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THE ABSORPTION AND METABOLISM OF ARSENIC IN FISH

by

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INTRODUCTION

It has been shown that fish contain arsenic both in the form of lipid soluble and water soluble arseno organic compounds (1—4). Experiments on the growth of single celled algae in cultures where radioactive arsenic has been added to the culture solution, showed that these algae were able to synthesise both lipid soluble and water soluble arseno organic compounds (5). It was considered that these organisms would be the main source of the arseno organic compounds demonstrated in the marine food chain; in organisms such as fish, shrimps and other invertebrates. However, if arsenic occurs in compounds which are essential to fish and other marine organisms, the possibility that they themselves are able to synthesise these compounds have to be considered. A better understanding of this problem would be of interest from both a biological point of view, and in connection with arsenic containing pollution and waste.

In order to study the absorption of inorganic arsenic by fish, two series of experiments based upon the use of inorganic radioactive arsenic were carried out, one with radioactive arsenic added to the feed and the other with the arsenic mixed into the water.

In the feeding experiments, two types of feed were used. One consisted of terrestrial feed compounds, and contained about 0.3 ppm arsenic, mostly present in the form of inorganic arsenic. The other was based on a marine feed composition containing about 15 ppm of arsenic in the form of arseno organic compounds. The aim of the experiment was to establish accumulation, respectively depletion of arsenic in the fish. If differences in the arsenic content between the two groups could be observed, a second experimental period with inorganic radioactive arsenic added to the feeds should make it possible to establish whether the absorption of radioactive (i.e. inorganic) arsenic is influenced by the level of arsenic in the fish. Two new groups of fish were started as controls on the same two diets a couple of days before radioactive arsenic was added to the two feed compositions during a feeding period of 14 days. In all four groups, both in the lipid and the non lipid phase, the content of synthesised radioactive arseno organic compounds were determined both during the feeding period and in the subsequent period where the pattern of depletion was investigated.

The arseno organic compounds produced by the fish were studied using molecular gel filtration of aqueous solutions produced from the fish, and further by thin layer chromatography of these fractions enriched in radioactive arsenic. In order to get information on the distribution of the radioactive arsenic in the fish in the depletion period, when the content of inorganic arsenic in stomach has been reduced to a negligible level, autoradiographic studies were carried out.

EXPERIMENTAL

Absorption of arsenic.

Rainbow trout (*Salmo gairdneri*) were used in all the experiments. The experimental conditions have been described earlier (6). Four groups with 35—40 fish in each group were used. The feed made up from terrestrial components consisted mainly of minced meat. About 2% carboxy methylcellulose (CMC) was used to hold the mixture together. The marine feed consisted of enzyme hydrolysed powdered defatted cod liver mixed with minced coalfish. CMC was also here used to hold the mixture together. The radioactive arsenic isotope As-74 with $T_{\frac{1}{2}} = 17{,}7$ d (As-74, AJS, 1P, Amersham, England) was sprayed on to the feed mixture and mixed well in before the CMC was added.

The activity of the feed was checked by measuring the specific activity (activity/g of feed) for 10 samples. The variation between these samples was less than 15%. The feed were given in small lumps which were eaten before they began to disintegrate. During most of the period when the two feed types were used, the fish in all groups exhibited a good appetite and normal growth. Only at the end of the period some stagnation in the growth was observed.

The groups fed terrestrial feed is called T1, and the groups fed marine feed is called M1. The two parallel groups started after about two months are correspondingly called T2 and M2.

Samples of fish were taken during the whole experimental periods, and stored at —20 °C prior to analysis.

In the experiments where the absorption of inorganic arsenic from the water was studied, the radioactive arsenic was added to the water using dosing pump (LKB, Sweden) operating at a speed of about 60 ml/hour. The radioactive solution was injected into the main water supply tube through an injection needle. The flow rate of the water through the aquarium during these experiments was 4 l water/min.

The analysis.

The fish selected during the feeding period with radioactive feed and likewise from the depletion period, were weighed and the radioactivity (counts/min, g fish) measured using a scintillation detector (3×3" NaI crystal). The fish were ground subsequently and homogenised in a blendor, and the lipids extracted using a chloroform/methanol mixture (v/v, 2/1) as extracting solvent. The extraction was carried out at room temperature under constant stirring for 2 hours. Some more water was then added and the chloroform phase was separated off. The chloroform-lipid phase was washed twice with distilled water, and the chloroform removed by evaporation.

The yield of lipids was determined by weighing. The radioactivity of the arsenic present as lipid soluble arsenic containing compounds was measured using a scintillation detector (2×2" NaI well type crystal).

The lipid phase extracted from some of the fish samples were analysed in order to obtain information as to the possible synthesis of lipid soluble arseno organic compounds, by observing the possible appearance of radioactivity in the polar fraction, as previously reported for fish lipids (2). The lipids were redissolved in chloroform, and transferred to a column (height: 15 cm, internal diameter: 1 cm) loaded with activated silica gel (Kieselgel 0.2—0.5 mm f. chrom, E. Merck A.G., Darmstadt, W. Germany). After washing with chloroform in order to remove neutral lipids still present in the solution the elution was carried out with respectively 10, 20 and 30% methanol in chloroform. The radioactivity was measured for each of these fractions.

After the extraction of the lipids from the homogenised raw fish, distilled water was added to the non lipid phase and the mixture boiled for 20 min. in an Erlenmeyer flask, whereupon the aqueous solution (the glue water) was filtered and the filter with the undissolved material washed once with distilled water. The washings were combined with the filtrate. All the samples of glue water produced from fish which had received radioactive feed and also fish taken up to 10 days after the feeding with radioactive arsenic was stopped, were eluted on an ion exchange resin (Dow 2 × 8, 200—400 mesh). The pH of the solution transferred to the column was 5—6, and distilled deionized water was used as eluting agent. The radioactivity in the aqueous solution was measured using the same counting equipment as used for the lipids. The ion exchange step is necessary in order to remove radioactive inorganic arsenic still present in the fish. The removal of inorganic radioactive arsenic was also studied by adding separately 6.6 N HCl to the glue water and to the solid phase. The mixture was heated to about 100 °C. The inorganic arsenic would during this procedure, evaporate as arsenic trichloride (7).

Molecular gel filtration of selected samples of glue water was carried out on a dextrane resin (Sephadex G-25 fine, Pharmacia fine Chemicals, Sweden). Before the collection of the fractions the eluate was recorded on an Uvicord spectrophotometer (LKB, Sweden) measuring the absorbance at 254 nm. A more detailed description of the gel filtration procedure used in this study is described elsewhere (3). After measuring each fraction, the most radioactive fraction produced by the gel filtration was analysed further by thin layer chromotagraphy. A system with cellulose substrate (1 mm thickness) and a developing solvent consisting of chloroform/methanol/ammonia in the ratio 2:2:1 was used (8).

Autoradiography.

The distribution of the synthesised arseno organic compounds was studied by use of autoradiographic technique. Selected samples of fish taken out after the period when the use of radioactive arsenic added to the feed had been discontinued, i.e. in the depletion period, were used in a study of how the radioactive arsenic was distributed in the fish. Samples of fish stored at —20 °C were cut up into sections, perpendicular to the backbone. The fish should during this operation be kept at a temperature of +4 °C or less. The sections were polished using fine grain emerypaper and placed in contact with a photographic emulsion (Ilford Industry G X-ray film). A thin layer of cellophane was placed between the fish sections and the photographic emulsion to prevent any of the compounds in the fish sample coming in contact with the film emulsion and thereby giving spurious darkening of the film. During the exposure the samples were placed in light tight boxes and kept at —20 °C. After an exposure period of about 2 1/2 months, the film was developed in the same way as ordinary X-ray film.

Activation analysis.

Glue water produced of fish samples taken from the four groups were evaporated to dryness at 105 °C. Samples of 100—200 mg were taken out and sealed in quartz ampoules, neutron activated together with an arsenic standard and analysed for arsenic using a method slightly modified which has been previously described (4). After the neutron irradiation, arsenic carrier was added and the organic matter was decomposed using sulphuric acid and hydrogen peroxide. The arsenic was then precipitated as arsenic sulphide and the γ-spectrum of As-76 recorded by a multichannel gamma ray spectrometer. It should here be noted that the radioactive arsenic isotope formed by neutron activation, As-76 with a half life of 26 h, has different nuclear properties from the As-74 isotope used in the feeding experiments.

RESULTS AND COMMENTS

Figure 1 shows how the radioactive arsenic mixed into the feed is absorbed by the fish from the four groups during the period in which they received radioactive arsenic added to the feed, and in the subsequent period when the addition of radioactive arsenic in the feed is

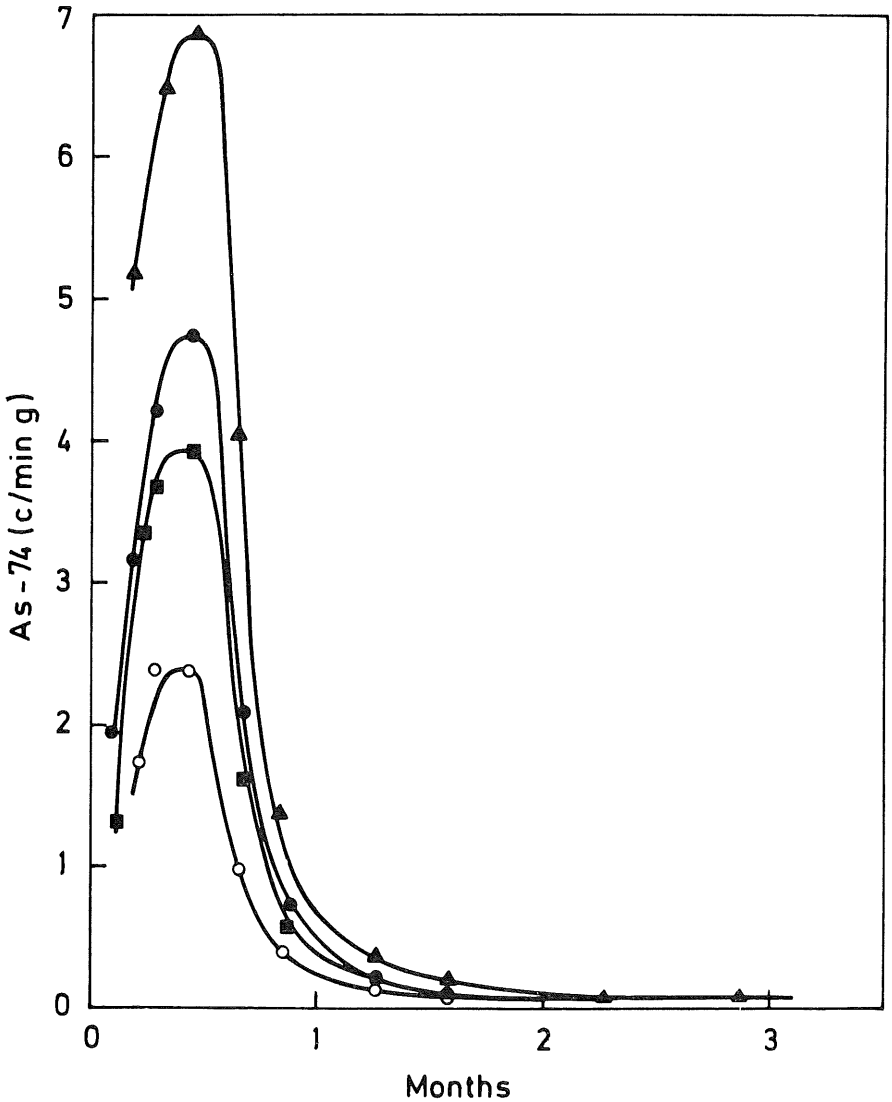


Fig. 1. The content of As-74 in fish taken from the four groups T1 (▲— ▲), MI (○— ○), T2 (●— ●) and M2 (■ — ■) in the period when feeding with As-74 was added to the feed and in the subsequent depletion period.

discontinued. In the beginning of the depletion period a steep decrease in the activity is observed. After 6—10 days the curves level out. At this time the contents of radioactive inorganic arsenic in the fish is negligible at least in the oil and in the aqueous extracts (glue water) produced by boiling fish samples. This is demonstrated for the glue water by ion exchanging and measuring the activity, i.e. arsenite-arsenate absorbed to the ion exchange resin. The same result is obtained for the oil when washing the oil and counting the oil between each washing procedures. The results of the HCl treatment of the solid phase of the same fish show, however, that there may be some inorganic arsenic or organic arsenic compounds decomposed to inorganic arsenic by HCl, located in the meal phase as the activity decreases 10—30% during this treatment. The results here are however rather scattered and may also be caused by individual variations between the different fish samples.

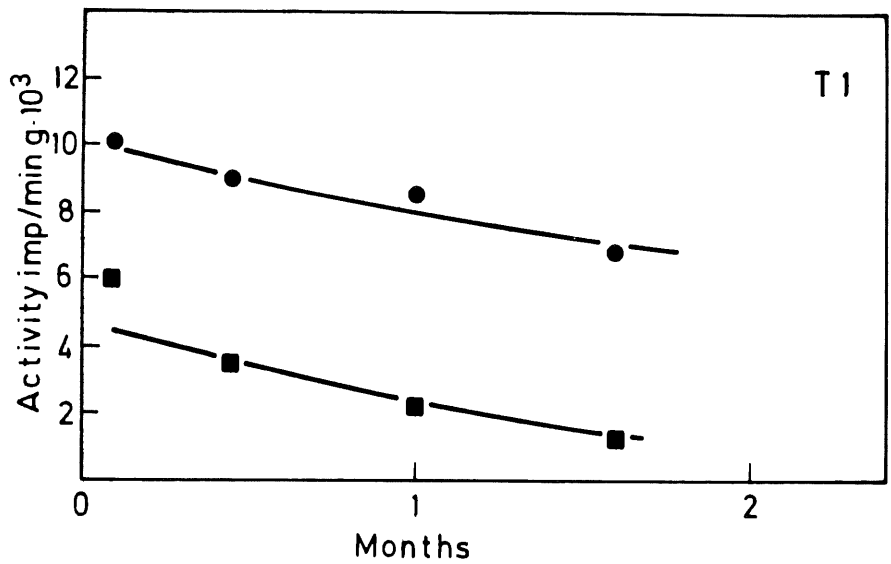
The results presented in Fig. 1 show further that the absorption of inorganic arsenic is influenced by the quantity of arseno organic compounds present in the feed. After a 2 months period of feeding prior to the addition of radioactive arsenic with the two different feeds, there is a significant difference in the absorption of inorganic (radioactive) arsenic in the two groups of fish. In the two groups starting with the two different feed compositions just before the radioactive arsenic was added to the feed, only a small difference in the uptake of arsenic is observed. The results indicate here that the uptake of radioactive inorganic arsenic mixed into the feed is dependent of the content of arsenic present in the fish, as discussed later, only a small fraction of this arsenic is converted by the fish to arseno organic compounds.

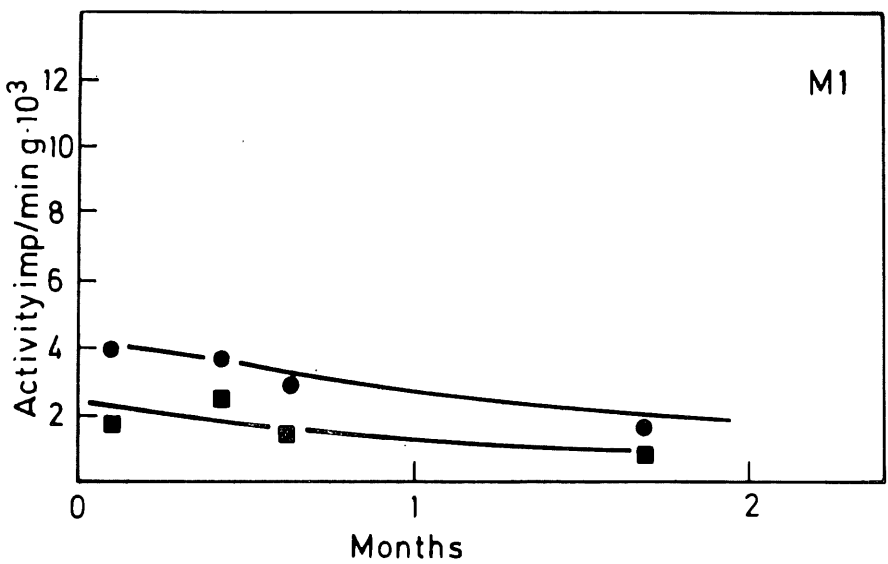
In Fig. 2a—d are shown the radioactivity of lipid soluble and water soluble arseno organic compounds present in the fish after the radioactive feed has been discontinued. The curves indicate that the lipid soluble arseno organic compounds have about the same biological half life, about 40—50 d, as the water soluble ones. However, taking into consideration that there may be considerable variations between the fish samples it is difficult to draw any definite conclusions concerning this matter.

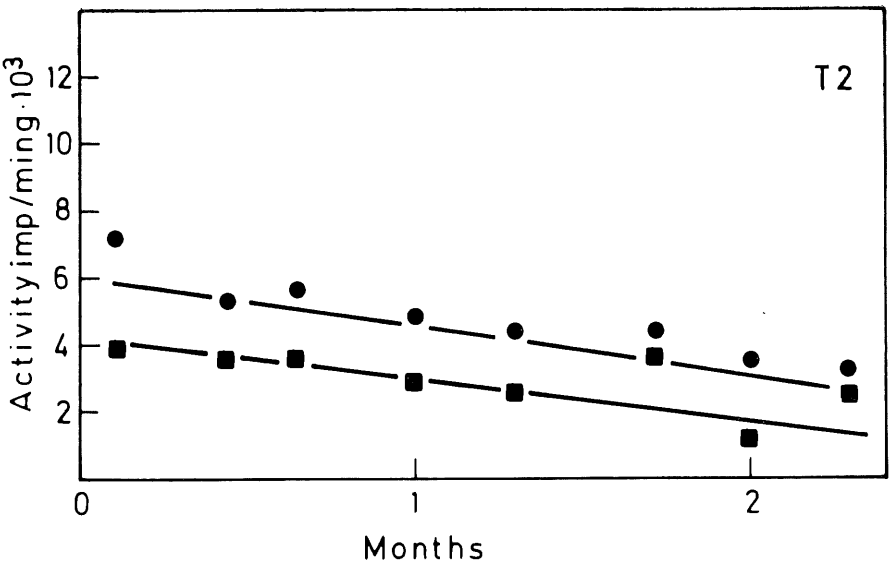
In the two groups, MI and TI, fed on marine and terrestrial feed which started 2 months before the addition of radioactive arsenic, there is a significant difference especially in the amount of radioactive lipid soluble arseno organic compounds. In the two other groups M2 and T2, only a small change in the level of radioactive water soluble arseno organic compounds is observed.

When comparing the arsenic activity from the arseno organic compounds present in the lipid and the non lipid phase of the fish samples

with the total amount absorbed, see Fig. 1 and 2a—d, it is observed tha- only a small fraction of the inorganic arsenic is converted to arseno organic compounds. The amount of inorganic arsenic in the terrestrial feed composition is less than 0.3 ppm and in the marine feed probably in the area of 1 ppm. As only a small fraction of this is absorbed and converted to arseno organic compounds in the fish, the results demonstrate







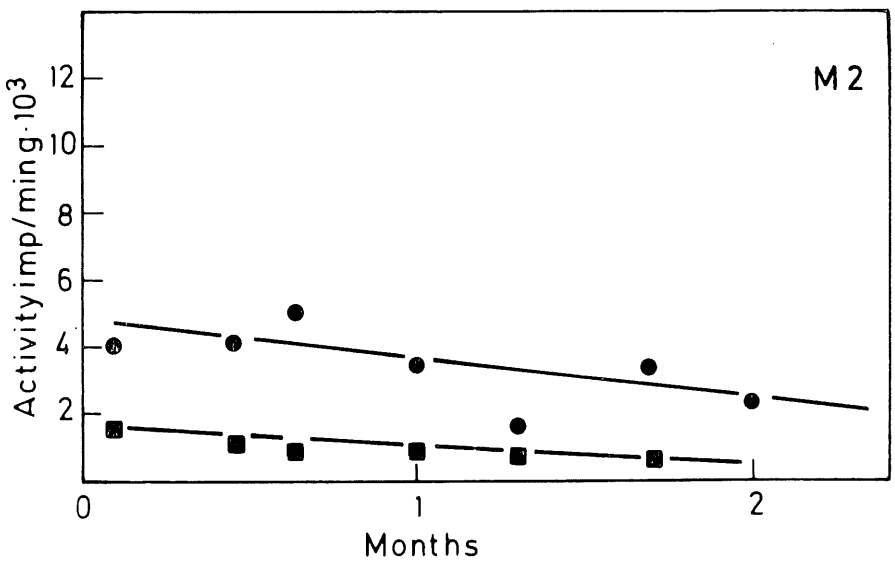


Fig. 2a—d. The content of radioactive (imp As-74/g matter) lipid soluble (● — ●) and water soluble (■ — ■) arseno organic compounds in fish taken from the four groups. Zero time represents the date when the addition of As-74 to the feed is discontinued.

that this source of arsenic represents a quite inessential contribution to the total quantity of arsenic found in fish. The arsenic present in the fish is therefore probably supplied from other marine organisms at lower stages in the marine food chain. This is also demonstrated by the results presented in Fig. 3. Here the results show that the two different feed compositions had a pronounced effect on the level of arsenic in the fish. Although there is some scattering in the determinations, the results show that after 1—2 months the groups fed on the marine feed have a considerably higher level of arsenic in the glue water than the other groups of fish fed on the terrestrial feed. Starting with about 20 ppm arsenic in dehydrated glue water in all groups, the M groups increase to 30—50 ppm while the arsenic content in the T groups decrease to about 10 ppm. These results suggest that the arseno organic compounds from lower stages in the marine food chain represent probably the main sources of arsenic present in fish.

The results of the molecular gel filtration experiments carried out on glue water extracts are presented in Fig. 4, where the absorbance at 254 nm of the eluate and the radioactivity registered (counts/min g dry material) for fractions obtained during the gel filtration are shown. They confirm that the water soluble arseno organic compounds synthe-

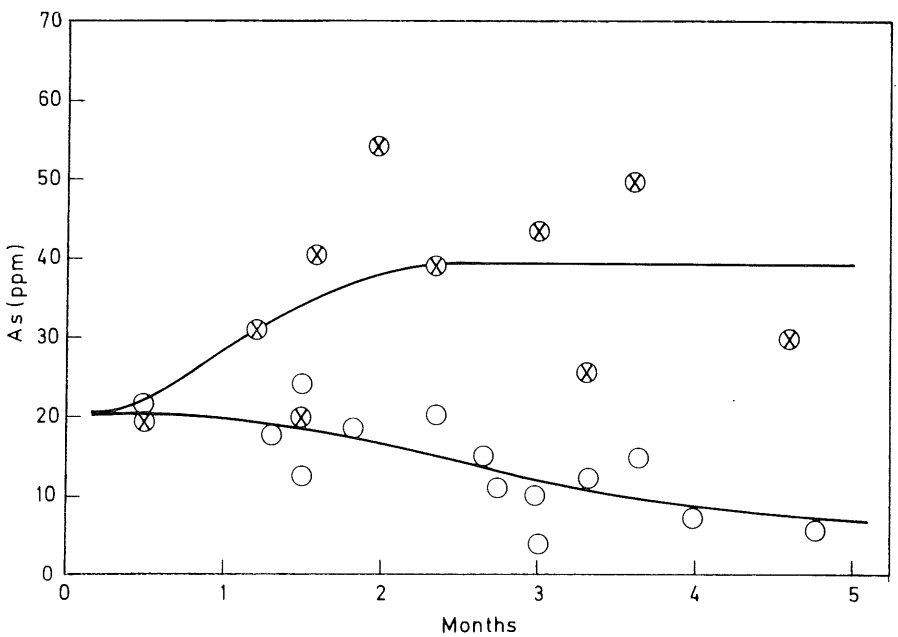


Fig. 3. The Absolute amount of arsenic in glue water produced from fish fed on marine (○ — ○) and terrestrial feed (× — ×).

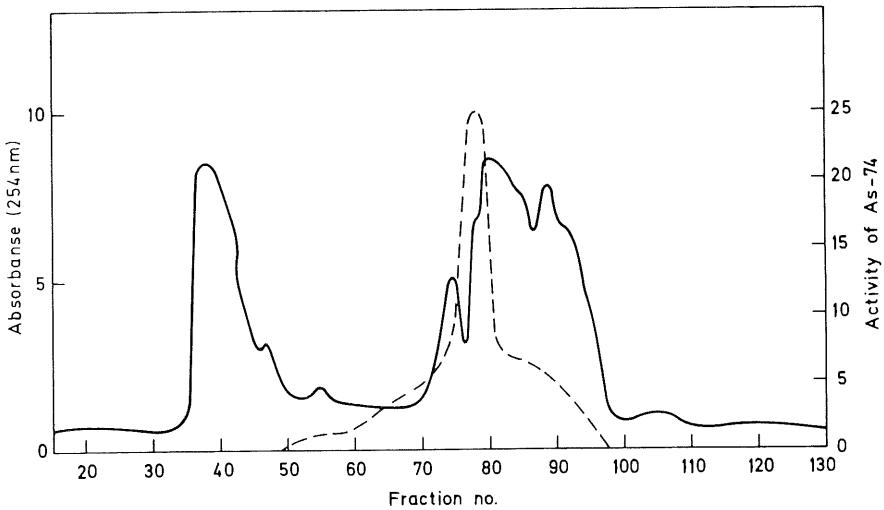


Fig. 4. The absorbance at 254 nm (———) and the radioactivity from As-74 (— — —) of molecular gel filtrated glue water. The glue water was produced from fish taken 6 days after the depletion started.

sised by the fish, are eluted in the same fractions as have been established for the main amount of organic bound arsenic in fish (3). Autoradiography applied on thin layer chromatograms of the molecular gel filtrated fractions with highest specific activity produced from fish taken in the depletion period, shows one radioactive arseno organic compound with a Rf value of about 0.30. Due to the low specific activity of the arseno organic compounds present in the glue water, only one radioactive arseno organic compound was detected. The Rf value, 0.30, corresponds to the results obtained when water soluble radioactive arseno organic compounds synthesised in single celled algae were analysed in the same way (5). Both the results from the molecular gel filtration and the thin layer chromatography indicate that the main water soluble arseno organic compound which is synthesised by the fish, is also synthesised by algae and probably by other marine organisms.

The results of the fractionation studies where the neutral part of the lipids was separated from the polar fractions on a silicagel column show that the arseno organic lipids synthesised by the fish follow the polar fractions. This is in agreement with previous results where the total amount of arseno lipids in fish were studied (2). Most of the radioactive arseno lipids was detected in the eluates using 20 and 30% methanol in chloroform as eluting agent.

Fig. 5—7 show how the radioactive arseno organic compounds are distributed in some selected sections of the fish. These compounds seem

especially to be enriched in the eyes, the throat and the gills, the pylorus organ and in some less distinct area. Due to a relatively rough technique used for preparing the samples and also the use of a fast X-ray-emulsion, a rather low resolution on the autoradiographs was obtained. Besides the organs already mentioned also the liver and the kidney exhibit a strong accumulation of radioactive arsenic immediately after the feeding of radioactive arsenic was discontinued. But this arsenic disappears faster from these organs in the depletion period than from the other organs. This may indicate that the synthesis or part of the synthesis of the arseno organic compounds occurs in the liver. No significant accumulation was observed in the fish muscle and in the skin. The strong concentration of arseno organic compounds in the eyes and in the throat and gills, that is in the most pronounced mucus membrane regions show that these compounds may have a bacteriostatic effect and is used by the fish to protect these area against microorganisms.

Fig. 8 shows the absorption of inorganic radioactive arsenic by fish when the arsenic was added to the water. Ordinarily the fish samples

Fig. 5. Autoradiograph of a section cut perpendicular on the back bone of the fish and through the eyes. The fish was taken 12 days after the depletion period started.

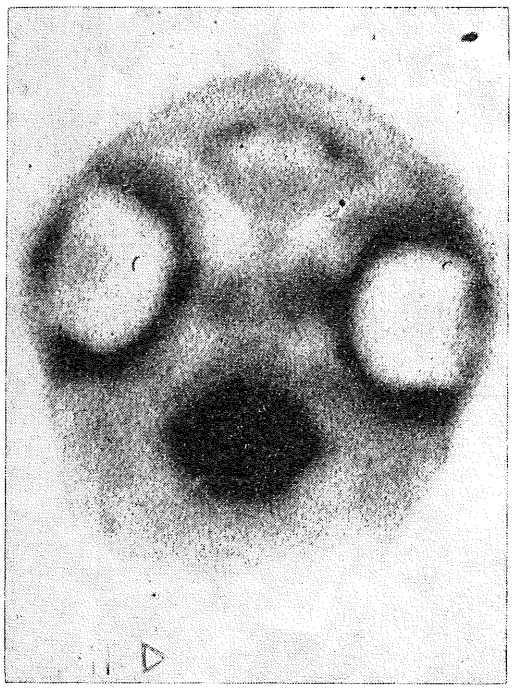


Fig. 6. Autoradiograph of a fish section prepared in the same way as in Fig. 5, but from a different area in the head of the fish. The fish taken 12 days after the depletion started.

were kept in inactive water for a period of 5 min. before they were taken out and the absorbed activity recorded. Some corresponding values for fish washed in inactive water for 1 day are also presented. These last results show that at least 25—30% of the absorbed activity is washed out during this period.

In fish samples selected from this latter series of experiments and which were treated in the same way as previously described, the presence of neither lipid soluble or water soluble arseno organic compounds was detected. Even though it was diffcult to achieve a sufficiently high specific activity in the water without using excessively large quantities of radioactive arsenic, it should nevertheless have been possible to detect radioactive arsenic in the oil phase if the synthesis of lipid soluble arseno organic compounds occurred in the same way as when arsenic was added to the feed. The results show also that some inorganic arsenic can be washed out, indicating at least to some degree a passive absorption. The activity of the water was in average 50 imp/min ml. Comparing this value with the amount absorbed in the fish ca. 0.4 imp/min g, it is evident that the absorption of arsenic is a rather inefficient or slow process.

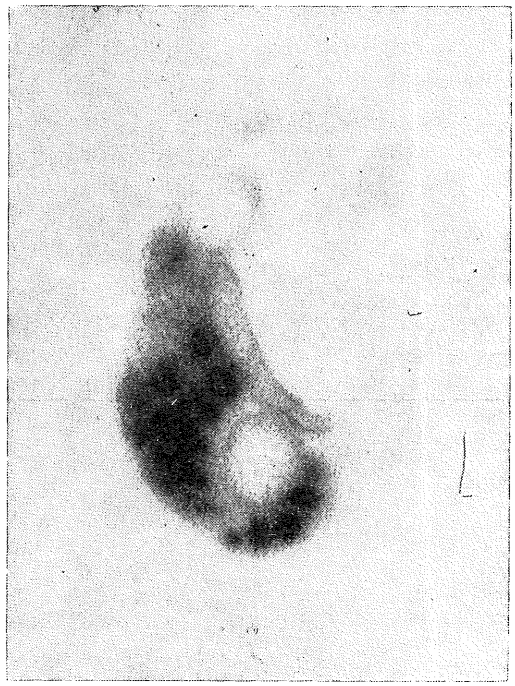


Fig. 7. Autoradiograph of a fish section prepared as in Fig. 5 taken from the stomach and through the pylorus organ. The fish was taken 12 days after the depletion started.

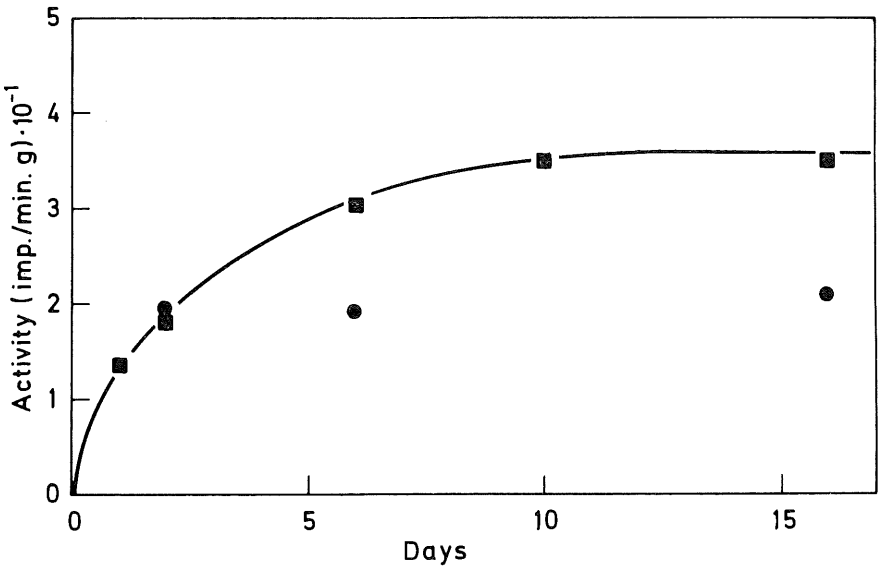


Fig. 8. The absorption by the fish of As-74 (imp As-74/g fish) added as inorganic arsenic to the water. The fish were kept in inactive water 5 min (■ — ■) and 1 day (———) before counting.

CONCLUSION

On the basis of the results obtained during these experiments it can be concluded that fish are able to synthesise both fat soluble and water soluble arseno organic compounds from the inorganic arsenic to be found in the feed eaten by the fish. However, this inorganic arsenic plays an insignificant role as source for the organic bound arsenic found in fish. The major part is supplied as already synthesised arseno organic compounds, from lower stages in the marine food chain. An accumulation of arseno organic compounds in specific organs shows that the compounds may possibly have a significance for fish. It is proposed that this may be as a bacteriostatic agent which acts to protect those regions of fish which are especially vulnerable to attack from microorganisms.

Inorganic arsenic present in the water will also be absorbed by the fish, but neither water soluble nor lipid soluble arseno organic compounds could be detected by the available methods used in this investigation.

ACKNOWLEDGEMENT

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SUMMARY

The absorption and metabolism of inorganic arsenic in rainbow trout (*Salmo gairdneri*) has been investigated by means of radioactive tracers. The results obtained show that the fish will absorb inorganic arsenic when this is mixed into the feed, and synthesise both lipid soluble and water soluble arseno organic compounds. The absorption of inorganic arsenic seems to depend upon the quantity of arsenic present in the fish. The absorption of inorganic arsenic decreases as the concentration of arsenic in the fish increases. When radioactive arsenic (As-74) is added to the water, absorption seems to take place through the skin and gills. This absorption, as far as can be determined by the methods used in this investigation, does not result in the formation of arseno organic compounds. When As-74 is mixed in the feed and thus absorbed through the digestive track, autoradiographic investigations indicate that arseno organic compounds are synthesised and accumulated in the eyes, the throat and gills and certain of the internal organs.

REFERENCES

1. LUNDE, G., J. Am. Oil Chem. Soc., 49, 44 (1972).
2. LUNDE, G., J. Am. Oil Chem. Soc., 45, 331 (1968).
3. LUNDE, G., J. Sci. Fd Agric., 21, 242 (1970).
4. LUNDE, G., Nature, 224, 186 (1969).
5. LUNDE, G. To be published.
6. BRÆKKAN, O. R., LAMBERTSEN, G., and ANDRESEN, J. (1971): Fiskeridir. Skr. Ser. Tekn. Undersøk. 5, No. 8, pp.
7. LUNDE, G. To be published.
8. BONDIVENNE, R. et Bush, N., J. Chromatog. 29, 349 (1967).