Histone Composition of Nucleosomes Isolated from Cultured Chinese Hamster Cells†

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ABSTRACT: Nuclei isolated from cultured Chinese hamster cells were treated with micrococcal nuclease and lysed, and the resulting chromatin subunit classes (nucleosomes) were purified by sedimentation and resedimentation through isokinetic sucrose gradients. Nucleosomes isolated from [^{3}H]thymidine-labeled cells were analyzed for DNA size using both polyacrylamide gel and electron microscopic techniques. Nucleosomes isolated from [^{14}C]lysine-labeled cells were analyzed for protein content using a sodium dodecyl sulfate-polyacrylamide gel system. The results from monitoring the [^{14}C]lysine in each protein indicate that, in the nucleosome classes (monomer through tetramer), the molar ratios of histones H2A, H2B, H3, and H4 are equivalent. Furthermore, in each population of the nucleosome classes monomer through tetramer, it was possible to demonstrate that this histone unit (H2A + H2B + H3 + H4) is present, on the average, in the amount of two for monomers, four for dimers, six for trimers, and eight for tetramers. This is direct experimental confirmation of the prediction of R. D. Kornberg [(1974) *Science 184*, 868] concerning the substructure of chromatin.

Recent experimental findings have provided convincing evidence that chromatin consists of a basic, repeating substructure. Mild treatment of nuclei or chromatin with deoxyribonucleases results in the release of defined chromatin subunits which have been referred to as "ν bodies" (Olins and Olins, 1974), "PS particles" (van Holde et al., 1974b), or "nucleosomes" (Oudet et al., 1975). A model has been proposed in which the monomeric subunit consists of two each of the histones H2A, H2B, H3, and H4 associated as an octamer with about 180–200 base pairs of DNA (Kornberg, 1974). It has been shown from gel analysis of the protein content of purified monomers that all four "core" histones are present and from densitometric analysis of the gels that the proteins quantitatively appear in roughly equivalent amounts (Bakayev et al., 1975; Oudet et al., 1975; Olins et al., 1976; Shaw et al., 1976). It has been estimated by inference from the histone: DNA mass ratio that there are two each of the core histones per chromatin subunit.

In this report, we quantitatively analyze the protein content of purified nucleosome classes, monomers through tetramers. Using a sodium dodecyl sulfate-polyacrylamide gel technique and nucleosomes radiochemically labeled to high specific activities, we demonstrate not only the molar equivalence of the core histones but also determine experimentally, rather than by inference, that each nucleosome population consists of an average of $2n$(H2A + H2B + H3 + H4) histone molecules, where n is the number of chromatin subunits per nucleosome class. These data provide direct experimental evidence supporting the octameric substructure of histones in chromatin subunits as predicted by Kornberg (1974).

Materials and Methods

Nucleosome Preparation and Isolation. All steps, including growth and radiochemical labeling of cultured Chinese hamster cells (line CHO) and nuclei preparation, were as previously described (Strniste and Rall, 1976). After the nuclei were isolated with Triton X-100, they were washed three times with isotonic saline, 1 mM $PhCH_2SO_2F$[1] (Sigma), and then resuspended in 0.34 M sucrose plus buffer A (containing polyamines instead of divalent cations to stabilize the nuclei) as described by Hewish and Burgoyne (1973). Micrococcal DNase treatment of nuclei (10^8 per mL for 3 min) was performed as described by Finch et al. (1975) and was terminated by the addition of Na_2EDTA to 1 mM. Fractionation of nucleosomes on isokinetic sucrose gradients was performed according to the procedure of Finch et al. (1975), except that the gradients contained 0.2 mM Na_2EDTA (pH 7), and individual fractions were monitored for radioactivity. Fractions representing individual nucleosome classes, monomer through tetramer, were pooled, dialyzed against 0.2 mM Na_2EDTA (pH 7)–1 mM $PhCH_2SO_2F$, reduced in volume by rotary evaporation, and rerun separately on isokinetic gradients as before. The purified individual nucleosome classes were again pooled, dialyzed, and stored at 0 °C.

Gel Electrophoretic Analysis of [^{3}*H*]*DNA.* Samples of [^{3}H]TdR-labeled nucleosomes containing 2–4 μg of DNA were lyophilized to dryness, redissolved in 10–15 μL of reservoir buffer (Loening, 1967) plus 1% $NaDodSO_4$–5% glycerol, and heated to 60 °C for 10 min before application to the gel. Polyacrylamide slab gels (9 cm in length) of 5% acrylamide (monomer:bisacrylamide = 19:1) with the buffer system of Loening (1967) were run for 4 h at 4 °C at 20 mA constant current. The gel slab was then stained for 15 min by immersion in 1 mM Na_2EDTA (pH 7)–20 μg/mL ethidium bromide, photographed under shortwave ultraviolet light illumination, and sliced for radioactive counting. Slices were incubated overnight in 2 mL of 15% H_2O_2–4% NH_4OH at 70 °C in tightly capped scintillation vials to dissolve the gel. After the addition of 3 mL of H_2O and 10 mL of PCS scintillation cocktail (Amersham/Searle), the samples were monitored for radioactivity in a Packard 2420 liquid scintillation spectrometer.

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[1] Abbreviations used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Na_2EDTA, disodium ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; CHO, Chinese hamster cell; mol wt, molecular weight; cpm, counts per minute; [^{3}H]TdR, [*methyl*-^{3}H]thymidine; [^{14}C]Lys, L-[U-^{14}C]lysine.

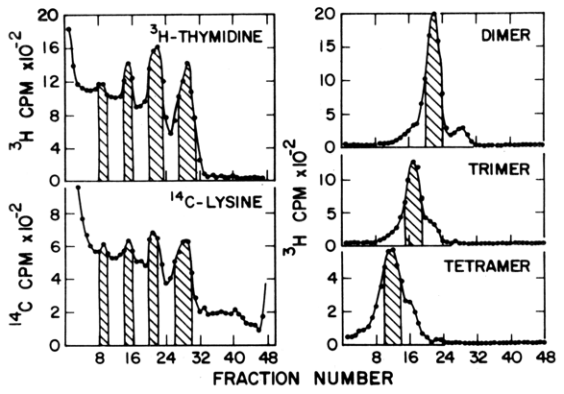

FIGURE 1: (Left) Isokinetic sucrose gradient sedimentation patterns of lysates of 3-min micrococcal DNase-treated, [^{3}H]TdR- or [^{14}C]Lys-labeled CHO nuclei. The shaded areas under the peaks represent that portion of each nucleosome class which was pooled and resedimented for further purification. (Right) Resedimentation patterns of isolated [^{3}H]TdR-labeled dimers, trimers, and tetramers on isokinetic gradients. The shaded areas under the peaks represent that portion of each nucleosome class which was pooled, dialyzed, and used in subsequent experiments. Sedimentation is from right to left.

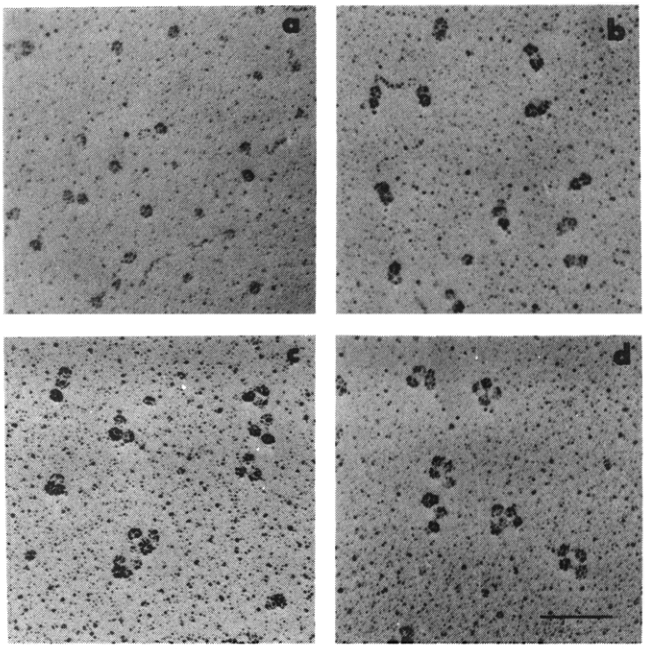

FIGURE 2: Electron micrographs of purified nucleosomes. (a–d) Monomers through tetramers, respectively. The bar denotes 1000 Å.

DNA marker fragments were prepared by digesting bacteriophage PM2 DNA with restriction endonuclease *Hin*dIII (Miles Laboratories) according to the procedure suggested by Miles Laboratories in their product bulletin. Aliquots of DNA fragments were prepared and analyzed on gels as described above. The number of PM2 DNA fragments produced by *Hin*dIII restriction enzyme was identical with that reported by Streeck et al. (1974) with their "late-eluting" *Hin* DNase.

Gel Electrophoretic Analysis of [^{14}C]*Lys Proteins.* Samples of [^{14}C]Lys-labeled nucleosomes containing 6–8 μg of DNA were lyophilized to dryness, redissolved in 20–30 μL of sample buffer, and boiled for 4 min just before application to the gel. Polyacrylamide slab gels (12.5% acrylamide and 28 cm in length) with the $NaDodSO_4$–Tris–glycine system of Laemmli (1970) were run for 4 h at room temperature (the apparatus was water cooled) at 20 mA constant current. The slab gel was stained according to Fairbanks et al. (1971), photographed, and then sliced for counting in the same manner as for the DNA gels.

Electron Microscopy and DNA Measurements. Purified nucleosomes were diluted into 50 mM ammonium acetate and then adsorbed onto glow-discharged, carbon-coated, specimen grids for about 1 min. The grids were washed with 2 drops of distilled water, stained with 2 drops of 1% uranyl acetate, and again washed with 2 drops of water. After air-drying, the samples were rotary-shadowed with Pt–Pd (80–20, E. Fullam, Inc., No. 1221) from a distance of 10 cm and a height of 1 cm. DNA samples isolated by treatment of nucleosomes with 3 M ammonium acetate or by extraction with chloroform:isoamyl alcohol were prepared for electron microscopic observation in a similar manner.

All samples were examined in a Phillips 200 electron microscope at an accelerating potential of 80 kV. Micrographs were taken at 35 000× on 35-mm film (Kodak, Fine Grain Release No. 5302) and calibrated with a diffraction grating (2160 lines per mm). The DNA contour length measurements were made on enlargements at a final magnification of 200 000×. Coordinates of points along individual DNA molecules were digitized by a graph pen, recorded by a PDP-8 computer, and processed by a PDP-11 computer to obtain the contour lengths of individual molecules. PM2 DNA (open, relaxed, double-stranded circles) with a molecular weight of 6×10^6 (Espejo et al., 1969) was determined to have a contour length of 3.05 ± 0.09 μm and was used as a standard in all measurements.

Results and Discussion

Purification of Nucleosomes. The sedimentation patterns of 3-min nuclease digests of either [^{3}H]TdR- or [^{14}C]Lys-labeled nuclei are shown in Figure 1. Note the similarities of both the DNA and protein distributions in the regions where the monomers through tetramers sediment. Note also that there is a region above fraction 32 where there is considerable protein but no DNA. Also presented in Figure 1 are sedimentation profiles of [^{3}H]TdR-labeled nucleosomes (dimers, trimers, and tetramers) rerun on isokinetic sucrose gradients. Nearly identical profiles were obtained with [^{14}C]Lys-labeled nucleosomes. Note the considerable contamination that is eliminated by resedimentation.

Electron micrographs of the purified nucleosome classes are shown in Figure 2. Since monomer fractions analyzed by electron microscopy after the first gradient run showed little evidence of contamination by dimers, they were not rerun on a second gradient.

Analysis of the DNA from Purified Nucleosomes. Polyacrylamide gel electrophoretic patterns of DNA isolated from purified nucleosome classes are shown in Figure 3. Note that, while each band is relatively free from contamination by the others, the broadness of the bands is quite large when compared with those of the *Hin*dIII restriction enzyme-treated PM2 DNA. This result indicates a rather wide range of DNA sizes within each of the purified classes of nucleosomes. Measurements of DNA contour lengths from electron micrographs of DNA isolated from the various classes of nucleosomes studied confirm that this is so. A summary of DNA sizes of nucleosomes isolated from a 3-min digest is presented in Table I.

Compton et al. (1976) have recently reported a DNA repeat length of 178 base pairs for nucleosomes isolated from Chinese hamster cells. Our results for the maximum repeat length of DNA, as determined by two independent methods, of about 183 base pairs agree favorably with this recent report, in light

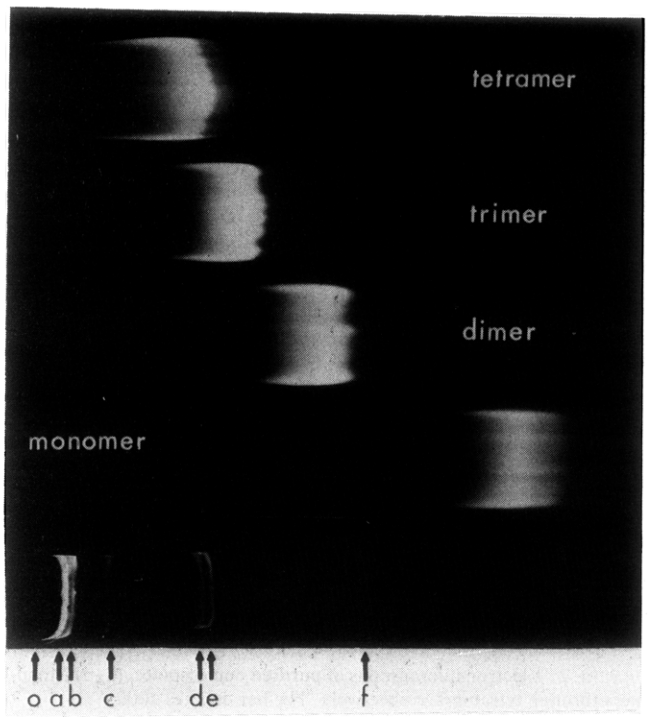

FIGURE 3: Polyacrylamide slab gel electrophoretic patterns of DNA from purified CHO nucleosomes (monomers through tetramers). Also shown is the pattern of PM2 DNA *Hin*dIII restriction endonuclease fragments: PM2 DNA fragments a–f have molecular weights of 28×10^5, 14×10^5, 7.9×10^5, 3.5×10^5, 3.5×10^5, and 1.4×10^5, respectively (see Streeck et al., 1974). A seventh fragment of 0.4×10^5 is not seen. (O denotes gel origin.)

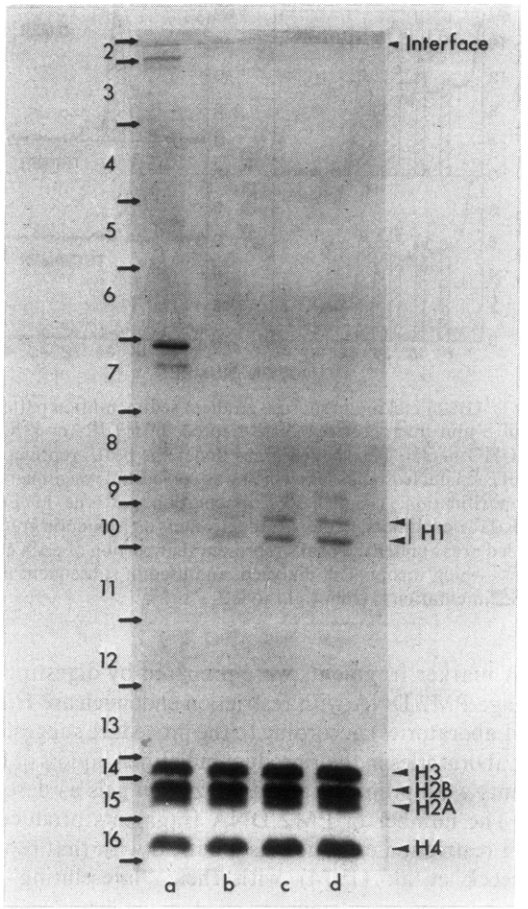

FIGURE 4: $NaDodSO_4$–polyacrylamide slab gel electrophoretic patterns of proteins from purified CHO nucleosomes. (Slots a–d) Gel, 12.5% of monomers through tetramers, respectively. Arrows (left side) refer to positions on the gel where slices (2 through 16) were made for radioactive assay (see Materials and Methods). Slice 1, which contains the stacking gel, is not included in the photograph.

TABLE I: DNA Sizes of Nucleosomes.[a]

	Monomer	Dimer	Trimer	Tetramer
Minimum	100 (110)	230 (230)	380 (385)	520 (530)
Maximum	190 (165)	380 (365)	540 (575)	720 (735)
Average	142	298	468	632
No. of molecules measured	233	198	166	125

[a] The data are given in base pairs, assuming 3.4 Å per base pair and 660 daltons per base pair. The numbers in parentheses were derived from polyacrylamide gels, all others are from electron microscopy measurements.

TABLE II: Ratios of the Core Histones as Determined by Their Lysine Content.[a]

	H3	H2A + H2B	H4
Monomer	0.266 (1.16)	0.545 (1.87)	0.189 (0.97)
Dimer	0.226 (0.99)	0.568 (1.95)	0.206 (1.06)
Trimer	0.223 (0.97)	0.567 (1.95)	0.210 (1.08)
Tetramer	0.249 (1.07)	0.534 (1.83)	0.217 (1.10)
Average	0.241 (1.05)	0.554 (1.90)	0.205 (1.05)

[a] For a hypothetical ratio of 1:(1 + 1):1 for H3:(H2A + H2B):H4, H3 (containing 13 lysine residues per molecule) would account for 0.228 of the total [^{14}C]Lys cpm of the four histones, H2A + H2B (containing 14 + 19 lysine residues per molecule) would account for 0.579 of the total, and H4 (containing 11 lysine residues per molecule) would account for 0.193 of the total.

of the fact that different experimental protocols were employed in isolation of the nucleosomes.

Analysis of the Proteins from Purified Nucleosomes. The $NaDodSO_4$–polyacrylamide gel patterns of the proteins from the various nucleosome classes are presented in Figure 4. Nonhistone proteins are barely in evidence (the histones account for 80–90% of the [^{14}C]Lys cpm). The presence of more nonhistones in the monomer fraction is most likely a contamination by non-DNA-bound proteins which overlap with the monomer fraction in the isokinetic gradient. Evidence for this can be seen in the sedimentation patterns of nucleosomes in which the protein is labeled, compared with the patterns of nucleosomes in which the DNA is labeled (see Figure 1). Also shown in Figure 4 is the manner in which the gel was sectioned for radioactivity counting. Since histones H2A and H2B were not considered to be sufficiently separated from each other, they were counted together in all subsequent results.

Determination of the Mole Ratio of Core Histones H2A, H2B, H3, and H4 in Nucleosomes. If it is assumed that the incorporation of [^{14}C]Lys by the core histones (H2A, H2B, H3, and H4) reflects the individual lysine composition of each histone, then it is possible to determine the ratio of any histone to another on a molecular basis. The lysine composition of CHO chromatin histones is known (see Gurley and Hardin, 1968) and agrees well with the published sequences of the same histones from other sources (DeLange and Smith, 1972). We can calculate for a hypothetical ratio of 1:(1 + 1):1 for H3:(H2A + H2B):H4 what the fraction of the sum of the [^{14}C]-Lys cpm of the four histones should be for each histone. This

TABLE III: Amount of Core Histone Unit[a] per Nucleosome.

	Monomer	Dimer	Trimer	Tetramer
Protein/DNA (w/w)	1.30	1.22	1.31	1.40
Corrected cpm of core histone unit	16408	14978	15035	14779
μg of core histone unit[b]	9.09	8.30	8.33	8.19
μg of DNA[c]	8.00	8.00	8.00	8.00
Mol wt of core histone unit[d]	54700	54700	54700	54700
Mol wt of DNA[e]	93400	197000	308900	417100
Moles of core histone unit $\times 10^{10}$	1.662	1.517	1.523	1.497
Moles of DNA $\times 10^{10}$	0.857	0.406	0.259	0.192
Moles of core histone unit/moles of DNA	1.94	3.74	5.88	7.80
Moles of core histone unit/moles of chromatin subunit	1.94	1.87	1.96	1.95

[a] Core histone unit defined as (H2A + H2B + H3 + H4). [b] Mass of core histone unit was obtained by dividing the corrected cpm by the average specific activity which, for this preparation, was 1805 ± 35 cpm per μg of protein. Since the core histones account for such a large percentage of the total protein in nucleosomes, the average specific activity of the total protein is probably very close to the average specific activity of these four histones. The other proteins present likely balance out to about the same specific activity, histone H1 being slightly above average and the nonhistones being slightly below average. [c] Amount applied to gel. [d] Obtained from the amino acid sequence of the four "core" histones (see DeLange and Smith, 1972). [e] Since it is difficult to assign an average DNA size from polyacrylamide gel data, the average DNA size used in the calculation for each nucleosome class was obtained from electron microscopic data. Allowing for magnification factors and assuming 3.4 Å per base pair, we determined the average number of base pairs of DNA in the monomer, dimer, trimer, and tetramer nucleosome classes to be 142, 298, 468, and 632, respectively, as shown in Table I. The average molecular weights were derived by multiplying the number of base pairs by 660, the average molecular weight of a base pair.

calculation and the actual result obtained for each of the core histones of the nucleosome classes studied are presented in Table II. The results agree favorably with the hypothetical ratio of 1-H3:(1-H2A + 1-H2B):1-H4 for all the nucleosome classes studied. This is in agreement with recent results of Olins et al. (1976), Shaw et al. (1976), and Simpson and Bustin (1976) who used densitometric scanning of stained gels to analyze the mole ratio of core histone proteins in purified monomeric nucleosome fractions.

Determination of the Number of Core Histone Units per Nucleosome. Now that we have shown that each nucleosome population contains a core histone ratio of one H3 to (one H2A and one H2B) to one H4, we wish next to determine how many of these units (H2A + H2B + H3 + H4) are present per nucleosome. We know the total ^{14}C cpm applied to each gel and the quantity of DNA applied to each. After slicing and counting the gel, we normalize the data to 100% recovery. In Table III are summarized the subsequent calculations which yield the number of core histone units in nucleosomes. The results indicate an average of two core histone units for monomers, four for dimers, six for trimers, and eight for tetramers. That is, the results state that there is an average of two histone units in a chromatin subunit, regardless of the particular nucleosome class studied.

Histone H1 Content in Nucleosomes. It is known that histone H1 is present in chromatin at only about half the amount of each of the other core histones (see Kornberg, 1974; Kornberg and Thomas, 1974). It has been proposed that, rather than being an integral part of the chromatin subunit itself, H1 occupies a site between subunits which is sensitive to nuclease digestion (van Holde et al., 1974a; Varshavsky et al., 1976; Whitlock and Simpson, 1976). It can be seen from Figure 4 that the amount of H1 protein increases as the number of chromatin subunits per nucleosome increases. We can calculate from the lysine content the ratio of histone H1 to the other histones using the same arguments as for Table II. Analysis of total CHO chromatin (no nuclease digestion) gave 0.97 H1 molecule per 2 each of the other "core" histones by this method—in good agreement with the "theoretical" 1.0 H1 molecule per 2 each of the other histones. However, analysis of nucleosomes shows an H1 content (per 2 each of the other histones) of only 0.32 in monomers, 0.54 in dimers, 0.64 in trimers, and 0.74 in tetramers. In other words, the results show an average of 1 histone H1 molecule for every 3 monomers, 1 H1 molecule per dimer nucleosome, 2 H1 molecules per trimer, and 3 H1 molecules per tetramer. The expected amount would be 1 per monomer, 2 per dimer, 3 per trimer, and 4 per tetramer if each class contained its full complement of histone H1.

If histone H1 were randomly distributed on the DNA or occupied only nuclease-insensitive sites, one would not expect to see these reductions from expectations in the H1 content of nucleosomes. Therefore, it would appear that H1 histone occupies a nuclease-sensitive site in chromatin, whereas the other histones do not.

After this manuscript was submitted for publication, Joffe et al. (1977) reported core histone ratios for chick erythroblasts using a technique similar to ours. We arrived at the same conclusion as these authors for the purified nucleosome classes monomers through tetramers from Chinese hamster cells and also used this technique to determine directly the total core histone composition for these same purified nucleosome classes.

Acknowledgments

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Occurrence of Fatty Acid Chlorohydrins in Jellyfish Lipids†

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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–

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