatogram of product of hydrogenation of toxic α 3.17

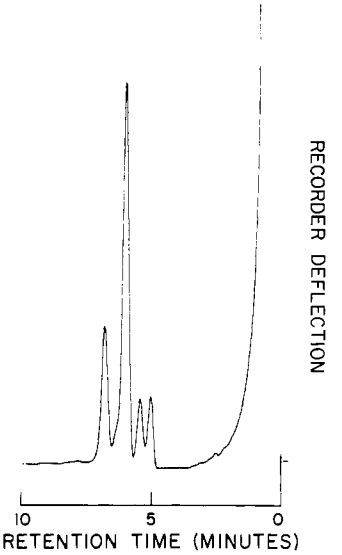


Figure 11. Gas chromatogram of a mixture of perhydrophenanthrene and the product of hydrogenation of toxic α 3.02

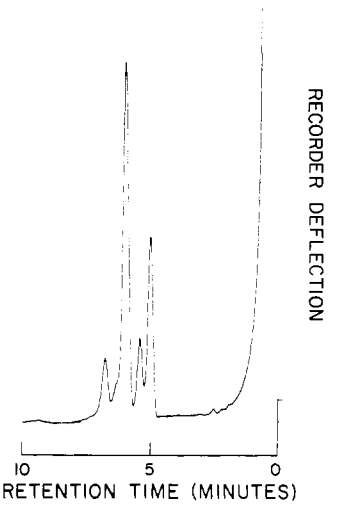
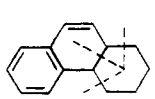


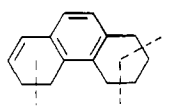
Figure 12. Gas chromatogram of a mixture of perhydrophenanthrene and the product of hydrogenation of toxic α 3.17

mixtures of these products with a sample of authentic perhydrophenanthrene are shown in Figures 9 to 12. These experiments provide strong evidence that the basic hydrocarbon structure of both compounds is that of a partially hydrogenated phenanthrene.

The mass spectral data for both α 3.02 and α 3.17 indicate that the preferred fragmentation of the parent molecule is loss of two units of $-C_2H_4Cl$, suggesting the presence of a dichlorocyclohexane ring in both compounds. The data also suggest that the aromatic ring, the presence of which is demonstrated by infrared and ultraviolet spectral data, is an end ring rather than the center ring. A high intensity peak in a mass spectrum indicates a high probability of formation of the particular fragment and thus implies that it arises by an energetically favorable process. The energy required for the production of a certain fragment from the molecular ion depends upon the energy of the bond ruptured, the stability of the positive ion formed, the stability of the neutral fragment, and the steric arrangement of atoms in the molecule. The most important of these factors appears to be stabilization of the positive charge of the fragment. If the hydrocarbon nucleus of the toxic compounds is 1,2,3,4,11,12 - hexahydrophenanthrene, one would expect fragmentation to occur as shown by the dotted lines:

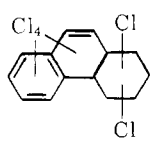


The carbonium ion formed at either carbon number 11 or 12 would be relatively stable since it is stabilized by spreading of the electron deficiency over the entire conjugated system. With the chlorine atoms (at carbon 1 or 2 and 3 or 4) present on the saturated ring, one can visualize the loss of two units of $-C_2H_4Cl$. If the center ring were aromatic, the most likely fragmentation would occur as shown:



The most stable carbonium ions in this case would be those with the positive charge on carbons 1, 4, 5, or 6. If a chlorine atom were present on carbon 2 or 3, loss of $-C_2H_4Cl$ is understandable. However, the loss of two such units is not readily explained. Similar arguments can be made if the double bond is at any of the other possible conjugated positions.

On the basis of these arguments, the authors hypothesize the general structure shown below.



Proof of the position of the aromatic ring and proper assignment of the positions of the chlorine atoms on the hydrocarbon nucleus must await further experimental work.

Acknowledgment

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MALT PASTEURIZATION

Sterilization of Barley Malt with Gamma Radiation

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This study has shown that barley malt can be effectively pasteurized with gamma radiations. When malts irradiated with 0.112 megarad of gamma rays were used in grain alcohol fermentations, saccharifying enzyme activity was not impaired, and no lactobacilli developed within 144 hours. Such malts should provide yields about equivalent to non-irradiated malts with some improvement in distillate quality and uniformity. Estimates based upon extrapolation of published costs for disinfestation of grain suggest a price from $1.79 to $2.77 per ton for pasteurization of grain with 0.1 megarad of gamma rays from cobalt-60.

OFF-FLAVORED DISTILLATES are occasionally produced during the processing of grain mashes to beverage alcohol. In many cases, these off-flavors can be traced to metabolic activities of bacteria that survive the saccharification step in mashing in which the mash is maintained at 145° to 148° F. for a short time. Barley malt is the principal source of these bacteria, which are mainly lactobacilli (*9*). It would be desirable to kill such bacteria before using the malt, but heating, which is the most common sterilizing technique, also inactivates enzymes of the malt. Other possible methods for controlling the growth of these contaminants include the use of antibiotics in the fermenting mashes (*4*) and presterilization of the malt with ionizing radiations. Such radiations selectively kill vegetative cells of bacteria at dosages that do not materially affect enzymes present in the grain. Cathode radiations have been investigated for this purpose (*10*); but gamma rays could be more effective since their penetrating ability is much greater, thus permitting more nearly uniform irradiation of the grain (*6*).

Materials and Methods

Malt samples were irradiated in the large, cobalt-60, gamma-ray source at the University of Michigan. This source has been adequately described elsewhere (*3*). For irradiation, the malt was sealed in tin cans and placed either in the center-well of the source or in locations around the periphery. The cobalt-60 was raised into the radiation room for the required time interval and then returned to the well beneath.

Radiation dosages were determined with Fricke dosimeters (*11*) placed in the center of test cans. When grain was irradiated outside the source, No. 2 tin cans were used. In these positions, gamma radiations came from one side only, so the cans were placed on turntables to produce essentially uniform dosages throughout the grain. For irradiations in the center-well, the grain was sealed into No. 1 picnic cans; here no turntables were needed.

The dose rate in the center-well was essentially uniform and amounted to approximately 0.23 megarad per hour during these experiments. (The terms rad and rep are used in this paper. Rad = dissipation of 100 ergs per gram of tissue; rep = 93 ergs per gram of tissue.) Outside the source, the dose rate varied as a function of distance from the cobalt-60 rods. By placing the cans on turntables at selected distances from the source, dose rates were obtained that permitted the desired amount of radiation to be delivered in the grain over an interval of 2 to 4 hours. The exact interval used varied between these time limits in the various irradiations.

The temperature of the barley was not precisely controlled during irradiation, but averaged about 5° C. Immediately after irradiation, the cans of barley malt were shipped to Peoria for analysis. Changes in numbers of lactobacilli and aerobic bacteria in the malt, as well as effects upon amylase activity and ethyl alcohol yields in test fermentations, were determined and evaluated as a function of radiation dosage.

Malt samples were aseptically ground in a Wiley mill to pass a 20-mesh screen. They were analyzed for α-amylase content and residual bacteria and were evaluated as saccharifying agents in laboratory scale, grain alcohol fermentations. Lactic acid organisms were determined in shake tubes of tomato juice agar as described by Garey *et al.* (*5*) and aerobic organisms by plating in nutrient agar. Colony counts were made after 48 hours of incubation at 37° C. Numbers of viable bacteria reported are the average of triplicate malt samples at each irradiation level.

α-Amylase was determined by a modification of the method of the American Society of Brewing Chemists (*1*) in which 0.47% sodium carbonate was used for extracting the enzyme. Values presented are the average of duplicate determinations expressed in dextrinizing units per gram.

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