variety Early Lethbridge showed slightly more yellowing of foliage than the others, and the reduction in rate of growth was slightly greater in this variety.

JOHN F. MOORE
Agricultural Research Department, Campbell Soup Company, Riverton, New Jersey

References and Notes

1. R. E. Larson and S. Paur, *Proc. Am. Soc. Hort. Soc.* 52, 355 (1948).
2. C. M. Rick, *Rept. Tomato Genet. Coöp.* 8, 51 (1958).
3. ———, *Proc. Am. Soc. Hort. Sci.* 46, 277 (1945).
4. M. M. Lesley and J.. W. Lesley, *ibid.* 71, 339 (1958).
5. L. Hafen and E. C. Stevenson, *ibid.* 67, 355 (1956).
6. The sodium 2,3-dichloroisobutyrate (FW-450) used in this study was supplied by the Rohm and Haas Company, Bristol, Pa.
7. F. M. Eaton, *Science* 126, 1174 (1957).

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Ionizing Energy as an Aid in Exchange Tritium Labeling

Abstract. The tritium labeling of organic compounds by the Wilzbach technique—that is, by simple exposure of the compound to tritium gas—is greatly accelerated by the simultaneous exposure of the system to a silent electrical discharge. The incorporation of tritium into benzene was increased by a factor of about 10^4 without undue decomposition. Cobalt-60 γ-rays were found to be far less useful for increasing the tritium incorporation.

The method described by Wilzbach (*1*) for the labeling of organic compounds by exposure to tritium gas has come into wide use. The compound to be labeled is exposed to subatmospheric pressures of tritium gas at room temperature for a few days. The radioactive gas is then removed, and the exposed compound is rigorously purified by recrystallization, distillation, or chromatography. By this technique, labeled organic compounds with specific activities of the order of 10 mc/g may be obtained.

It has been shown that reactions with recoiling tritons from the labeling gas are not the principal process by which the organic substrate becomes labeled (*2*). The most likely process appears to be reactions between ionized or excited organic molecules and the tritium gas, a supposition furthered by the work of Ahrens *et al.* (*3*), in which was measured the product distribution obtained by exposing hydrocarbons to tritium-labeled hydrogen and to γ-radiation. It therefore seemed to us that the exchange labeling could be greatly speeded up by providing the system with an external source of ionizing energy. For this purpose we applied, separately, a silent electrical discharge and γ-rays from a Co^{60} source to a benzene-HT system (*4*). The electrical-discharge work recalls a recent publication by Wolfgang *et al.* (*5*), in which organic compounds were irradiated with T^+ and T^+_2 ions accelerated with a d-c voltage. In our experiments we used a high-voltage alternating current whose sole purpose was to provide a greater number of excited molecules; no ion accelerations were involved.

Tritium was kept in the form of uranium tritide, UT_3, from which the tritium gas could be obtained by heating the tritide to 450°C. The vacuum line and associated apparatus used to transfer the tritium to the organic substrate were similar to those recently described in *Nucleonics* (*6*).

The effect of the silent electrical discharge was determined in the following way: Benzene (600 μl) was exposed to 40 mc of tritium (partial pressure of $H_2 + HT$, 210 mm) in the apparatus shown in Fig. 1. The volume of the space occupied by the benzene vapor was about 60 cm^3; therefore, only 4 percent of the benzene was in vapor form and the remainder was in liquid form at the bottom of the tube. A silent discharge (alternating current, 20 kv, 1 ma) was passed through the system for 1 hour. The hydrogen-tritium gas was removed, and the irradiated benzene sample was purified to constant specific activity by vapor-phase chromatography. Two passes through a 5-ft paraffin column were enough to accomplish this; further passes through paraffin, Silicone, Carbowax, or Ucon columns led to no change in the specific activity. After the paraffin-column purification, 0.67 mc was found to have been incorporated into 500 μl of the purified benzene. Very little decomposition of the benzene was evident in this experiment. The isotopic percentage of the tritium used was only 0.1, and under these conditions the Wilzbach technique alone (that is, without the accompanying electrical discharge) would have given a total activity of only about 4×10^{-5} mc (or 0.04 μc) in the 500 μl of benzene. It is apparent that, with more tritium in the gas, very high specific activities can be obtained by the electric discharge method.

In a second experiment (with the entire assembly in a horizontal position to spread out the liquid benzene) passage of the current for 5 hours (46.5 mc, 256 mm partial pressure of the H_2-HT gas) resulted in the incorporation of 5.56 mc into a 200-μl aliquot portion of the purified benzene. However, in this experiment only 200 μl of the original 600 μl of the benzene was recovered. Extensive decomposition (roughly 50 percent) was observed to have taken place. Consequently, the conditions of the first experiment, with a shorter time of exposure to the electric discharge, are to be recommended.

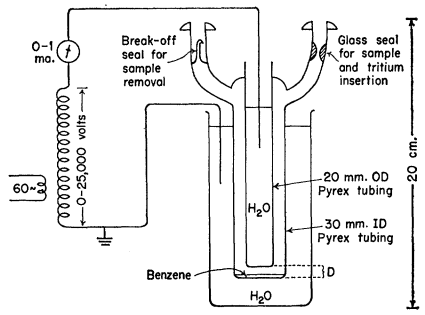

Fig. 1. Electrical discharge apparatus.

Further experiments were performed to test whether a change in the distance *D* (Fig. 1) through the liquid benzene would alter the amount of radioactivity incorporated under conditions otherwise identical. No significant differences were observed between an experiment performed with $D = 5$ mm and another with $D = 25$ mm.

To determine the effect of γ-radiation on the incorporation of tritium into benzene, the following experiment was performed: In each of two similar glass vessels (40-ml volume) were placed 600 μl of benzene and a mixture of H_2 and HT (specific activity 2.014 mc/cm^3 at standard temperature and pressure; total activity 25 mc). The total pressure in each vessel was 330 mm (benzene, 80 mm; H_2-HT, 250 mm). The vessels were then glass-sealed. One vessel was allowed to stand for 24 hours at room temperature. The total energy expended in this vessel by the tritium was 4.7×10^{17} ev, and the energy absorbed by the gases would be almost equal to this amount (*7*). The other vessel was irradiated for 24 hours at room temperature with γ-rays from a Co^{60} source. The total dose delivered to the gases was 2.7×10^{19} ev. The vessels were then opened, through break-off seals, on a vacuum line. The hydrogen-tritium gas was removed, and the irradiated benzene samples were purified to constant specific activity by vapor-phase chromatography. In the "control" benzene a total of 0.565 μc was incorporated; in the γ-irradiated sample a total of 3.85 μc was incorporated. The tritium labeling in the irradiated sample was therefore greater by a factor of 6.8.

A second experiment was performed in exactly the same way as the first except that in both vessels the partial pressure of the H_2-HT was 70 instead of 250 mm, and the total activity was 7 instead of 25 mc. As before, the partial pressure of the benzene was 80 mm. The total energy expended in (and absorbed by) the control vessel was 1.3×10^{17} ev. The gases in the γ-irradiated vessel received

an additional 2.7×10^{19} ev. The total incorporations obtained were 0.16 μc for the control and 1.11 μc for the γ-irradiated sample; thus, the incorporation was increased by a factor of 6.9. This factor is similar to that found in the previous experiment, and the total activities incorporated in the two experiments are, therefore, proportional to the HT concentrations.

These experiments indicate that the γ-radiation does, as expected, increase the rate of tritium labeling. However, the factor of increased labeling (6.8 or 6.9) is much lower than the increase in energy delivered to the system (57 in one case, 207 in the other). Consequently, it appears that the increased labeling is obtained only at the expense of molecular destruction—that is, the γ-radiation increases the ratio of destruction to labeling. It is thus inferior to exposure to higher tritium specific activities as a means of increasing the amount of labeling for a given exposure time.

RICHARD M. LEMMON
BERT M. TOLBERT*
WALTER STROHMEIER†
IRVILLE M. WHITTEMORE
Lawrence Radiation Laboratory, University of California, Berkeley

References and Notes

1. K. E. Wilzbach, *J. Am. Chem. Soc.* 79, 1013 (1957).
2. P. Reisz and K. E. Wilzbach, *J. Phys. Chem.* 62, 6 (1958).
3. R. W. Ahrens, M. C. Sauer, J. E. Willard, *J. Am. Chem. Soc.* 79, 3285 (1957).
4. The work described in this report was supported by the U.S. Atomic Energy Commission.
5. R. Wolfgang, T. Pratt, F. S. Rowland, *J. Am. Chem. Soc.* 78, 5132 (1956).
6. *Nucleonics* 16, 62 (1958).
7. L. M. Dorfman, *Phys. Rev.* 95, 393 (1954).

* Present address: Department of Chemistry, University of Colorado, Boulder.
† Appointment supported by the International Cooperation Administration under a program administered by the National Academy of Sciences. Present address: Institut für Physikalische Chemie, Universität Würzburg, Würzburg, Germany.

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Bilirubin Inhibition of Heme Biosynthesis

Abstract. The conversion of protoporphyrin and iron to heme is catalyzed by a soluble enzyme prepared from rat liver. This reaction is inhibited by bilirubin, and initial kinetic studies suggest that the inhibition is due in part to a competition between protoporphyrin and the bile pigment. Implications of this finding in hyperbilirubinemia are mentioned.

Hyperbilirubinemia, a condition in which the blood concentration of unconjugated (indirect) bilirubin is markedly increased, is a frequent clinical problem in the newborn infant. In these cases hyperbilirubinemia may be accompanied by serious brain injury. Since brain tissues are very sensitive to even mild states of anoxia, any interference with their ability to carry out biological oxidation might result in cellular damage. Bilirubin has already been implicated experimentally in this way through its inhibition of aerobic oxidation and uncoupling of phosphorylation in both brain and liver tissue (*1*). A more specific, but perhaps related, biochemical effect of bilirubin is described in this report (*2*).

In this laboratory we have been studying the mechanism of the reaction by which iron and protoporphyrin combine to form heme (*3*). The reaction is the last step in heme biosynthesis and is catalyzed by a soluble enzyme found in such varied tissues as rat and chick embryo liver, beef heart, chicken erythrocytes, and rat brain. On comparing the structure of one of the substrates, protoporphyrin, with bilirubin it can be seen that they are both tetrapyrroles with identical side chains and that they can exist in spatially similar arrangements. The work of Granick and Gilder (*4*) with *Hemophilus influenzae* has shown that the propionate groups of protoporphyrin attach to the apoenzyme and that the vinyl groups are essential for iron incorporation. This being the case, it is not difficult to visualize how bilirubin could act as a competitive inhibitor of protoporphyrin utilization, since reduction of the γ-methene bridge between the pyrroles bearing the propionate groups would allow restricted rotation but still have little effect on their relative spatial positions, whereas complete removal of the α-methene bridge could so affect the spatial arrangement of the vinyl groups that they would be nonfunctional.

In order to test this hypothesis we used the soluble enzyme preparation obtained from rat-liver mitochondria and both the isotopic and spectrophotometric assays reported previously (*3*). Only one change was made in these procedures—the reaction was stopped after only 20 minutes' incubation to assure that measurements were made at a constant reaction rate. Bilirubin (*5*) was dissolved in 10-percent sodium carbonate, adjusted to *p*H 8 with HCl, and diluted to a concentration of 0.007*M*.

As is shown in Fig. 1, bilirubin inhibited simultaneously the utilization of protoporphyrin and the uptake of iron-59, the latter to a slightly greater extent. Even though the enzyme was in a crude form, preliminary kinetic studies were carried out in an effort to learn whether bilirubin was inhibiting primarily through competition with protoporphyrin. By varying the concentrations of these two compounds and keeping iron

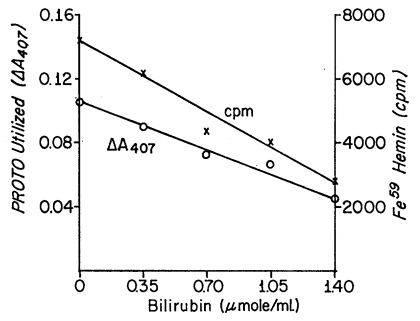

Fig. 1. Effects of bilirubin on heme biosynthesis as measured simultaneously by protoporphyrin utilization and by iron-59 incorporation.

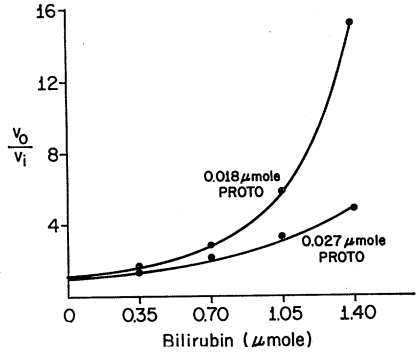

Fig. 2. Kinetics of bilirubin inhibition at different protoporphyrin concentrations.

constant, the data plotted in Fig. 2 were obtained. These data suggest competitive inhibition, since the curves for different substrate (protoporphyrin) concentrations are in the proper relation and intersect the ordinate at unity. However, the curves are not straight lines but increase approximately logarithmically with increasing inhibitor concentration. Thus, there may be competitive inhibition with protoprophyrin with a second effect superimposed, perhaps involving the iron directly. As an inhibitor of this reaction bilirubin is quite active, since 50-percent inhibition is obtained with a bilirubin concentration of $10^{-3}M$ and a bilirubin-protoprophyrin molar ratio of 53.

In conclusion, it should be mentioned that bilirubin inhibits this same reaction in rat-brain homogenate. While the data presented briefly here do not explain the varied clinical manifestations of hyperbilirubinemia, they do show that one action of bilirubin in this disease may be inhibition of the biosynthesis of heme, the prosthetic group of numerous enzymes essential for biological oxidation.

ROBERT F. LABBE
MERLIN R. ZASKE
ROBERT A. ALDRICH
Department of Pediatrics, School of Medicine, University of Washington, Seattle