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# THE PROPERTIES OF SOME EMULSIFIERS IN THE DIGESTIVE FLUIDS OF INVERTEBRATES

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**Abstract**—1. Curves for surface tension vs. concentration of emulsifiers from the intestinal juice of several invertebrates and their dependence on the salt concentrations have been determined.

2. In *Cancer pagurus* L. the composition of the intestinal contents is favourable for a low critical micellar concentration of the emulsifiers and thus for solubilization and absorption of lipids.

3. An emulsifier in the gut of an insect (*Dyticus*) is demonstrated.

4. The possible advantage of vertebrate bile as compared to the invertebrate emulsifiers is discussed.

## INTRODUCTION

IN THE intestinal tract of vertebrates conjugated bile salts emulsify the fats which ensures better contact with lipase. The fats are split up into glycerol, free fatty acids and monoglycerides. Above a certain critical micellar concentration (CMC)* of the bile acids they form micelles which take up the insoluble fatty acids and so enable their absorption. Mono-olein is helpful in this process: it lowers the CMC of the bile acids and is itself taken up into the micelles. The CMC of pure conjugated bile acids lies between 5 and 7 mM/l in 0·15 M NaCl, and in the presence of mono-olein at 0·5–4 mM/l (Hofmann, 1963; Hofmann & Small, 1967). According to Hofmann & Borgström (1962) the concentration of the bile acids in the intestine after a test-meal (in man) is about 0·3%, that is 5·5 mM/l, and as mono-olein is always present in sufficient amount, solubilization of the split-off fatty acids is ensured.

It has never been widely studied how the situation is in this respect in the intestinal tract of invertebrates. As their food contains fat and lipases have been found, fatty acids are formed and probably mono-olein. One of the properties of bile-salts and other emulsifiers is that, present in small amounts (1 per cent or much less), they considerably lower the surface tension of water against air and the interfacial tension of their aqueous solutions against lipids. Now it has been found long ago that the intestinal contents of various invertebrates have a surface tension

* The following abbreviations will be used in this paper: CMC = critical micellar concentration; CMT = critical micellar temperature; PDST = potassium decanoyl-sarcosyl-taurine; STCh = sodium taurocholate; SDS = sodium dodecanoyl-sulphate = sodium lauryl sulphate; TLC = thin-layer chromatography; $\gamma$ = surface tension in dyn/cm.

even lower than that of bile. It therefore occurred to me that emulsifiers could be present in these invertebrates which play the part fulfilled by the bile in vertebrates. This supposition could be proved for the fresh-water crayfish (*Astacus leptodactylus*) (Vonk, 1935) and for some other invertebrates (*Cancer* and other decapod crustaceans, *Helix pomatia*, *Aplysia limacina* and *Holothuria tubulosa*). No such emulsifiers were found in *Loligo species* and *Mya arenaria* (Vonk, 1962).

The solubility of these emulsifiers in water (+), ethanol (+) and ether (−) was the same as that of conjugated bile acids. Moreover, like bile acids, they precipitate protein in acid solution. On hydrolysis by means of 2 N NaOH or 0·25 N HCl the emulsifiers of the crustaceans were found to split off taurine with the exception of *Eriocheir sinensis* from which sulphate was obtained. On these grounds the emulsifiers were considered provisionally as bile acids or bile alcohols.

However, Van den Oord (1965) and Van den Oord *et al.* (1965) purified the emulsifiers of the crab *Cancer pagurus* chromatographically and found them to be a mixture of fatty acyl-sarcosyl-taurines. The chief component (62·5 per cent of the mixture) contains the fatty acid C 12:1 (*cis* form), 6·6 per cent is 10:0 and 4·4 per cent is 14:3; 7·2 per cent could be 24:0. Their synthesis by *Cancer* was proved by injection of 1-$C^{14}$-acetate (Van den Oord 1966).

Thus the emulsifiers of *Cancer* are of the type of the well-known industrial detergents acyl-taurines, with the difference that in *Cancer* sarcosine is inserted between fatty acid and taurine. It is probable that the emulsifiers of other crustaceans will prove to be of the same type. For those of the Holothuroidea, which are not related to crustaceans, this is less certain.

Many questions concerning these substances are still unanswered. How is the medium composed in which they are acting? This composition must influence their physico-chemical properties. Of the latter nothing is known, although it can be expected that they will resemble those of detergents like sodium lauryl-sulphate (sodium dodecanoyl-sulphate, SDS). An impression on this point can be obtained by comparing the properties of the original juice with those of the purified emulsifiers or with those of one of the synthesized emulsifiers.* As a proof of the general occurrence of these substances, their presence is demonstrated in the intestinal tract of a carnivorous insect larva (*Dytiscus* sp.).

## MATERIALS AND METHODS

Intestinal juice of *Cancer pagurus* from the North Sea was obtained by the method originally indicated by Jordan for *Astacus*, as recently described by Vonk (1960). The juice was collected between 19 October and 16 February. In spring and summer its content of emulsifiers diminishes (Van den Oord, 1965). About 2 ml juice per animal can be obtained. As the animals had been fasted for some days after their capture the juice did not contain much insoluble material. It was centrifuged clear and stored in frozen condition. The total amount collected was 450 ml; its pH (radiometer) was 6·08.

Surface tension was determined by means of a Traube stalagmometer with a content of about 6 ml at temperatures between 20 and 25°C. The surface activity of water at 20°C being 72·75 dyn/cm and that at 25°C 71·97, $\gamma$ is not strongly temperature-dependent. The

* The latter procedure has been followed in this paper.

difference of 0·78 dyn/cm is just at the limit of exactitude of our method. As the effects of dilution and addition of salts far exceed these values, small temperature effects due to variations of temperature of 2–3°C during the experiments could be neglected.

N-(N-decanoyl-sarcosyl)-taurine as the potassium salt (PDST) was synthesized by the method of Norman for the preparation of conjugated bile acids (Van den Oord, 1965; Van den Oord *et al.*, 1965). The decanoic acid used contained (gas chromatography) about 4 per cent of homologues, mostly C 12:0. The result of the synthesis was controlled by hydrolysis. The synthesized product showed no traces of decanoic acid in TLC (method of Freeman & West, 1966) and no traces of either taurine, sarcosine or sarcosyl-taurine (method of Jones & Heathcote, 1966). After hydrolysis all these components could be demonstrated by TLC. If the hydrolysis was weak, also the peptide sarcosyl-taurine was found present. The synthesized PDST in 1% solution showed no traces of turbidity or opalescence at prolonged cooling (0°C), so that CMT must be below 0°.

For the analysis of the juice (method Douglas-Sauermann, 1935) proteins were precipitated by addition of 9 vol. of 96% ethanol. After standing for some days the solution was filtered and the filtrate which contains the emulsifiers was evaporated. The residue was dissolved in water (about the original volume). It was acidified to pH 2 with 4 N HCl and ethanol was added (a third of the volume). Subsequently it was extracted three times with xylene and two times with petroleum ether (b.p. 60–80°C). The extract was washed with water, dried with sodium sulphate, evaporated and the weight of the lipid residue was determined. In other experiments the original juice, after acidifying, was directly extracted with a mixture of methanol–chloroform (1 : 2, v/v). The amount of lipids found in this way was nearly the same as with the first method; for obtaining the emulsifiers from the aqueous solution, the first method is to be preferred. From the lipid fraction TL chromatograms (Freeman & West, 1966) were made.

The aqueous solution was neutralized and evaporated *in vacuo* to remove the alcohol and traces of the other organic solvents. After dissolving the residue in water to the original volume of the juice, the surface tension is nearly as low as that of the original juice. It must be noted that this solution still contains calcium chloride and magnesium chloride, as both salts are fairly soluble in alcohol. The precipitated and superficially dried protein precipitate has, after solution in water, nearly the surface tension of this solvent.

The intestinal juice of the *Dytiscus* larvae was collected after feeding the animals, killing them with chloroform and removing the intestine. Collecting the juice of *Holothuria* has been described by Vonk (1962).

## RESULTS

### *Composition of the medium (intestinal contents)*

This composition is represented in Table 1. The following comments upon this table can be made. The pH is rather characteristic of the species or genus. In other genera the pH may be much lower, being lowest in *Homarus* (pH 4·8; Vonk, 1962). The slight deviation of pH to the acid side must result from the slightly acid reaction of salts like $CaCl_2$ and $MgCl_2$. In fact when proteins are removed by precipitation with alcohol and the residue of the evaporated alcoholic filtrate is dissolved in water the pH of this solution is 4·73. In the original juice the pH is higher because of the buffering by proteins. Phosphates cannot greatly influence pH, as their concentration is only *ca.* 0·002 M, and titration curves (not reproduced here) show that the buffer capacity is just lowest around pH 6 (from pH 5·0 to 7·0).

Addition of the percentages of the determined compounds gives 6·34 per cent against 7·54 per cent for the dry weight. The compounds corresponding to the

non-protein nitrogen, some undetermined compounds and some losses may account for this discrepancy. The amount of magnesium is somewhat lower than in sea water; that of calcium is double the value in sea water.

TABLE 1—COMPOSITION OF THE INTESTINAL CONTENTS OF *Cancer pagurus* L.

| | | |
|---|---|---|
| Insoluble material (centrifuged) | 0·144% | |
| Specific gravity | 1·030 | |
| pH (glass electrode Radiometer) | 6·08 | |
| Surface tension ($\gamma$) at 22°C | 36·5 dyn/cm | |
| Dry weight | 7·54% | |
| Ash | 3·818% | |
| Protein (6·25 × N-content alcohol precipitate) | 1·26% | |
| Non-protein nitrogen | 0·13% | |
| Lipids (extracted with xylene) | 0·44% | |
| Lipids (extracted directly with methanol chloroform 1 : 2) | 0·40% | |
| Free taurine (Pentz *et al.*, 1957) | 0·087% | |
| Taurine bound to emulsifiers | 0·22% | |
| Emulsifiers (calculated as dodecenoyl-sarcosyl-taurine-Na) | 0·70% | Sea water |
| Na (flame photometer) | 1·23% | 1·056% |
| K (flame photometer) | 0·04% | 0·04% |
| Ca (flame photometer) | 0·08% | 0·04% |
| Mg (flame photometer) | 0·09% | 0·127% |
| Cl (gravimetric) | 2·232% | 0·1898% |
| Inorganic P as $PO_4$ | 0·018% | Variable (Sverdrup *et al.*, 1942) |
| $CO_2$: 0·85 vol.% | 0·015% | 0·01397% |

The analysis was carried out with 460 ml of intestinal juice and thus is the mean value of the juices of about 250 animals collected between the months of October and February. Percentages are of fresh weight (w/v).

The isolated lipids were submitted to TLC according to Freeman & West (1966). The results, together with those for *Holothuria* (which will be discussed later on), are summarized in Table 2.

We see that cholesterol is present in a fair amount. Monoglycerides and phospholipids are also present. This is important because these play a part in the formation of micelles and in the lowering of the CMC. On the whole, the picture does not deviate essentially from that given by Hofmann & Borgström (1962) for the intestinal contents of man.

*Properties of the emulsifiers and influence of the medium*

Measuring of the surface tension ($\gamma$) in dependence of the concentration is a means for determining CMC and investigating the influence of various agents on $\gamma$ and on the situation of CMC. First, we investigated these influences on one of the synthesized emulsifiers of *Cancer*, i.e. decanoyl-sarcosyl-taurine. For comparison

we used a preparation of pure sodium taurocholate, kindly provided by Professor Haslewood, London.

TABLE 2—LIPID COMPONENTS OF A XYLENE EXTRACT OF THE INTESTINAL JUICE OF *Cancer pagurus* AND OF *Holothuria* INTESTINAL CONTENTS

| | Lipids of *Cancer* | Lipids of *Holothuria* |
|---|---|---|
| Hydrocarbons | + + | + + |
| Cholesterol esters | − | − |
| Triglycerides | + | + |
| Diglycerides | − | ± |
| Cholesterol | + + + | + |
| Long-chain fatty acids | + | + + |
| Short-chain fatty acids | − | |
| Monoglycerides | + | ± |
| Phospholipids (base-line) | + | + |

± = slight concentration.

Some of the results are shown in Table 3. We see that the influence of pH on $\gamma$, if any, is very slight. This fact is due to the strongly acid character of these compounds. In weak acids, like octanoic acid, pH strongly influences the surface

TABLE 3—INFLUENCE OF pH AND OF SALTS ON THE SURFACE TENSION OF SYNTHESIZED PDST AND OF PURE STCh

| | PDST | | Na taurocholate | |
|---|---|---|---|---|
| Solvent | pH | $\gamma$ in dyn/cm | pH | $\gamma$ in dyn/cm |
| Water | 7·07 | 69·0 ± 0·2 | 5·92 | 64·3 ± 0·4 |
| Primary phosphate and secondary phosphate, 1 : 1 | 6·80 | 57·0 ± 0·2 | 6·78 | 53·2 ± 0·3 |
| Primary phosphate | 3·71 | 56·7 ± 0·2 | 4·64 | 54·0 ± 0·1 |
| Secondary phosphate | 8·59 | 56·3 ± 0·2 | 8·68 | 52·8 ± 0·2 |
| Water (theoretical value) | | 72·46 | | 72·46 |

Fifteen mg PDST (0·0039 mM) or 20 mg pure STCh (0·0036 mM) were dissolved in 10 ml doubly distilled water or in 10 ml 0·15 M phosphate buffer. T = 22°C.

tension (Matijević & Pethica, 1957). However, the influence of salts is very important; 0·15 M phosphate lowers the surface tension appreciably. It may be seen from Fig. 1 that this is primarily an effect of the valence of the added cations, as is well known from the literature (e.g. Durham, 1961). We see there that for taurocholate a decrease in $\gamma$ of about 8·4 dyn/cm is reached at a concentration of 4 mM calcium chloride/l, whereas in Table 3 with sodium phosphate a concentration of

150 mM gives a decrease of $\gamma$ of 11 dyn/cm. A more detailed account of these effects is given later on (Fig. 2).

Moreover, from Table 3 we get an impression of the errors in the determination of $\gamma$. In most cases for each determination the drops of a given volume of the stalagmometer were counted 5 times. The standard error of the determination is between 0·2 and 0·4 dyn/cm, i.e. between *ca.* 0·2 and 0·7 per cent. The error in making dilutions of the fluids is, of course, much smaller.

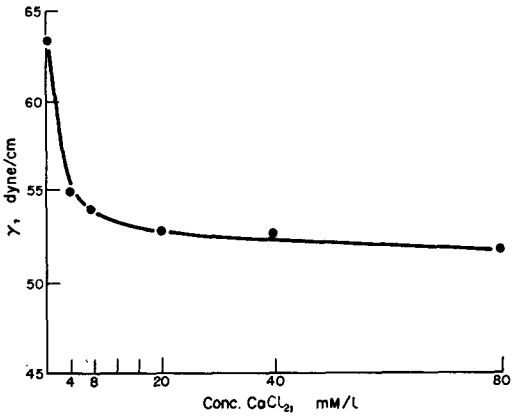


FIG. 1. Influence of $CaCl_2$ on the surface tension of pure sodium taurocholate.

Curves of the relations between the concentration of the emulsifiers and the value of $\gamma$ are given in Fig. 2. For the curve of the pure decanoyl-sarcosyl-taurine salt (PDST) we see that at concentrations of 20–40 mM/l rather low surface tensions are reached, lower than these of pure taurocholate (STCh) which was determined for comparison. Neither curve shows a very definite CMC, although we might estimate this for the taurocholate at 20 and for the PDST at 30 mM/l. However, a rather sharp CMC at 12·5 mM/l appears if $\gamma$ is determined for PDST in the presence of 0·15 M phosphate buffer and 0·08% $CaCl_2$. An (uncompleted) curve for the same compound with only 0·15 M phosphate lies somewhat more to the right. A still lower and more definite CMC (at 3·5 mM/l) is given by the curve obtained from the dissolved residue of the alcoholic extract of the intestinal juice of *Cancer*. The concentrations in this case have been calculated from the value for PDST given in Table 1. This extract still contains the natural amount of $CaCl_2$ and the lipid fractions mentioned in Table 2. It is known from the literature that small amounts of lipids (especially mono-olein, but also fatty acids) may lower CMC considerably (Hofmann, 1963; Hofmann & Small, 1967).

A curve for the original juice could not be exactly determined by this method, as in the undiluted fluid the presence of protein through its viscosity somewhat affects the result, but an idea is given in Fig. 3. Gamma of the undiluted juice was determined by means of the balance method and proved to be 36·5 instead of 40 as found by the stalagmometer. This point is indicated at J in Fig. 2. For deter-

mination of the whole curve by this method the amount of material was not sufficient. As, moreover, for dilutions of 5 times and more the curve coincides with that of the protein-free juice, we may safely assume that the shape of these two curves will not be very different.

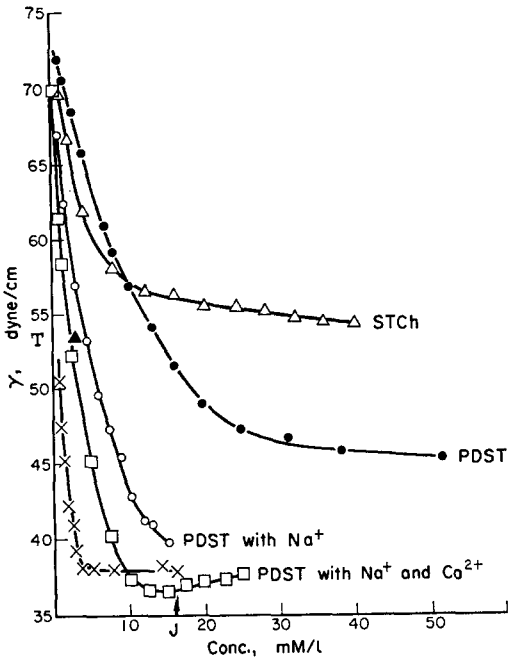


FIG. 2. Relation between the surface tension against air ($\gamma$) of emulsifiers and their concentration. Δ, Pure sodium taurocholate, pH 5·92, T = 21°C. ▲, At T on ordinate: *idem* with 0·15 M sodium phosphate, pH 6·78. ●, Synthesized PDST in water, pH 7·0, T = 21°C. ○, Same in 0·15 M NaCl, pH 7·25, T = 23°C. □, Same in 0·15 M NaCl and 0·02 M $CaCl_2$, pH 1·79–2·47 (diluted), T = 20°C. ×, Evaporated alcohol extract of gut contents of *Cancer*, dissolved in water, pH 4·73, T = 25°C. Top of arrow at J on abscissa, undiluted original juice with balance method.

*Occurrence of emulsifiers in other invertebrates*

The presence of emulsifiers in the gut could be demonstrated for an insect: the larva of *Dytiscus marginalis*. From nineteen animals a total of *ca.* 1 ml of dark-brown intestinal juice could be obtained. With a small stalagmometer the surface tension could be determined at several dilutions (see Fig. 3). The results were verified by measuring the capillary rise of the fluids. Both methods gave nearly the same results. We see that $\gamma$ of the original fluid is low (35 dyn/cm) and that on dilution the curve has nearly the same shape as that of *Cancer*.

After hydrolysis no taurine or glycine could be demonstrated by means of TLC, but a precipitate of $BaSO_4$ appeared after addition of $BaCl_2$. The identity

of the barium sulphate was proved by reduction with charcoal powder and soda at high temperature. After extraction with water the solution gave dark spots when placed on filter paper saturated with lead acetate (Vogel, 1960). It is therefore not improbable that these emulsifiers are alcohols combined with sulphate.

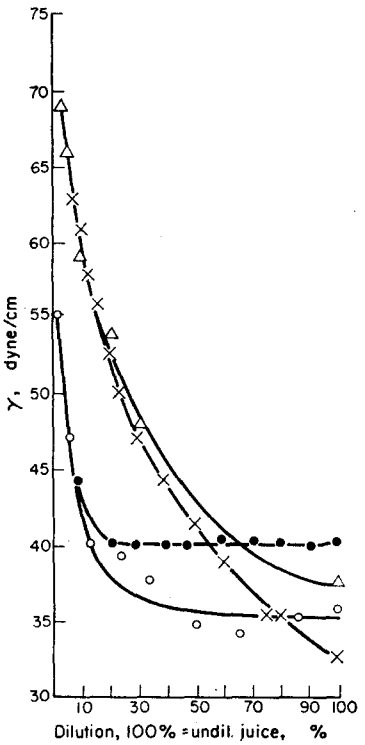


FIG. 3. Same relation as in Fig. 2. ●, Original juice of *Cancer*, pH 6·05, T = 20°C. Definite CMC at 20 per cent of original concentration ( = 3·5 mM/l SDST). ○, Larva of *Dytiscus marginalis*, original juice, T = 20°C. ×, *Holothuria tubulosa*, residue alcohol extract redissolved in water to volume of original juice, pH 7·27, T = 27·5°C. Δ, Other sample without lipids, pH 5·1, T = 22°C.

Sulphate is also formed on hydrolysis of the emulsifiers in the intestinal juice of *Holothuria tubulosa*, as could be demonstrated in the same way as related above. A chromatographic analysis of the lipids in the holothurian juice is given in Table 2. Their total amount is less than in *Cancer*: 0·2 per cent against 0·4 per cent. Two curves showing the relation between $\gamma$ and the concentration of the juice are given in Fig. 3. A definite CMC is not to be observed. On diluting $\gamma$ rises rather rapidly. The intestinal juice of *Aphrodite aculeata* has also a surface tension as low as that of *Cancer*. However, on measuring with the balance method the tension changed during the measurement. This could point to the splitting of an ester by means of an enzyme. Moreover, after extracting a water–alcohol extract of the juice with

xylene, no surface active agent remained in the aqueous phase, which also points to an ester.

Lack of material prevented the continuation of these experiments for the present.

## DISCUSSION

It has been demonstrated in this paper that sodium taurocholate and one of the synthesized emulsifiers (PDST) found in the intestinal juice of *Cancer* are influenced in the same way by salts: their presence causes a decrease in surface tension, dependent on the concentration of the salt. Bivalent cations have a much stronger influence than monovalent cations. The pH has no influence. [All this is in accordance with what is known from colloid chemistry about such effects on other anionic detergents. SDS has been investigated elaborately in this respect (see Durham, 1961).] Moreover, the curves relating concentration vs. surface tension of intestinal juices of different invertebrates and of STCh and PDST (the latter two in the presence of 0·15 M monovalent cations) show a much steeper slope than the curves of STCh and PDST do without additions. The curves of the substances in their natural environment thus reach a lower surface tension and show a lower CMC than the curves of the pure substances. It has been shown further (Table 1) that the natural environment of the emulsifiers (the intestinal juice), especially of *Cancer*, contains indeed the salt concentrations and amounts of lipids favourable for a low CMC and thus for formation of micelles.

The work of Van den Oord *et al.* (1965) has proved that the emulsifiers in the gut of *Cancer* are of another type than that of the bile acids of vertebrates. Although both contain taurine, the compounds with which it is conjugated are steroids (bile acids) in vertebrates, whereas they are some kinds of fatty acids in *Cancer*. Moreover, in *Cancer* sarcosine is inserted between the fatty acids and the taurine. From chromatographic behaviour it could be deduced that this insertion is equivalent to an elongation of the fatty acid. On the other hand, the presence of a double bond in the principal emulsifier of *Cancer* gives rise to a stronger polar character than would be the case for a saturated acid.

The finding that emulsifiers of other invertebrates on hydrolysis split off either taurine or sulphate makes it probable that they too will be of the type of the *Cancer* emulsifiers, although especially in groups systematically far removed from crustaceans other possibilities may be realized.

All vertebrates possess bile acids or bile alcohols conjugated with taurine or sulphate (Haslewood, 1962, 1964) and these have been found nowhere in invertebrates. Thus the interesting problem arises what advantage the development of bile acids may have for the organization of the vertebrate phylum. One advantage could be a greater power of solubilization—a greater efficiency. Some experiments of Hofmann (1963) might point in this direction. Hofmann found that for the solubilization of mono-olein conjugated bile acids are 3·5–4 times more effective than SDS and 4–6 times more than sodium oleyl taurate (mol/mol). These latter emulsifiers are easily comparable to those of *Cancer*. But similar experiments for

fatty acids are lacking. Moreover, the amounts of the emulsifiers present in the intestinal contents must be compared. These are, for example, in man 5·5 mM/l (Hofmann & Borgström, 1962) whereas in *Cancer* they are 0·7 per cent, i.e. 17·6 mM/l, which would compensate for the greater effectiveness of the bile acids. However, in many other invertebrates these concentrations would be 2 or 3 times smaller, according to the amount of taurine or sulphate set free on hydrolysis. This question can therefore not be decided at present. Determining the amount of solubilization in the juice of *Cancer* and other invertebrates is hardly possible because of their strong colour and much more material than available in our investigation would be necessary.

Another possible advantage of bile acids over fatty acyl-taurines could be the following. Recently the particle size of several emulsifiers has been determined (review by Hofmann & Small, 1967). It has been found that the particle size of straight-chain emulsifiers like SDS (expressed as molecular weight, $M$, or aggregation number) is appreciably larger than that of conjugated bile acids. So $M$ is for unhydrated SDS in 0·15 M NaCl 116,000 and for sodium taurodeoxycholate 11,000–12,000. Moreover, it has been found in a few experiments that the increase in size on solubilization is much greater for SDS than for conjugated bile acids. If these experiments could be confirmed, we would have here a definite advantage of bile acids over straight-chain emulsifiers. A difference in size by a factor of 10 would greatly facilitate the passage of particles through the intestinal wall by means of pinocytosis or some such mechanism.

Connected with the appearance of bile in the vertebrate phylum is perhaps the ability of vertebrates to synthesize cholesterol from which the bile acids are derived, whereas this synthesis seems to be impossible in the phylum of arthropods (Sedee, 1961; Van den Oord, 1964; Zandee, 1967) and is lacking in several other invertebrate groups (Voogt, 1967). The amounts of bile acids secreted by vertebrates are high (i.e. when compared to the amount of steroid hormones). Such a need might not be met by the cholesterol taken up with the food, so that synthesis of cholesterol becomes necessary.

These suppositions may be the subject of further investigations.

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