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TESTS OF THE METHODS BY WHICH PAPER CHROMATOGRAPHY OF FISH MUSCLE CAN BE USED AS A TAXONOMIC AID

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During recent years work has been done with the aid of paper chromatography for the identification of fish (BUZZATI-TRAVERSO & RECHNITZER 1953, DANNEVIG 1955, 1956). The different species and even subspecies or populations show constant though often small differences in the combinations of the amino acids, which are the building stones of the proteins in the muscle.

It was considered worth while to try this biochemical identification method on the taxonomically very troublesome fish genus, *Coregonus*, the whitefish.

In the lake from which the material was obtained there are three or four different forms of whitefish, with particular local names.

Before starting on the main taxonomic work it was found necessary to do some preliminary tests of the methods as used. For these initial tests material was used from only one of the local forms, the "vintersik", which was caught during the spawning time.

One-dimensional descending paper chromatography was used in the usual manner. Two solvent systems were tried. The first comprised 2 parts of n-propanol, and 1 part of 1 % ammonia. The second, which was a mixture of n-butanol, glacial acetic acid and water in the ratio 4 : 1 : 5 (BUZZATI-TRAVERSO & RECHNITZER 1953), gave the better results. The time of running was about 24 hours at 20° C. during which time the solvent front almost reached the bottom of the paper. The chromatograms were sprayed with a 0.2 % solution of ninydrin in 96 % ethanol, and kept for five minutes at 105° C., to develop the ninhydrin-positive patterns.

The usual method of preparing paper chromatograms for taxonomic use with fish is to cut out a small piece of muscle, about 2 mm^3, from a certain area of the fish, and press it on to the paper. This will give a spot

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of about 8—10 mm. in diameter which is allowed to dry, after the remains of the muscle have been removed.

It was thought that possibly this sampling technique might not give all the substances that could be shown by the ninhydrin reaction. A comparison was therefore made between this sampling method and a method using a sample of the same muscle which was homogenized and centrifuged. Muscle (2 gm.) to which was added 4 ml. of distilled water, was homogenized in a "Potter-Elvehjem" homogenizer, cooled in ice. To the homogenate an additional 2 ml. distilled water was added, and the whole then centrifuged until the supernatant was completely clear. This test was repeated, using the same amount of 0.1 M phosphate buffer pH 7.0, instead of distilled water. A suitable amount of the supernatant (25 µl) from each of these two tests was then placed with a Carlsberg pipette on the same chromatogram as samples from the first method. On developing the chromatograms, both methods gave the same result. It therefore appeared that the first method (direct pressing out), which is certainly simple and easy to use, was quite adequate. The method using a Carlsberg pipette and the supernatant has the advantage that the same amount of material is accurately transferred each time. However, experience shows that the first method also transfers the same amount of material fairly accurately each time. This method can also be used in field work, where it has also been established that dried chromatograms can be stored for months when kept dry.

In previous work in this field entirely fresh material has always been used, and it has been pointed out that this is necessary so that postmortal changes of the muscle shall not have any influence. However, it became of interest to examine how and when the chromatograms changed in the case of fish that had been stored for some time, as it is not always possible to work with newly-killed fish. Tests were therefore made after various periods of time, on fish kept at various temperatures (—10°, 5°, 8—10°, 18—20° C.), 4—6 ungutted fishes being used at each temperature. Here the first method of direct pressing out was used in preparing the chromatograms. The fish were kept in plastic bags to prevent dehydration. On the same chromatogram a freshly-killed fish was also tested. At —10° C. the chromatograms showed no change, even after the fish had been kept for a month. At 5° C. a change appeared after 6 days, at 8—10° C. after 5 days and at 18—20° C. after $2^1/_2$ days. The changes manifested themselves by some of the spots increasing in intensity, while the separation became continuously less distinct. Eventually the whole chromatogram became one band. However, in some cases the first change consisted in the weakening of a particular spot. The strong increase in certain components has been considered due to their increased production by bacterial attack.

Ranke & Bramstedt (1954) in corresponding investigations using paper electrophoresis found a change after 5 days, with extracts from the white muscle of the coalfish fillet kept at 2 to 4° C. They obtained the same results with ungutted freshwater fish kept under the same conditions. In subsequent work (1955) the same authors have also used paper chromatography and found changes in the content of several free

amino acids from ling and coalfish fillet during several days at 2 to 4° C. The time before the appearance of the first change is not given exactly, but the article seems to indicate that it is less than 5 days. In the same connection JONES (1954a), using a series of chromatograms, has found increased amounts of two free amino acids in the earliest spoilage phase, before bacterial attack. In these investigations he used ethanol (75 % v/v) extracts of muscle from gutted and iced cod, and two-dimensional paper chromatography. This increase was also found at 0° C. and 15° C. in sterilized muscle homogenates and cell-free aqueous muscle extracts. The exact time when the first increase was observed is not indicated, but later work (JONES 1954 b) states that early changes occur during the first 4 days, with fish stored in ice, and that the changes are primarily caused by the effect of autolytic enzymes. Later changes result from the additional effects of spoilage micro-organisms.

According to the results of JONES (op.cit.) it is possible that if we had used two-dimensional chromatography the time before the first change appeared in our experiments would have been somewhat reduced. However, some time must elapse before the enzymes manage to break down the proteins to peptides and amino acids, and thus disturb the pattern obtained for freshly-killed fish.

In conclusion it would seem that half the times observed in our experiments can be considered safe time limits when preparing paper chromatograms for taxonomic purposes. This applies to temperatures above zero, while fish frozen to — 10° C. and below can certainly be used after some weeks without risk of any change in the chromatograms due to decomposition while in storage. This applies of course only to those cases in which newly-killed fish are not available, otherwise quite fresh material is to be desired.

To test the stability of the chromatographic pattern from this form of whitefish, "vintersik", a series of chromatograms were taken of about 40 newly-killed fish; they all showed the same pattern.

With regard to the different forms of whitefish, a difference in the chromatographic pattern from one of these forms has so far been observed. This work is continuing and will be published later.

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