4. A. Yalçin, *Urbanization and Labour Problems in Turkey* (Cambridge, Mass., 1959).
5. H. Z. Ulken, *Intern. Soc. Assoc. Working Paper No. 3, Suppl.* (London, 1958), p. 1.
6. O. C. Sarc, *Rev. Fac. Sci. Econ. Univ. Istanbul* **9**, 52 (1947–48).
7. ———, *Intern. Soc. Assoc. Working Paper No. 3, Suppl.* (London, 1958), p. 4; R. D. Robinson, *Publ. Opinion Quart.* **22**, 401 (1958).
8. Data were gathered from November 1959 to April 1960.

8 July 1960

## Potassium Dihydrogen D$_s$L$_s$-Isocitrate

*Abstract.* The monopotassium salt is to be preferred to the lactone as the final product of the synthesis of isocitric acid by the method of Fittig and Miller.

In 1889, Fittig and Miller (*1*) described the synthesis of isocitric acid from sodium succinate and chloral by condensation in the presence of acetic anhydride. The resulting trichloromethylparaconic acid was hydrolyzed with barium hydroxide, and the isocitric acid produced was liberated from the barium salt, dehydrated, and isolated as its lactone. Fittig and Miller's procedure was studied in detail in 1946 by Pucher and Vickery (*2*), and several modifications were found advantageous. A yield of lactone free from alloisocitric lactone of approximately 60 percent was obtained in moderately large-scale operations. Further slight modifications of procedure have since been described by Deutsch and Phillips (*3*) and by Kato and Dickman (*4*), but without improvement in yield or convenience.

The excellent properties of monopotassium L$_s$-isocitrate obtained from the leaves of plants of the family Crassulaceae (*5*) suggested that the troublesome lactonization step could be avoided if the monopotassium salt were isolated as the final product of the synthesis. It has been found that the yield of trichloromethylparaconic acid can be increased from about 73 percent (*2*) to about 80 percent, and that the reaction goes more smoothly if a liberal excess over 1 molar proportion of chloral is used during the condensation. If hydrolysis of the acid is carried out with an excess of barium hydroxide, and the insoluble barium isocitrate is filtered from the hot solution, most by-products and impurities remain in the filtrate. The monopotassium salt is then easily isolated as described by Vickery and Wilson (*5*).

Trichloromethylparaconic acid is prepared as follows. Twenty grams of anhydrous sodium succinate, 16.0 ml of chloral (33 percent excess over 1 equivalent) and 12.6 ml of acetic an-

hydride (1 equivalent) are heated in an oil bath for 1 hour at 140°C under a reflux condenser with mechanical stirring. The reaction mixture turns black and becomes viscous, but remains fluid. The tar produced is dissolved in 200 ml of hot water, boiled with 15 g of decolorizing carbon, and the solution is filtered and concentrated *in vacuo* to about 120 ml when separation of sodium salts makes further concentration difficult. The solution is heated to dissolve the salts and 30 ml of concentrated HCl are added. The dark red oil which separates is induced to crystallize by chilling the solution and stirring it with a rod. The mixture is chilled overnight, and the crystals are filtered, pressed down hard in the funnel, and washed with a little ice water. After thorough drying in a vacuum desiccator, the yield is 22 to 24 g (72 to 78 percent). A little more of the acid can be recovered, usually as a few drops of oil, by extraction from the mother liquor with ether. The ether extract is washed with water before being concentrated.

Monopotassium D$_s$L$_s$-isocitrate is then prepared as follows. The crude crystalline trichloromethylparaconic acid, together with the material extracted by ether from its mother liquor, is added slowly to a hot solution of 120 g (20 percent excess over 6 equivalents) of barium hydroxide octahydrate in 150 ml of water, and the thick suspension of barium isocitrate is boiled under reflux with mechanical stirring for an hour in an oil bath. The boiling hot solution is filtered on Whatman No. 3 filter paper covered with a thick layer of Celite, and the precipitate is washed with boiling water. The barium salt is then suspended in cold water and decomposed with a slight excess of sulfuric acid, and the monopotassium salt is isolated by crystallization at $p$H 3.50 as described by Vickery and Wilson (*5, 6*). The yield of this salt is considerably improved if 25 percent of alcohol is added to the concentrated aqueous solution, but the addition of too much alcohol may lead to contamination of the product with alloisocitrate: yield, 14 to 16 g (49 to 56 percent). Most preparations contain between 49 and 50 percent of potassium L$_s$-isocitrate as determined by the isocitric dehydrogenase method of Ochoa (*7*). Once recrystallized from hot water after addition of 25 percent of alcohol, the salt is essentially pure. The solution from which the monopotassium salt is isolated contains isocitric acid and alloisocitric acid in the ratio of approximately 10:1 as determined by chromatographic analysis on Dowex 1 by the method of Palmer (*8*) with formic acid as eluent.

Monopotassium D$_s$L$_s$-isocitrate crystallizes from water in tiny needles which collect in nodular masses and adhere strongly to glass unless the solution is stirred during crystallization. When crystallized slowly from alcohol-containing mother liquors, it forms fascicles of small flattened rhombic needles often aggregated into masses. The salt is soluble in its own weight of water at boiling temperature. The solubility at 0°C of a three-times recrystallized specimen is close to 8 g in 100 ml of water and 1.5 g in 100 ml of 25-percent alcohol. It is thus appreciably more soluble than monopotassium L$_s$-isocitrate. It decomposes at 175° to 176°C with evolution of gas (*9*).

H. B. VICKERY
*Department of Biochemistry, Connecticut Agricultural Experiment Station, New Haven, Connecticut*

**References and Notes**

1. R. Fittig and H. E. Miller, *Ann. Chem. Liebigs* **255**, 43 (1889).
2. G. W. Pucher and H. B. Vickery, *J. Biol. Chem.* **163**, 169 (1946).
3. D. H. Deutsch and R. E. Phillips, *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1957), vol. 3, p. 421.
4. H. P. Kato and S. R. Dickman, *Biochem. Preparations* **3**, 52 (1953).
5. H. B. Vickery and D. G. Wilson, *J. Biol. Chem.* **233**, 14 (1958).
6. ———, *Biochem. Preparations* **7**, 72 (1960).
7. S. Ochoa, *J. Biol. Chem.* **174**, 133 (1948); J. R. Stern, *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1957), vol. 3, p. 428.
8. J. K. Palmer, *Conn. Agr. Expt. Sta. Bull.* **589** (1955).
9. This work was supported in part by a grant from the National Science Foundation.

7 July 1960

## Nucleic Acids in Some Deuterated Green Algae

*Abstract.* In order to determine whether the replacement of hydrogen by deuterium in living organisms is accompanied by changes in amounts and distribution of the cellular components, a preliminary cytochemical investigation has been made on deuterated *Chlorella vulgaris* and *Scenedesmus obliquus*. Cytoplasmic ribonucleic acid is more widely distributed and occurs in higher quantities in deuterated than in nondeuterated algae. Nuclei of deuterated cells are more irregular in shape, and mitotic figures appear with greater frequency in the deuterated organisms.

As part of a study of the effects of deuterium on biological systems, we have made a cytochemical study of some deuterated green algae. The cultivation of *Chlorella vulgaris* and *Scenedesmus obliquus* in 99.6-percent D$_2$O under conditions that lead to essentially fully deuterated organisms has been previously described (*1*). The organisms used in the studies described here were grown in D$_2$O for protracted peri-

ods of months and years. There is thus every reason to believe that the nucleic acids have the same isotopic composition as the D$_2$O medium and that the deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are therefore essentially fully deuterated. The organisms were grown on a rotating shaker and therefore usually occurred as single cells.

Deuteration of the algae results in morphological changes. Size measurements were made with an ocular micrometer on cells chosen at random. Both hydrogen- and deuterium-containing *Chlorella vulgaris* are spherical. The deuterated *C. vulgaris* has a mean diameter of $5.42 \pm 0.033 \mu$ compared with $5.14 \pm 0.041 \mu$ for the hydrogen (protium) prototype. The deuterated organisms are thus substantially larger than the hydrogen organisms. In the case of *Scenedesmus obliquus*, both size and shape are altered by deuteration. Measurements were made along the widest and the longest parts of the organisms. Deuterated *S. obliquus* had a mean length of $7.99 \pm 0.050 \mu$ as compared with $6.46 \pm 0.056 \mu$ for the hydrogen organisms, while the mean widths were $3.91 \pm 0.044 \mu$ for the deuterated organisms and $4.16 \pm 0.030 \mu$ for the hydrogen-containing *S. obliquus*. The deuterated *S. obliquus* are thus longer and thinner than their prototypes.

Cytochemical reactions were performed after fixation by freeze-substitution. A cell suspension was spread on a slide and immersed in liquid nitrogen. The slide was then rapidly moved to chilled absolute alcohol. The slides could be stored by clearing them in xylene and coating them with paraffin.

Conclusions regarding the amount and distribution of the nucleic acids were based on agreement of results from several staining reactions and extraction procedures.

The nucleic acid stains used were modifications of the following: Azure B (2), Feulgen (3), Methyl Green (4), and pyronin (4). Under the conditions which were employed, material that stained with both Methyl Green and with the Feulgen reagent and which was removed by treatment with 0.1-percent deoxyribonuclease (buffered at $p$H 6.5) was regarded as DNA. Pyronin-staining material which was removed by extraction with 0.2-percent ribonuclease (buffered at $p$H 6.0) was regarded as RNA. Azure B stains both sorts of nucleic acids as well as some other basophilic substances. The various basophilic substances are reputed to acquire different colors by Azure B staining. However, the presence of chlorophyll in the algae made determination of exact colors, especially in the blue-green region, difficult. Azure

B staining, therefore, was simply regarded as indicative of basophilia.

When such basophilic material was removable by treatment with 5-percent trichloroacetic acid it was regarded as nucleic acid. That portion of the Azure B staining material which was removed by ribonuclease treatment was regarded as RNA. The deoxyribonuclease-removable, Azure B positive material was thought to be DNA.

The enzyme reactions were controlled by treating slides with buffers identical to those in which the enzymes were dissolved under the same conditions of time and temperature. In this study the assumption has been made that the staining characteristics of deuterated compounds are the same as

for the corresponding hydrogen compounds. Although this is very likely the case, it is a point that requires verification for more quantitative studies.

A comparison of stained cells of deuterated and nondeuterated algae reveals that staining for both RNA and DNA is less localized in deuterated cells. This is the case for both *Scenedesmus obliquus* and *Chlorella vulgaris*, but the situation is seen in its simplest form in a comparison of deuterated and nondeuterated *S. obliquus*. In nondeuterated *S. obliquus*, DNA (Feulgen, Methyl Green, and Azure B stainable; deoxyribonuclease and trichloroacetic acid extractable) is confined to and fills the peripheral nucleus. The nucleus
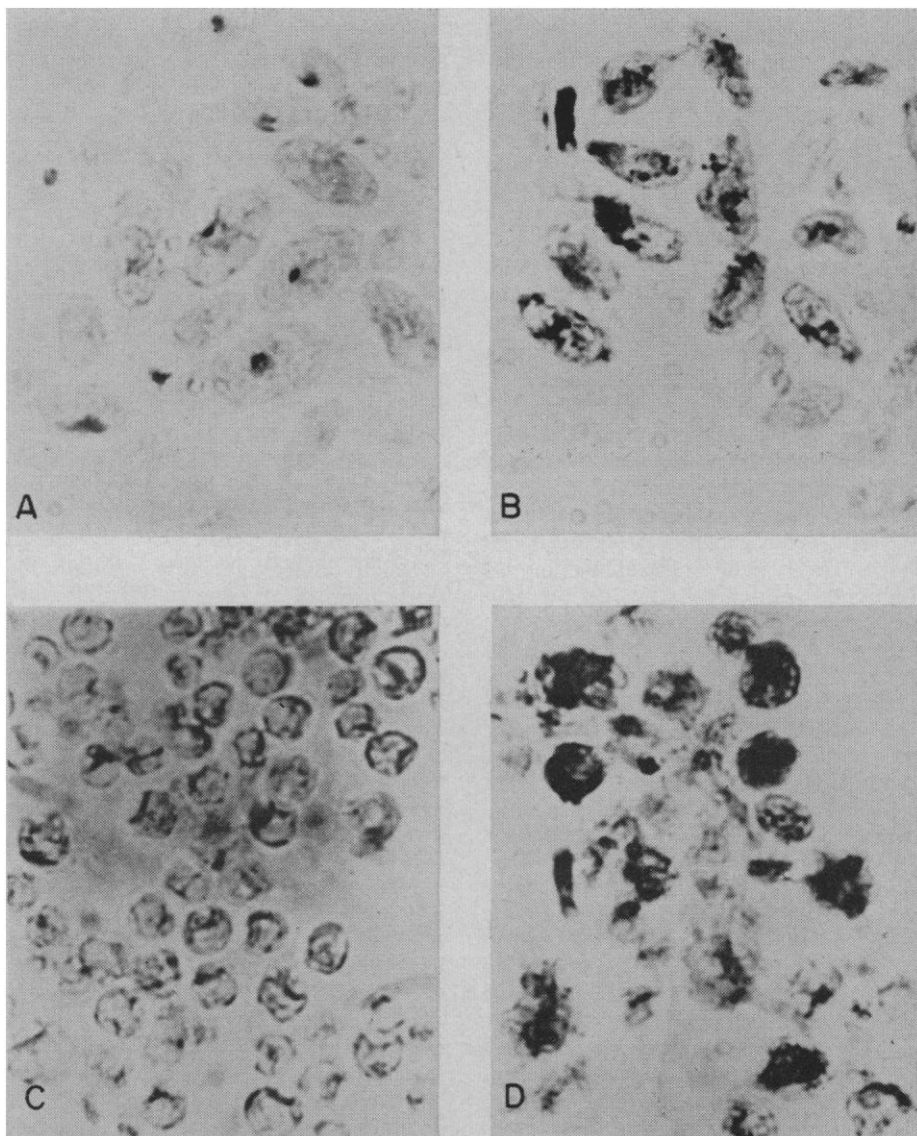


Fig. 1. Algae stained for both DNA and RNA with Azure B ($\times$1700). (*A*) Nondeuterated *Scenedesmus obliquus*. The nucleic acids are largely concentrated in the nucleus. (*B*) Deuterated *S. obliquus*. More cytoplasmic RNA is present and the cell nuclei are more irregularly shaped. (*C*) Nondeuterated *Chlorella vulgaris*. The strongly basophilic peripheral structure is the chloroplast. The nucleus is more centrally located and is stained less intensely than the chloroplast. (*D*) Deuterated *C. vulgaris*. Far more cytoplasmic basophilia occurs than in the nondeuterated organisms.

contains some RNA (pyronin and Azure B stainable; ribonuclease and trichloroacetic acid extractable) as well. The small amounts of cytoplasmic RNA are localized close to the nucleus. In corresponding deuterated cells, the nucleus is larger and appears to contain greater amounts of DNA; more striking than this, however, is the occurrence of far larger amounts of cytoplasmic RNA which is much more widely distributed throughout deuterated cells (Fig. 1, A and B).

The widespread dissemination of nucleic acids throughout the deuterated cells is also observed in Chlorella vulgaris. The chloroplasts of C. vulgaris, unlike those of S. obliquus, contain (according to the previously mentioned criteria) both DNA and RNA. The presence of larger amounts of cytoplasmic nucleic acids in deuterated C. vulgaris as compared to the nondeuterated organisms is clearly visible in Fig. 1 (C and D).

The basophilia shown in these photomicrographs is caused by both DNA and RNA. In C. vulgaris DNA fills the nucleus and occurs at the periphery of the chloroplast. Ribonucleic acid occurs in the same regions as DNA, but is also found more centrally in the chloroplasts and in nonchloroplast cytoplasm. This is true of both deuterated and normal cells. However, the nucleic acid-containing structures of deuterated algae are larger and stain more intensely than do their hydrogen counterparts.

Deuterated algae generally show increased heterogeneity of chloroplast shape. The typical single chloroplast of the nondeuterated cells was, in the deuterated cells of both S. obliquus and C. vulgaris, replaced by a great many varieties of chloroplast shape and number.

Some individuals in both deuterated and nondeuterated C. vulgaris appear to be multinuclear. Occasionally a nuclear configuration which resembles a telophase was observed in C. vulgaris. This configuration was observed with so much greater frequency in deuterated cells than in nondeuterated cells that it seems as though nuclear division had been delayed by deuteration. Since in these cells telophase is the easiest stage of mitosis to recognize, it cannot be said at this time that other stages of mitosis are not also affected by deuteration. That the nucleic acid-containing structures have grossly distorted shapes in deuterated algae may be only a reflection of the larger amounts of nucleic acid present, but it is probable that other factors are involved.

Although mitosis appears to be delayed in the algae, it is clearly not prevented. The situation here is clearly

894

different from the arrest of mitosis of Arbacia eggs caused by high concentrations of $D_2O$ as described by Gross and Spindel (5). But even in the case of the green algae, the effects of isotopic substitution are already evident on the cytochemistry of the organisms (6).

E. FLAUMENHAFT
Hiram College, Hiram, Ohio
S. M. CONRAD
J. J. KATZ
Argonne National Laboratory,
Argonne, Illinois

**References and Notes**

1. H. L. Crespi, S. M. Archer, J. J. Katz, Nature 184, 729 (1959); W. Chorney, N. J. Scully, H. L. Crespi, J. J. Katz, Biochim. et Biophys. Acta 37, 280 (1960).
2. M. H. Flax and M. Himes, Physiol. Zool. 25, 297 (1952).
3. C. Leuchtenberger, in General Cytochemical Methods, vol. 1, J. F. Danielli, Ed. (Academic Press, New York, 1958), pp. 219–278.
4. N. B. Kurnick, Stain Technol. 27, 233 (1952).
5. P. R. Gross and W. Spindel, Science 131, 37 (1960).
6. This work was carried out under the auspices of the U.S. Atomic Energy Commission. E. Flaumenhaft acknowledges with thanks support from the National Institutes of Health.

20 June 1960

## Staining of Skin with Dihydroxyacetone

Abstract. The reaction of skin with dihydroxyacetone to produce a brown "artificial tan" appears to proceed through combination with free amino groups in skin proteins, and particularly by combination of dihydroxyacetone with the free guanido group in arginine.

Much interest centers around the mechanism of staining of skin by solutions of dihydroxyacetone, a three-carbon keto sugar. Our interest in this sugar arose from observations on excretion of it following ingestion of the substance in a diagnostic test for glycogen-storage disease (1). Urine specimens obtained from children after dihydroxyacetone-tolerance tests (2) gave several conspicuous spots on chromatograms resolved in isopropanol–ammonia–water solvent treated with diazotized sulfanilic-acid sodium-carbonate reagent (Pauly reagent). No reaction was observed when the same reagent was applied to duplicate chromatograms resolved in butanol–acetic acid–water solvent. This reagent ordinarily reacts with substances which contain an active hydrogen, an aromatic phenol or amine, or substances with imidazole nucleus. An area at $R_F$ 75 (in isopropanol–ammonia–water) turned brown on standing a short time in air, more rapidly on heating. It seemed likely that a reaction occurred between dihydroxyacetone and ammonium hydroxide to form new compounds.

Analyses, by the micro-Kjeldahl method, of areas of the chromatogram which gave positive reactions with Pauly reagent and blank areas indicated that the substance at $R_F$ 75 contained nitrogen. The stability of the reaction products was confirmed when the substances were eluted from chromatograms run in isopropanol–ammonia–water and rechromatographed in butanol–acetic acid–water solvent with no apparent change. Reactions of two principal substances derived from interaction of dihydroxyacetone and ammonium hydroxide are given in Table 1.

These observations led to the conjecture that the staining of skin by dihydroxyacetone might occur through combination with basic groups of skin proteins, or with free amino acids on the surface of the skin. Aqueous solutions of the sugar with ammonium hydroxide alone produced a brown color. The reactions with amino acids and related compounds are shown in Table 2. Arginine was the most reactive, with the appearance of a dark brown color within 30 minutes. Mixtures of dihydroxyacetone with glycine, lysine, and histidine also gave brown to yellow colors.

Chromatography of the mixtures of amino acids with dihydroxyacetone showed that while a positive test for arginine was obtained with ninhydrin, there was no reaction with aniline-phthalate, a sensitive reagent for sugars, at the position usually occupied by dihydroxyacetone. However, in the mixture of arginine and dihydroxyacetone another compound was formed which was positive both with ninhydrin and aniline-phthalate. In the case of glycine at least two additional ninhydrin-positive, aniline-phthalate-positive substances were formed. These reaction products, unlike those obtained from dihydroxyacetone and ammonium hydroxide, did not give a strong positive test with Pauly reagent. Similar observations were made of reaction mixtures of lysine, alanine, proline, and hydroxyproline, even though in

Table 1. Reactions of substances obtained from combination of dihydroxyacetone (DHA) and ammonium hydroxide.

| | Substance | | DHA |
|---|---|---|---|
| | A | B | |
| $R_F$ INH* | 0.75 | 0.88 | 0.70 |
| $R_F$ BuAc† | .48 | .70 | .60 |
| Pauly reagent | Red-orange | Red | — |
| Ninhydrin | — | — | — |
| Aniline-phthalate | — | — | + |
| Phosphomolybdate | — | — | + |
| Arsenomolybdate | — | — | + |
| Brown color on standing in air | + | — | + |
| N (micro-Kjeldahl) | + | — | — |

* Solvent: isopropanol-ammonia-water.
† Solvent: butanol-acetic acid-water.