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Occurrence of Fatty Acid Chlorohydrins in Jellyfish Lipids†

Robert H. White‡ and Lowell P. Hager*

ABSTRACT: Fatty acid chlorohydrins are characterized as lipid components of an edible jellyfish. The four isomers 9-chloro-10-hydroxypalmitic acid, 10-chloro-9-hydroxypalmitic acid, 9-chloro-10-hydroxystearic acid, and 10-chloro-9-hydroxystearic acid were identified by gas chromatography–mass spectrometry comparison of the methyl esters and their trimethylsilyl derivatives with known synthetic samples. Two additional isomers, 11-chloro-12-hydroxystearic acid and 12-chloro-11-hydroxystearic acid, were also found in the lipid by the identification of the expected mass spectral fragments of the trimethylsilyl (Me_3Si) derivative of their methyl esters. These six isomeric compounds represented approximately 1.4% of the total extractable jellyfish lipid and were released from the lipid as methyl esters by boron trifluoride–methanol treatment. These isomers account for only about 30% of the organic chlorine in the lipid. Evidence is given that the remaining organic chlorine is also present as fatty acid chlorohydrins containing more than one hydroxyl group.

Monohydroxyl fatty acids are found to occur widely in nature. Their structures, in terms of stereochemistry, length of carbon chain, position of the hydroxyl group, and the organisms from which they are isolated, are quite diverse (Downing, 1961; Pohl and Wagner, 1972; Fulco, 1974). In this paper we report the occurrence of a new series of hydroxyl fatty acids which contain organic chlorine adjacent to the OH group, i.e., chlorohydrins. These jellyfish chlorohydrins were discovered as a result of our survey of marine organisms for halometabolites (Hager et al., 1974). We originally noted high levels of organic chlorine containing compounds in lipid extracts of jellyfish which were collected in Falmouth Bay and assayed at the Woods Hole Biological Laboratories, Woods Hole, Mass. The following six isomeric acids, 9-chloro-10-hydroxypalmitic, 10-chloro-9-hydroxypalmitic, 9-chloro-10-hydroxystearic, 10-chloro-9-hydroxystearic, 11-chloro-12-hydroxystearic, and 12-chloro-11-hydroxystearic, were identified as their methyl esters from a species of jellyfish (phylum Cnidaria, class Scyphozoa). These acids represent the first examples of organic chlorine compounds isolated from this animal class. They add to the growing list of halogenated compounds derived from fatty acids (Mooney et al., 1972; Fenical, 1975).

Experimental Section

Materials

Frozen jellyfish distributed by the Japan Food Corp., San Francisco, Calif., was obtained at a local market. The thin-layer chromatography (TLC)[1] plates were purchased from Brinkmann Instruments, Inc. Methyl palmitoleate was obtained from Applied Science Laboratories. All other chemicals were reagent grade and were obtained commercially.

Methods

Isolation of Lipids. Lipids were extracted from the thawed jellyfish by homogenization with 16 volumes of benzene–

† From the Rodger Adams Laboratory, Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received May 17, 1977.* This work was supported by grants from the National Science Foundation (NSF BMS 71-01280) to L.P.H. and by a grant from the National Institutes of Health (HEW PHS CA 11388) for the University of Illinois Mass Spectrometer Facility.

‡ Present address: Department of Biochemistry, Rice University, Houston, Texas 77001.

[1] Abbreviations used: TLC, thin-layer chromatography; Me_3Si, trimethylsilyl; GC, gas chromatography; MS, mass spectrometry.

methanol (1:1, v/v) in a Virtis 45 tissue homogenizer (Model 16-900), following by filtration to remove undissolved material. Water (5 parts) was then added and, after shaking, the lipid was recovered from the benzene layer. Fatty acid methyl esters were prepared by heating 80 mg of this lipid extract with 2 mL of 14% boron trifluoride–methanol and 1 mL of benzene for 3 h at 50 °C in a capped tube (Morrison and Smith, 1964). After cooling and the addition of water, the methyl esters were recovered from the benzene layer.

Preparative TLC was run on 20 × 20 cm silica gel F-254 glass plates (2.5-mm thick). The desired areas were scraped from the plate, packed in small columns, and eluted with benzene–methanol (2:1, v/v).

Analyses of Lipids. The original lipid extract and subsequent fractions were assayed for organic halogen content as previously described (White and Hager, 1977). Glycerol analysis of the lipid fractions was performed by the method of Wells and Dittmer (1965). Organic phosphate and hexose analyses were performed as described by Dittmer and Wells (1969a,b).

Synthesis of Known Fatty Acid Chlorohydrins. An equal mixture of 9-chloro-10-hydroxystearic acid and 10-chloro-9-hydroxystearic acid was prepared by epoxidation of oleic acid with peracetic acid in acetic acid followed by the addition of HCl in ether to form the chlorohydrin. The resulting product was repeatedly crystallized from hexane at 0 °C to the point where gas chromatography of the methyl ester gave only one peak as either the free hydroxyl or the trimethylsilyl (Me_3Si) derivative. The resulting pair of fatty acid chlorohydrin isomers was converted to methyl esters by reaction with boron trifluoride–methanol as described by Metcalfe and Schmitz (1961). Oxidation of a portion of the fatty acid chlorohydrin with Kiliani reagent (Kiliani, and Merk, 1901) led to the isolation of the chloroketone. Anal. Calcd for $C_{18}H_{33}O_3Cl$ (332.9): C, 64.93; H, 9.99; Cl, 10.65. Found: C, 64.80; H, 9.92; Cl, 10.68.

An equal mixture of 9-chloro-10-hydroxypalmitic acid and 10-chloro-9-hydroxypalmitic acid methyl esters was prepared from a 25-mg analytical grade sample of palmitoleic acid methyl ester. This compound was converted to the epoxide by reaction in methylene chloride with an excess of *p*-chlorobenzoic acid. The epoxide was then converted to the chlorohydrin by reaction with HCl in ether.

Preparation of Me_3Si Derivatives. Me_3Si ethers were prepared by reacting ~2 mg of the synthetic fatty acid methyl esters or lipid methyl esters with 0.1 mL of a mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (5:5:1, v/v/v). After 1 h at room temperature, 1 mL of water was added to the above mixture which was then extracted with hexane. The hexane layer was dried over sodium sulfate and reduced in volume for subsequent analysis.

Gas Chromatography and Mass Spectrometry. Gas chromatography–mass spectrometry data were obtained with a Varian CH-7 mass spectrometer interfaced with a Varian series 1700 gas chromatograph. All spectra were recorded at an ionization potential of 70 eV. Separations were effected with 12 ft × 1/8 in. i.d. glass columns containing either 3% OV-17 or 3% OV-225 on Gas Chromosorb Q and temperature programmed as indicated in the text. The injection port temperature was maintained at 280 °C, the separator at 300 °C, and the ion source at 270 °C.

Results and Discussion

The isolated jellyfish lipids contained 0.48% chlorine and a trace of bromine (0.0034%). All of the chlorine appeared to be covalently bound to organic molecules in the lipid, because control experiments in which radioactive chloride ($^{23}Na^{36}Cl$) was added to crude lipid extract did not lead to the incorporation of detectable levels of radioactivity. A total of 227 mg of lipid was obtained from 113 g of jellyfish tissue. Fractionation of a portion of jellyfish lipids into simple and complex lipid classes after the method of Börgstrom (1952) led to no significant fractionation of the organic chlorine; i.e., both fractions still had a chlorine content of approximately 0.48%. In an attempt to improve this fractionation, 50 mg of the lipid was applied to a preparative TLC plate and chromatographed in the solvent system benzene–methanol–acetic acid (95:5:1, v/v/v). After development of the plate, it was divided into eight sections from which the lipid was eluted with benzene–methanol (1:1, v/v), weighed, and assayed for organic chlorine. Table I summarizes the results of this analysis.

TABLE I: Analysis of Preparative TLC of Total Jellyfish Lipids for Organic Chlorine.[a]

Fraction no.	Area of plate eluted			% organic chlorine	% of total material isolated	% of organic Cl isolated
	R_f	to	R_f			
1	0.00		0.027	1.37	4.7	12.5
2	0.027		0.25	1.01	13.6	26.4
3	0.41		0.81	0.86	6.4	11
4	0.35		0.41	0.13	37.3	9.4
5	0.31		0.35	0.71	16.4	22.7
6	0.25		0.31	1.11	4.7	10.7
7	0.81		0.89	0.098	2.7	0.50
8	0.89		1.0	0.25	14.2	6.8

[a] Original sample was 0.48% chlorine. The solvent system used was benzene–methanol–acetic acid (95:5:1). See text for details.

Of the eight fractions assayed, five had been enriched in chlorine and three had been reduced. No single fraction contained the major portion of the chlorine. These data are consistent with the occurrence in the lipid of several different chlorine containing compounds of varying polarity. However, the distribution of organic chlorine was dramatically different when the jellyfish lipids were first treated with boron trifluoride–methanol to esterify fatty acids present in the extract.

Following boron trifluoride–methanol treatment, 80 mg of the fatty acid methyl esters derived from the lipid was fractionated by preparative TLC. With the solvent system hexane–diethyl ether–acetic acid (90:10:1, v/v/v), 4.3 mg of lipid and 30% of the chlorine were recovered in a fraction having a R_f value of 0.22–0.38. This fraction, which will be referred to as the hydroxy acid fraction, contained 4.0% organic chlorine and produced nine major GLC peaks when examined on a 3% OV-17 column programmed from 215 to 290 °C at 4 °C/min (Figure 1). None of these peaks showed the presence of chlorine in the mass spectra of the organic fragments; however, peaks were present at *m/e* 36 ($H^{35}Cl$) and *m/e* 38 ($H^{37}Cl$) with ratios of approximately 3:1 for peaks D, E, F, H, and I. A strong similarity exists among the mass spectra obtained for peaks D and E, and H and I. The ions at *m/e* 253 ($M^+ - HCl - OCH_3$) and *m/e* 266 ($M^+ - HCl - H_2O$) seen in D and E occur 28 mass units higher in H and I at *m/e* 281 (M^+ HCl – OCH_3) and *m/e* 294 ($M^+ - HCl - H_2O$). This implies that D is an isomer of E, and H is an isomer of I and that the two sets differ by a C_2H_4 unit. Peak F had the retention time and mass spectrum of an isomer of peak D but 14 (CH_2) mass units higher. Intense peaks at *m/e* 74 and groups of ions spaced at mass intervals of *m/e* 14 for each of these peaks are typical of fatty acid methyl esters. When the TLC polarity of this fraction and its strong infrared absorption at 3400 cm^{-1} were

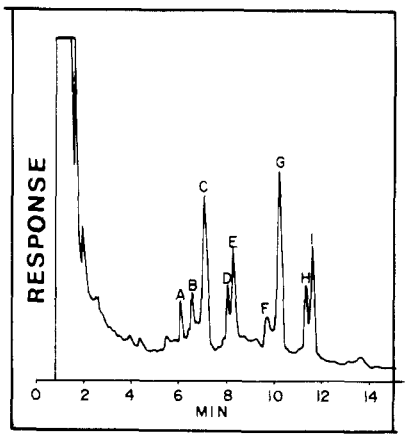

FIGURE 1: Gas chromatographic trace of hydroxy acid fraction from boron trifluoride–methanol treated jellyfish lipids. Conditions are as described in the text. Mass spectra were obtained for each lettered peak. Chlorine was indicated only in peaks D, E, F, H, and I. The sample contained 4.0% organic chlorine.

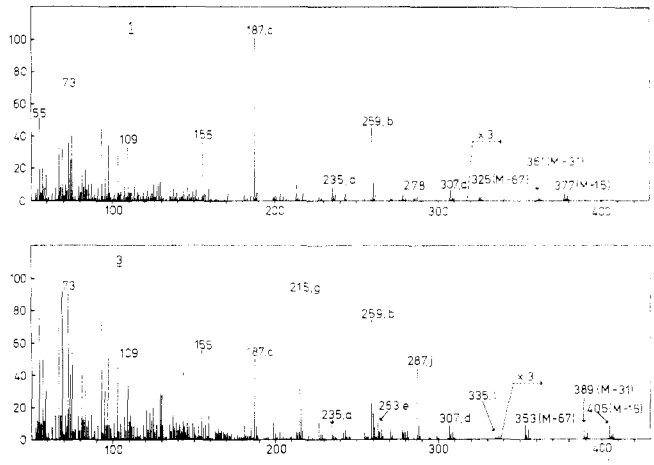

FIGURE 3: Mass spectra of peaks 1 and 3 of the GC trace shown in Figure 2. Molecular ions were not observed but fragments corresponding to M-CH_3, M-OCH_3 and M-HCl, -OCH_3 were observed. Each spectrum is generated by several different isomers in each GC peak.

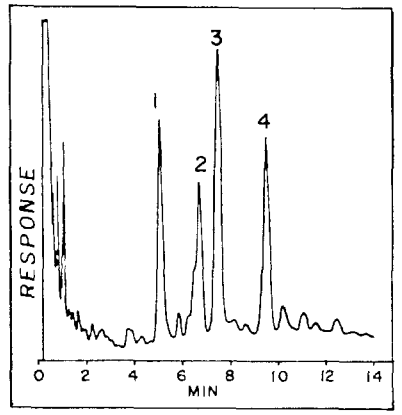

FIGURE 2: Gas chromatographic trace of the Me_3Si derivative of the hydroxy acid fraction from boron trifluoride–methanol treated lipids. Chlorine was present only in peaks 1 and 3.

considered, it was clear that we were dealing with hydroxylated fatty acids.

In order to determine the number and position of the hydroxyl groups on the fatty acid methyl esters, they were converted to their corresponding Me_3Si derivatives. Figure 2 shows the GC trace produced by separation of the Me_3Si derivative of the hydroxy acid fraction on a 3% OV-225 column programmed at 4 °C/min from 200 to 245 °C.

Mass spectra of the major peaks showed only 1 and 3 to contain chlorine since only these two peaks showed the characteristic *m/e* 36 and 38 isotope peaks of chlorine.

The mass spectrum of peak 1 (Figure 3) has ions containing one chlorine at *m/e* 377 and one at *m/e* 361 corresponding, respectively, to the loss of a methyl and a methoxy group from the molecular ion at *m/e* 392. In addition, an ion present at *m/e* 325 (containing no chlorine) is consistent with the loss of both HCl and methoxy from the molecular ion. The mass spectrum of peak 3 (Figure 3) shows this same series of ions, but increased by 28 *m/e*. These are the expected fragments for the Me_3Si derivatives of the methyl esters of stearic and palmitic acids having one chlorine and one hydroxyl group.

Considering the similarity of peaks D and E, and H and I (Figure 1) and the reduction of the number of peaks in Figure 2, we expect peak 1 to contain the Me_3Si derivatives of compounds D and E, and peak 3 to contain the Me_3Si derivatives of compounds H and I. Thus, the mass spectrum of each peak should have fragments corresponding to more than one isomer.

In the spectrum of peak 1, strong ion intensities at *m/e* 187 and *m/e* 259 support the presence of ion fragments $CH_3(CH_2)_5CHOSi(CH_3)_3^+$ and $CH_3OOC(CH_2)_7CHO\text{-}Si(CH_3)_3^+$. Fragments containing one chlorine atom are present at *m/e* 307 and *m/e* 235. These support the occurrence of a pair of isomers corresponding to the Me_3Si derivatives of 9-chloro-10-hydroxypalmitic acid methyl ester and 10-chloro-9-hydroxypalmitic acid methyl ester. Likewise, in peak 3 there are ions at *m/e* 215 and *m/e* 259 and ions at *m/e* 263 and *m/e* 307 with one chlorine atom supporting the presence of the Me_3Si derivatives of the methyl esters of the two isomer molecules 9-chloro-10-hydroxystearic acid and 10-chloro-9-hydroxystearic acid.

Confirmation of these structures was made by comparing their mass spectral and their GC characteristics with those of known compounds. Coinjection of the synthetic mixture of the methyl esters of 9-chloro-10-hydroxypalmitic acid and 10-chloro-9-hydroxypalmitic acid with the hydroxy acid fraction gave an increase in only peak D (Figure 1). Injection of the Me_3Si ethers of these known compounds gave a single peak with the same retention time as peak 1 in Figure 2. The mass spectrum of this compound obtained by direct insertion is shown as spectrum A in Figure 4. Strong ion intensities appearing at *m/e* 187 and *m/e* 259 and the presence of chlorine in ions at *m/e* 235 and *m/e* 307 are the same as in the mass spectrum of peak 1 in Figure 2 confirming the presence of these known isomers in the hydroxy acid fraction.

In like manner, coinjection of the synthetic mixture of 9-chloro-10-hydroxystearic acid methyl ester and 10-chloro-10-hydroxystearic acid methyl ester with the hydroxy acid fraction gave an increase in only peak H of Figure 1. Coinjection of the Me_3Si derivatives of these known isomers with the Me_3Si derivative of the hydroxy acid fraction caused an increase only in peak 3 (Figure 2). The mass spectrum of these compounds obtained by direct insertion is shown as spectrum B in Figure 4. Strong ion intensities appearing at *m/e* 215 and *m/e* 259 and the presence of chlorine in ions at *m/e* 263 and *m/e* 307 are the same as in the mass spectrum of peak 1 in Figure 2.

The occurrence of two more isomers in addition to the above compounds can be inferred from mass spectrum 3 (Figure 3)

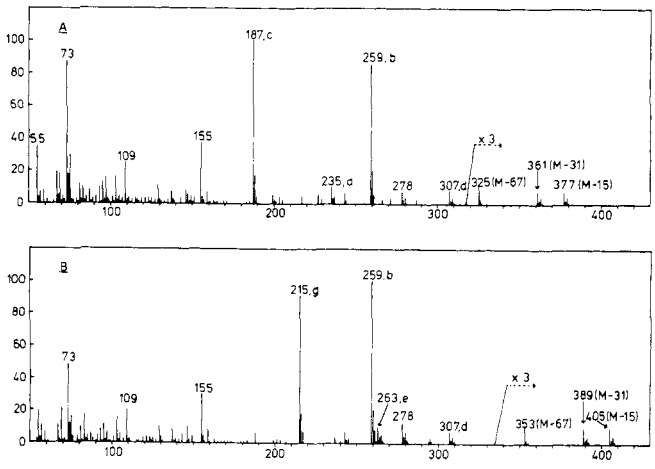

FIGURE 4: Mass spectra of the Me_3Si derivatives of known compounds. Spectrum A is produced by the Me_3Si derivative of a mixture of 9-chloro-10-hydroxypalmitic acid methyl ester and 10-chloro-9-hydroxypalmitic acid methyl ester and spectrum B by the corresponding stearic acid derivatives.

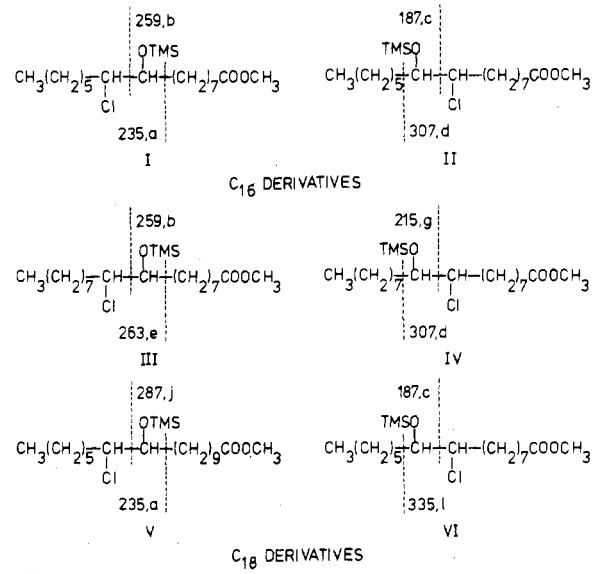

FIGURE 5: Proposed fragmentation of the Me_3Si derivatives of the fatty acid chlorohydrin methyl esters identified in the jellyfish lipids. Compound I and II are in peak 1 of Figure 2. Compounds III, IV, V, and VI are in peak 3 of Figure 2.

and the observed fragmentations of the known compounds. In mass spectrum 3 there are additional intense ions which are not present in the 9-10 stearic acid derivatives. Among these are ions at *m/e* 187 and *m/e* 287, which would be those expected from a mixture of the Me_3Si derivatives of 11-chloro-12-hydroxystearic methyl ester and 12-chloro-11-hydroxystearic methyl ester. Final confirmation of their presence comes from the expected one chlorine containing fragments at *m/e* 235 and *m/e* 335. A summary of the fragmentations for all the characterized fatty acid derivatives is shown in Figure 5.

As stated previously, only 30% of the total chlorine in the boron trifluoride treated lipid was accounted for in the hydroxy fatty acid fraction. The remaining 60% of the original organic chlorine was found at the origin. This material was separated further by preparative TLC in the solvent system benzene–methanol–acetic acid (95:5:1, v/v/v) into four additional halogen containing fractions: (1) R_f 0.00 to 0.17, 0.1% Cl; (2) R_f 0.17 to 0.27, 1.2% Cl; (3) R_f 0.27 to 0.32, 2% Cl; and (4) R_f 0.32 to 0.50, 0.12% Cl. Infrared spectra of these fractions were all very similar indicating the presence of hydroxyl groups, 3400 cm^{-1}, alkane protons, 2950 cm^{-1}, and ester carbonyls, 1745 cm^{-1}. The occurrence of organic chlorine in these more polar fractions could have two possible explanations. They could be fatty acid chlorohydrin methyl esters which contain more than one hydroxyl group per molecule or, alternatively, they could represent monochlorohydrin fatty acids which are still bound to polar lipid constituents due to incomplete transesterification in the boron trifluoride-methanol treatment. If this latter explanation were correct, then one would expect to find polar lipid constituents in these same fractions. Analysis showed each fraction to contain less than 0.02% organic phosphate and less than 1.0% hexoses. Analyses for glycerol did show a small amount of glycerol (1.29%) in fraction 2. GC–MS analysis of this fraction as the Me_3Si derivative demonstrated that the glycerol could be accounted for by the occurrence of a small amount of the α monoglycerides of the saturated C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, and C_{20} fatty acids. Thus it is concluded that the more polar chlorinated fractions are best represented by fatty acid methyl esters which contain additional free OH group(s) in addition to the chlorohydrin group. GC–MS analyses of the more polar chlorinated fractions as the Me_3Si derivatives failed to produce mass spectra which could be assigned to any one of the postulated structures, perhaps because of the large number of isomers present.

Additional support that the chlorine in these fractions and all the chlorine in the original lipid was present as chlorhydrins comes from the loss of organic chlorine upon alkaline treatment. Thus, treatment of the freshly extracted lipids or subsequent fractions with 0.5 M methanolic NaOH at 30 °C for 10 min followed by acidification and extraction gave lipids which were free of chlorine. This results from the rapid, base catalyzed elimination of HCl from the chlorohydrins to give the epoxides. The fact that all the chlorine is lost during this treatment eliminates the possible presence of nonchlorohydrin chlorine such as that found in the chlorosulfolipids from *Ochromonas danica* (Elovson and Vagelos, 1970).

One possible mode of biosynthesis of these compounds involves the addition of HCl to the appropriate cis or trans fatty acid epoxide. In an analogous manner to the reported enzymatic hydration of epoxy fatty acids (Niehaus et al., 1970), the cis epoxides would be expected to give the threo isomers and the trans to give the erythro isomers. Since, at present, only the cis-epoxy fatty acids have been reported in nature (Pohl and Wagner, 1972), we may expect these chlorohydrins to be threo isomers. It is clear, however, that, if this is the mode of biosynthesis of these chlorohydrins, there is little selectivity as to which carbon of the epoxide the chlorine resides upon. This fact emerges from the experimental data where we see similar intensities of ion fragments from each isomer of each pair of derivatives, i.e., 9-chloro-10-hydroxystearic and 10-chloro-9-hydroxystearic acid.

This apparent equal abundance of each of these positional isomers requires one to consider a possible nonbiological origin of these compounds. This could possibly result from nonenzymatic reactions occurring during the processing and frozen storage of the tissue prior to analysis; however, the original isolation of chlorinated lipids from fresh living tissue of the White Sea Jelly (*Auritia aurita*) in the Woods Hole survey argues strongly against a nonbiological origin for the chlorohydrins.

An alternate biological mechanism could involve the direct hydroxylation of a saturated fatty acid, a process well characterized in other eucaryotes (Morris, et al., 1966; Heinz et al., 1969; Kolattukudy and Walton, 1972), followed by subsequent α chlorination. This mechanism has recently been

postulated for the biosynthesis of the chlorosulfolipids in *Ochromonas danica* (Elovson, 1974) based on ^{18}O experiments.

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CORRECTIONS

Relaxation Phenomena in Aqueous Dispersions of Synthetic Lecithins, by Tian Yow Tsong* and Minoru I. Kanehisa, Volume 16, Number 12, June 14, 1977, pages 2674–2680.

On page 2678 in Table II, α_2 should read α_1 and α_1 should read α_2, with all the numerical values under these columns untouched.

Evidence for Site Equivalence in the Reaction Mechanism of Horse Liver Alcohol Dehydrogenase with Aromatic Substrates at Alkaline pH, by Charles F. Weidig, Herbert R. Halvorson, and Joseph D. Shore,* Volume 16, Number 13, June 28, 1977, pages 2916–2922.

On page 2919, column 2, the equation at the bottom of the page should read:

$$\beta = E_0 \left(\frac{1}{1 + k_4'/k_h(1 + k_{-h}/k_3)} \right)^2$$

Characterization of Collagen Precursors Found in Rat Skin and Rat Bone, by Barbara D. Smith,* Keith H. McKenney, and Thomas J. Lustberg, Volume 16, Number 13, June 28, 1977, pages 2980–2985.

On page 2980, second column, line 8 after Experimental Procedures should read: for 30 min in approximately 2 L of 150 mM *NaCl–50 mM* Tris-HCl–20 mM. The words in italics were omitted.

Phosphoglycerate Mutase from Wheat Germ: Studies with Isotopically Labeled 3-Phospho-D-glycerates Showing That the Catalyzed Reaction Is Intramolecular, by John A. Gatehouse and Jeremy R. Knowles,* Volume 16, Number 14, July 12, 1977, pages 3045–3050.

On page 3047, column 1, the equation at the bottom of the page should read:

$$I \ln \left[1 - p\left(1 + \frac{a}{a'}\right) + \frac{x}{a'} \right] - \ln \left(1 - \frac{x}{a}\right) = 0$$

Pig Liver Phosphofructokinase: Asymmetry Properties, Proof of Rapid Association-Dissociation Equilibria, and Effect of Temperature and Protein Concentration on the Equilibria, by John L. Trujillo and William C. Deal, Jr.,* Volume 16, Number 14, July 12, 1977, pages 3098–3104.

On page 3101, line 16 of the caption for Figure 4 should read p stands for polymer, $s^c_m = s^0_m[1 - k_m c_m - 0.5(k_m + k_p)c_p]$, $s^c_p =$, and line 17 should read $s^0_p[1 - k_p c_p - 0.5(k_m + k_p)c_m]$ and the values . . . . The slash mark in each line should be deleted.