On the Structure of the Emulsifiers in Gastric Juice from the Crab, Cancer pagurus L*

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The presence of surface active compounds in the gastric juice of some decapod Crustacea was established by Vonk (1). Early work on the structure of the emulsifiers in the gastric juice of the crab, Cancer pagurus L, and of the crayfish, Astacus astacus L, indicated that these might be taurine-conjugated bile acids (2). However, later investigations by Vonk (3) have demonstrated several differences between the properties of the emulsifiers and those of bile salts. In a recent communication (4) it was reported that no bile salts could be detected in extracts of crab gastric juice when analyzed by chromatographic and mass spectrometric techniques. However, material with emulsifying properties was isolated and shown to yield fatty acids, sarcosine, and taurine upon hydrolysis. The present report describes the purification of the emulsifiers from the gastric juice of the crab, Cancer pagurus L, and presents evidence that these compounds are fatty acylsarcosyltaurines.

MATERIALS AND METHODS

Gastric juice was collected from freshly caught crabs as described by Vonk (2, 3). A bent glass tube was introduced through the mouth and into the stomach, and the stomach contents were carefully sucked into the tube. The juice was freeze-dried and stored at -20°C until used. Sarcosine, sarcosine, and decanoic acid were purchased from Fluka, Buchs, Switzerland. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden, and silica gel G from Merck, Darmstadt, Germany.

Chromatographic Methods—Reversed phase partition chromatography (5, 6) was carried out with Phase Systems D (100 ml of 1-butanol as stationary phase and 300 ml of water as mobile phase) and F3 (10 ml of heptane per 40 ml of chloroform as stationary phase and 195 ml of methanol per 105 ml of water as mobile phase). Hydrophobic Hyflo Super-Cel was used as support for the stationary phase. Thin layer chromatography of the emulsifiers was performed on silica gel G with phase systems developed by Ganshirt, Koss, and Morianz (7) for the separation of taurine-conjugated bile acids. The water-soluble hydrolysis products of the emulsifiers were chromatographed on thin layer plates with 1-butanol-acetic acid-water, 4:1:2, or ethanol-ammonia-water, 90:5:5, as moving phase (8). These products were also analyzed by two-dimensional paper chromatography according to the method of Redfield (9).

Gas chromatography was performed with a Becker gas chromatograph, type 4038 E (Becker, Delft, Holland) with a 1.5-m or a 5-m column packed with ethylene glycol adipate polyester (20%) and phosphoric acid (2%) on Chromosorb W, 60 to 70 mesh. The carrier gas was hydrogen, and the column temperature was 197°C. The methyl esters of the fatty acids were prepared with freshly distilled diazomethane in ether-methanol, 9:1. The fatty acid methyl esters were identified and quantitated according to Farquhar et al. (10) and Carroll (11).

Mass Spectrometry—The compound gas chromatography-mass spectrometry instrument developed by Ryhage (12) was used for the direct mass spectrometric analysis of gas chromatographic effluents.

Isolation of Emulsifiers—Freeze-dried gastric juice was extracted with hot 95% ethanol and the ethanol extract was evaporated to dryness on a 90°C water bath under reduced pressure (about 10 mm of Hg). The residue was dissolved in water, acidified with dilute hydrochloric acid, and extracted three times with water-saturated 1-butanol. The combined butanol extracts were washed with water until neutral and the solvent was evaporated according to Farquhar et al. (10) and Carroll (11). The residue was dissolved in water, 2.5 ml/10 mg of residue, and the solution was mixed well with 1.5 times its volume of Sephadex G-25 powder. Ether was added to the mixture to make a thin slurry which was stirred for 2 hours. The ether layer was removed and the Sephadex-water mixture was then stirred for an additional 2 hours with a solution of ethyl acetate-ethylene chloride, 1:1, and finally for 30 minutes with methylene chloride. The Sephadex was collected on a glass filter and was extracted with 95% aqueous ethanol. The ethanol extract was taken to dryness. A colorless residue exhibiting marked surface active properties was obtained. Table I summarizes some data on samples of gastric juice purified as described above.

EXPERIMENTAL PROCEDURE AND RESULTS

Identification of Sarcosine and Taurine—The purified emulsifiers were hydrolyzed with 1 m sodium hydroxide in 50% aqueous ethanol for 12 hours at 110°C in a sealed steel bomb. The saponification mixture was acidified with 0.1 m hydrochloric acid and was extracted twice with ether. The combined ether extracts were washed with water until neutral and the solvent was evaporated.
The residue of the ether extract was shown to consist of a mixture of fatty acids and these results will be described below. The water phase was taken to dryness and the residue was analyzed by paper and thin layer chromatography. Two-dimensional paper chromatography as well as thin layer chromatography showed the presence of two main components which had exactly the same mobilities as sarcosine and taurine, respectively. Sarcosine was isolated from the residue of the water phase by extraction with ethanol. The ethanol was evaporated and the solid was crystallized from water-acetone-methanol chloride, yielding crystals with a melting point of 168° (reported m.p. 171-172° (13)) with no depression upon admixture with authentic sarcosine hydrochloride. The free acid had a melting point of 209°, with decomposition (reported m.p. 210.5-211.5°, with decomposition (14)). Taurine was isolated from the material remaining after ethanol extraction by dissolving this residue in water and passing the solution through a column of Dowex 50-X8 (hydrogen form; column size, 200 X 10 mm). The column was eluted with water until the effluent was neutral. All of the effluent was combined and the solvent was evaporated, yielding a residue consisting predominantly of taurine. Taurine was isolated from the ether-acetone phase by crystallization from methanol-water. The sodium salt of N-acetyltaurine melted at 234-236° (reported m.p. 235-236° (15)). Data on amounts of sarcosine and taurine in the hydrolysis mixtures are given in Table I.

Identification of Fatty Acids—The residue of the ether extract of the hydrolysis mixture was analyzed by gas chromatography. Fig. 1 shows a gas chromatogram of fatty acids isolated from Sample 3 (cf. Table I). A number of compounds with retention times characteristic of fatty acids methyl esters were found. Together, Compounds A, B, and C accounted for about 75% of the material that emerged from the column. These acids were tentatively identified as methyl decanoate, dodecenoate, and tetradecatrienoate, respectively. For further identification the samples were analyzed in the compound gas chromatography-mass spectrometry instrument. Fig. 2 shows a chromatogram of the same sample. The tentative identification of Compound A as methyl decanoate (mol. wt. 186) was confirmed. The spectrum of methyl decanoate (Fig. 3) shows typical peaks at M-31 and M-43 and prominent peaks at m/e = 74 and m/e = 87. Further interpretation of the peaks is given in a report by Ryhage and Stenhagen (16). The tentative identification of Compound B as methyl dodecenoate (mol. wt. 212) was also confirmed.

The spectrum of methyl dodecenoate (Fig. 4) shows a peak at M-32 typical of unsaturated fatty acid methyl esters with the double bond at a distance of more than 3 carbon atoms from the carboxyl group (cf. Reference 17). The position of the double bond in the dodecenoic acid was established in the following manner. A sample of the fatty acid fraction liberated upon hydrolysis of the emulsifiers was chromatographed on a column of hydrophobic Hyflo Super-Cel with Phase System F3. Fig. 5 shows this chromatogram. The dodecenoic acid was eluted with its peak at 57 ml of effluent. This material was collected and the solvent was evaporated. Dodecenoic acid, 22 mg, was oxidized by the method described by Jones and Stolp (18), yielding 12 mg of glutaric acid, identified as its dimethyl ester by mass spectrometry (cf. Fig. 6 and Ryhage and Stenhagen (19)). The infrared spectrum (taken in KBr with a Perkin-Elmer instrument, model 221) of the isolated dodecenoic acid had no prominent band at 965 cm\(^{-1}\) but a band at 720 cm\(^{-1}\), indicating the presence of a cis-double bond (20). Concerning Compound C the mass spectrum (Fig. 7) showed a molecular peak at 236 which agrees with the tentative identification of this compound as methyl tetradecatrienoate but the spectrum was notably devoid of typical peaks in the higher mass region. It appears therefore that the identification must be considered tentative.
FIG. 2. Gas chromatogram of the methylated fatty acid fraction from Sample 3 (cf. Table I) with the compound gas chromatography-mass spectrometry instrument. Column, 19% ethylene glycol succinate polyester on Gas-Chrom P, 100 to 140 mesh; column temperature gradient, 5.6° per minute starting at 100°; helium inlet pressure, 1.8 kg per cm². The arrow indicates that a mass spectrum was recorded.

FIG. 3 (top). Mass spectrum of Compound A
FIG. 4 (bottom). Mass spectrum of Compound B

FIG. 5. Chromatogram of fatty acid fraction from a sample of hydrolyzed emulsifiers. Column, 13.5 g of hydrophobic Hyflo Super-Cel; Phase System F3.

However, the result of the hydrogenation of the fatty acid mixture, giving a marked increase in the amount of tetradecanoic acid (cf. Table II), supports the tentative structure for Compound C.

Table II summarizes the composition of the main fatty acids in three samples of gastric juice (Samples 1, 2, and 3, Table I). Except for the fatty acids, 10:0, 12:1, and 14:3, the structures assigned are based only on chromatographic properties.

Nonidentity of Emulsifiers with Acylsarcosine and Acyltaurine—Attempts to separate the individual components in the mixture of emulsifiers by partition or ion exchange chromatography failed. On thin layer chromatography the emulsifiers moved as a single, tailing band. The possibility that the emulsifiers were composed of a mixture of fatty acylsarcosines and fatty acyltaurines could be ruled out by comparison with synthetic samples. Conjugates of fatty acids and amino acids were synthesized on
the 0.5-m mole scale according to the procedure described by Norman (21) for the preparation of conjugated bile acids. The conjugates were purified by reversed phase partition chromatography. Phase System D was used for purification of taurine conjugates. The potassium salts of the conjugates were crystallized from ethanol-water. Potassium deconoyltaurinate gave the following elementary analysis

\[ C_{12}H_{21}NO_3SK \]

Calculated: C 45.4, H 7.6, N 4.4
Found: C 45.2, H 8.0, N 4.4

Potassium dodec-5-enoyltaurinate prepared with dodec-5-enoic acid isolated from hydrolyzed emulsifiers as described above gave the following analysis

\[ C_{14}H_{29}NO_3SK \]

Calculated: C 49.0, H 7.6, N 4.1
Found: C 48.8, H 8.4, N 4.1

| Table II
Composition of fatty acid fraction* |
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<tr>
<td>Fatty acid</td>
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* Values less than 1% of total fatty acids have not been listed.
† The number preceding the colon designates the number of carbon atoms in the fatty acid chain and the number following the colon designates the number of double bonds.

Decanoylsarcosine, the preparation of which is described below, was found to be much less polar than the emulsifiers in various chromatographic systems. Decanoyltaurine and dodec-5-enoyltaurine moved somewhat faster than the emulsifiers in the thin layer chromatographic systems of Ganshirt, Goss, and Morianz (7). The stability of the taurine conjugated fatty acids differed considerably from that of the emulsifiers. As already noted by Vonk (3) the emulsifiers are rather labile towards acid whereas hydrolysis of taurine conjugated fatty acids requires the use of molar solutions of hydrochloric acid at reflux temperature.

Isolation of Sarcosyltaurine—When purified emulsifiers were treated with 0.2 M aqueous hydrochloric acid at 90°, fatty acids were liberated. In an experiment designed to isolate the products formed, 72 mg of purified emulsifiers were hydrolyzed under these conditions for 16 hours. After this period of time, 22 mg of fatty acids were isolated by ether extraction. Of the water-soluble part, amounting to 48 mg, 13 mg could be extracted with ethanol and were found to consist predominantly of starting

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**Fig. 6.** Mass spectrum of dimethyl glutarate, isolated after oxidation of dodec-5-enoic acid.

**Fig. 7.** Mass spectrum of Compound C.
Evidence for General Structure of Fatty Acylsarcosyltaurine—The experimental evidence obtained indicates that the emulsifiers are composed of a number of compounds having the general structure of fatty acylsarcosyltaurine. Since it was not possible to isolate any of the components in the mixture of emulsifiers, a direct comparison with synthetic samples could not be made. However, the properties of a synthetic sample of decanoylsarcosyltaurine were compared with those of the mixture of the emulsifiers. In the synthesis of decanoylsarcosyltaurine a slight modification of the procedure of Norman (21) was made. Decanoylsarcosine was prepared as described for glycine-conjugated bile acids, and was purified by chromatography with Phase System F2 (6). For the coupling of decanoylsarcosine with taurine a 100% molar excess of taurine was used (cf. Reference 21). Decanoylsarcosyltaurine was purified by chromatography with Phase System D (5). The potassium salt of this compound gave the following analysis

\[
\text{C}_{12} \text{H}_{24} \text{N}_{2} \text{O}_{8} \text{S}\text{K}
\]

Calculated: C 46.4, H 7.5, N 7.2

Found: C 46.2, H 7.6, N 7.0

The emulsifiers present in the gastric juice of the crab, Cancer pagurus L, have been isolated. It was not possible to separate the individual components in the mixture of emulsifiers. Hydrolysis of the purified emulsifiers yielded fatty acids, sarcosine, and taurine. Dodec-5-enoic acid constituted about 50% of the fatty acid fraction. Mild acid hydrolysis of the emulsifiers resulted in the liberation of fatty acids and sarcosyltaurine. The general structure of fatty acylsarcosyltaurine has been assigned to the emulsifiers.

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REFERENCES
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