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The Estimation of Free Sugars in Skeletal Muscle of Codling (*Gadus callarias*) and Herring (*Clupea harengus*)

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(Received 6 August 1957)

Data relating to free sugars in the skeletal muscle of fishes are scarce. Moreover, many of the early reductometric studies (e.g. MacPherson, 1932) lacked specificity and it is now well-established that large excesses of amino nitrogen such as occur in extracts of fish muscle, cause serious inaccuracy with such procedures (Hewitt, 1938; Strange, Dark & Ness, 1955). Tarr (1954) has described the chromatography of concentrated ethanolic extracts, followed by direct photometry of the sprayed papers to evaluate sugars in the flesh of some species of the Canadian Pacific seaboard. In this Laboratory, both interference by 'salt' and fat and low recoveries of ribose accompanied a similar manipulation of extracts of codling and herring muscle. The present paper shows that the loss of ribose results from Maillard (1912) type reactions with amino nitrogen.

In attempting to overcome these difficulties use had been made of a number of techniques employed in current biochemical practice. The removal of salts by electrodialysis (Consden, Gordon & Martin, 1947) and ion-exchange resins is a commonplace in the literature. Partridge (1948) used the latter principle to remove substances interfering with the chromatography of sugars in apple juice but Hulme (1953) demonstrated that strongly basic resin in the OH^- form employed (in conjunction with strongly acid resin in H^+ form) limited the recovery of glucose. Phillips & Pollard (1953) favoured a resin mixture containing strongly basic resin in CO_3^{2-} form, which did not degrade glucose. Strange *et al.* (1955) in a study reported while the

present work was in progress, removed the amino acids of casein hydrolysate by a mixture containing a weakly basic resin in OH^- form before the reductometric determination of glucose in media.

This paper describes the effects of various procedures on the recovery of free sugars from extracts of muscle and reports the danger of serious artifacts which may have relevance to sugar analysis in other biological extracts and fluids. A short preliminary communication of some of the work has appeared (Jones, 1956a).

EXPERIMENTAL

Materials

Codling. These were landed alive from R.V. 'Keelby' and maintained in aerated sea water until killed by a blow on the head. For post-mortem studies the fish were gutted and packed in ice at an ambient temperature of 2.5° . Sterile codling muscle was dissected from the anterior-dorsal part of the fillet, immediately after death, under aseptic conditions: for studies on autolysis, samples of muscle (5 cm. $\times$ 2 cm. $\times$ 1 cm.) were stored at 0° in sealed Petri dishes.

Herring. These fish, 14–20 hr. dead and stored at about 0° , were made available through the kindness of the Marine Laboratory, Scottish Home Department, Aberdeen.

Resins. Amberlite resins IR-120, IR-4B (Rohm and Haas Co., Philadelphia, Pa., U.S.A.); Dowex 50; Dowex 2 (Dow Chemical Co.); sulphonated polystyrene, nominal divinyl content 4.5% (PSX), De-Acidite FF (Permutit Co. Ltd.). The resins were used in the form and mesh-size reported in the text.

Nucleotides, ribosides and sugar phosphates. Adenosine mono-, di- and tri-phosphates were supplied by the Sigma

Chemical Co., St Louis, Mo., U.S.A. Ribosides, adenosine and inosine were supplied by L. Light and Co. Hexose diphosphate (Ba salt) was presented by Dr C. Long. Glucose 6-phosphate was supplied by the Sigma Chemical Co.

Chromatographic solvents. These were redistilled before use.

Methods

Preparation of extracts. (a) Trichloroacetic acid extracts: muscle (10 g.) was macerated into 30 ml. of 10% (w/v) trichloroacetic acid (TCA) and centrifuged. (b) Ethanol extracts: muscle (10 g.) was macerated into 32 ml. of ethanol and the residue re-extracted three times into 80% (v/v) ethanol to a total extract volume of 250 ml. This procedure assumed 80% (w/w) of water in the muscle. Moisture was determined on parallel samples to correct subsequent calculations on the 80% (w/w) basis. (c) Chloroform-separated ethanol (CSE) extracts: chloroform was added in 3:1 (v/v) ratio to portions of ethanol extracts prepared as above. The upper, aqueous phase which separated contained the free sugars. Awapara (1948) used a similar technique for free amino acid analysis: it is useful in removing fat and the little protein soluble in 80% (v/v) ethanol.

Lyophilization. This was carried out over P_2O_5 in a centrifugal freeze-drying apparatus.

Reducing-sugar estimations. The method of Somogyi (1945) was used.

'Direct' chromatography of extracts. (a) Portions (10 ml.) of TCA extracts were continuously extracted with ether to remove excess of acid and lyophilized. They were stored and applied to chromatograms as described below. Chromatograms were developed (decending, 16–24 hr.) in phenol- NH_3 (no HCN), collidine and butanol-acetic acid (Partridge, 1948) at 25°. (b) Ethanol extracts (50 ml.) were concentrated at 12 mm. Hg to 1 ml. at a bath temperature of 35°. This is essentially the procedure of Tarr (1954). Portions were chromatographed as above. (c) CSE extracts (8 ml.) were lyophilized and stored at -2° until immediately before use. Water (150 μ l.) was added and portions were applied by Agla microsyringe on to Whatman no. 1 paper in successive 1 μ l. volumes; spots were dried in a stream of cold air to minimize undesirable reactions (see Results).

Chromatography of electrolytically desalted extracts. TCA and CSE extracts were desalted in an apparatus (Shandon Scientific Co.) based on that of Consden *et al.* (1947). The chamber of this apparatus has no provision for cooling other than the flow of chilled sulphuric acid. To minimize undesirable reactions the temperature was maintained below 40° by switching off the current periodically. Desalted extracts were lyophilized, stored and chromatographed as described above.

Chromatography of extracts after removal of salt and amino compounds by ion-exchange resin. Systems of mixed resins (all 1:1, w/w) tested were: (a) Amberlite IR-120 (H^+ , 100–200 mesh): Dowex 2 (CO_3^{2-} , 200–300 mesh); (b) Amberlite IR-120 (H^+ , 100–200 mesh): Amberlite IR-4B (OH^- , 60–100 mesh); (c) PSX (H^+ , 100–200 mesh): De-Acidite FF (CO_3^{2-} , 100–200 mesh); (d) Dowex 50 (H^+ , 200–400 mesh): Amberlite IR-4B (OH^- , 60–100 mesh).

Resins were prepared in the desired form in columns and sucked just free from surface water at the pump. Excessive drying was avoided as absorption of water from extracts

under such conditions leads to high sugar values. The resins were stored in sealed bottles to avoid evaporation. With different mesh sizes, resins may be recovered by differential decantation.

Resin mixtures (2 g.) were shaken in batches at room temperatures (about 18°) with CSE or ether-extracted TCA extracts equivalent to 2 g. of tissue. Deionized suspensions were centrifuged and samples lyophilized before chromatography as described above.

Evaluation of sugar chromatograms. After drying at 60° papers were sprayed on both sides with aniline hydrogen phthalate–butanol (Partridge, 1949). The paper was heated for 10 min. at 105° and sugar spots (ribose and glucose were identified in the extracts) were eluted into 2.0 ml. of acetic acid for absorptiometric evaluation against chromatogram standards at 480 $m\mu$. Blank values of equivalent areas adjacent to spots were determined at the same time. This procedure was similar to that of Baar (1954) in that spots were eluted into acetic acid. It differed in the lower quantity of sugar estimated (2–20 μ g. in comparison with 25–200 μ g.) and that chromatograms were sprayed rather than immersed in aniline hydrogen phthalate reagent, which gave lower 'blank' values and reduced diffusion of the spots. In all quantitative measurements standard sugar solutions and extracts have been subjected to identical preparatory treatments. The accuracy was ± 5 –6% at the 5–10 μ g. level.

Amino acid analyses. Amino acids were estimated by two-dimensional chromatography and ninhydrin spot-elution (Jones, 1955).

RESULTS

Chromatography of untreated extracts. The direct chromatography of lyophilized CSE extracts, vacuum-concentrated ethanol extracts and ether-extracted TCA extracts gave chromatograms with poor resolution in solvents other than butanol-acetic acid (cf. Block, Durrum & Zweig, 1955). A brown pigmentation formed at the origin during the application of clear extract. The pigment did not migrate in the solvents tested and darkened after spraying and heating. Controls showed that this was not due to reaction with aniline hydrogen phthalate reagent. Glucose and occasional traces of ribose were identified in extracts of muscle from codling 10 days *post mortem*. When glucose and ribose were added at levels equivalent to 20 mg./100 g. of tissue to extracts of muscle from codling, both freshly killed and iced for 10 days, satisfactory recoveries of glucose (93–108%) were obtained, but those of ribose ranged from 7 to 16%. Low recoveries of ribose were accompanied by increases in the density of brown pigment at the origin of the chromatograms and it seemed reasonable to suppose that loss of ribose could result from reactions between the sugar and muscle amino compounds (Maillard, 1912). These would readily occur under the conditions of low humidity during the successive applications of extract concentrate to the chromatogram. The largest sources of amino

nitrogen present in lyophilized CSE extracts are ammonia, trimethylamine, amino acids and anserine (Jones, 1954a).

Effects of electrolytic desalting. Ammonia and trimethylamine are particularly reactive with sugars and if these were the sole agents of ribose destruction it appeared that they might be removed by electrolytic desalting. During the course of such treatment of TCA and CSE extracts it was noted that they turned first pink and then brown in colour; the brown pigment remained at the origin during chromatography. Levels of glucose relative to those of untreated TCA and CSE extracts (muscle from codling stored in ice for 10 days) were similar at 8.0–8.4 mg./100 g. of tissue, whereas, in contrast with zero recoveries of ribose from controls, 3.1 and 5.2 mg./100 g. were recovered from electrolytically desalted CSE and TCA extracts. These results could not be interpreted as proving that substances interfering with the determination of ribose had been removed: 'browning' had increased considerably and, while other factors [e.g. degradation of 1-methylhistidine (Jones, 1956b)] could cause this, there was a possibility that although ribose and amino acids were reacting, the sugar was also being liberated from bound forms. This possibility was confirmed in experiments in which 0.9% NaCl solution (8 ml.) was desalted in the presence of 5 mg. of adenosine or inosine with and without a synthetic amino acid mixture (taurine, 7.5 mg.; glycine, 0.41 mg.; alanine, 0.35 mg.; glutamic acid, 0.163 mg.; 1-methylhistidine, 0.136 mg.; leucine, 0.154 mg.; valine, 0.081 mg.; β -alanine, 0.025 mg.; aspartic acid, 0.045 mg.; serine, 0.028 mg.; lysine, 0.045 mg.; cysteine acid, 0.025 mg.) equivalent to the free amino acid composition of fresh codling muscle (Jones, 1954b). Ribose was liberated from both adenosine (121 μ g.) and inosine (95 μ g.) when treated alone and these levels were reduced to 51 and 39 μ g. in the presence of amino acids.

Removal of interfering substances by ion-exchange resins. It appeared that loss of ribose could be due to reaction with muscle amino acids in the absence of ammonium and trimethylammonium ions. To remove interfering amino compounds and salts, while at the same time eliminating the liberation of sugars from bound forms, treatment with mixtures of ion-exchange resins was attempted. CSE extracts of codling muscle (5 days *post mortem* at 0°) were lyophilized and chromatographed in butanol-acetic acid. Portions of the same extracts (equivalent to 2 g. of tissue) were shaken with resin mixtures, as described in the Experimental section, before lyophilizing and chromatography. With both strong acid (H^+)-strong base (CO_3^{2-}) and strong acid (H^+)-weak base (OH^-) systems con-

siderable improvement in the quality of chromatograms and the recovery of ribose was obtained. After a 15 min. shaking period with the different resin mixtures, extracts of codling muscle (5 days *post mortem*) still contained some 90% of the original taurine (120 mg./100 g. of tissue) together with traces of aspartic and glutamic acids. This residual amino nitrogen was not significantly Maillard-reactive under the conditions of analysis: recoveries from 2 mg. of ribose and 2 mg. of glucose in 50 ml. of water containing 200 mg. of taurine, 8 mg. of glutamic acid and 4 mg. of aspartic acid were 95 and 98%, after lyophilizing and chromatography in butanol-acetic acid.

The actions of the resin mixtures on a number of muscle metabolites containing glucose and ribose were examined. No free sugar was liberated from solutions of adenosine triphosphate, adenosine diphosphate, adenylic acid, diphosphopyridine nucleotide, inosinic acid, tested at physiological concentration (Jones & Murray, 1957) with a 15 min. shaking period. Nor were fructose 1:6-diphosphate, glucose 6-phosphate or inosine hydrolysed with liberation of free sugar where 5 ml. of 0.1% solutions were shaken with mixtures of 2 g. of resin. Adenosine was hydrolysed by 2 g. of IR-120 (H^+)-IR-4B (OH^-) to the extent of 300 μ g./hr. from 10 ml. of 0.2% solution. The highest concentration of this riboside measured in fresh or spoiling codling muscle in this Laboratory is less than 0.4 mg./100 g. of tissue and the riboside has not been detected in herring. The maximum inaccuracy from this cause in codling muscle was therefore +1.5 μ g. of ribose/100 g. of muscle. This was well within the standard error of the chromatographic procedure in muscle samples where the artifact could occur.

From the preceding experiments it appeared that desalting and removing amino compounds by suitable mixtures of ion-exchange resins provides a basis for reasonably accurate analysis of free sugars in codling muscle. Results of such analyses (Fig. 1, Table 1) indicate the presence of variable amounts of glucose and the absence of ribose in the muscle of codling killed in an exhausted condition, with a liberation of ribose after death: analyses with the other resin mixtures listed in the Experimental section were very similar. Reductometric analysis of untreated extracts of fresh and spoiling muscle gave values of the order of 450 mg. of glucose equivalent/100 g. Recovery experiments (Table 2) with extracts of herring indicated that the procedure was also satisfactory for that species, an observation recently confirmed by more extensive studies (Dr R. B. Hughes, personal communication). Results with the other resin mixtures were again similar. Ribose and glucose values were of the same order as those of codling.

On different fish, dead for 14–20 hr. and kept at 0°, values ranged from 5.1 to 38.2 mg. of glucose/100 g. and 1.9–6.2 mg. of ribose/100 g. (12 samples).

DISCUSSION

The skeletal muscle of fishes and many other marine animals contains very high levels of 'extractive' nitrogen present largely in amino form. Such quantities of amino nitrogen are known to interfere with reductometric analysis (e.g. Hewitt, 1938) and the present paper shows that their presence may also result in low recoveries of ribose in chromatographic analysis owing to Maillard reaction during the application of extracts to the paper. Such reactions would be most likely to occur under the near-dry conditions obtaining during successive applications in 'building-up' spots and in the presence of naturally occurring cation catalysts. A finding (Tarr, 1954) that a similar procedure is satisfactory may be explained by the greatly differing quantitative and qualitative patterns of nitrogenous substances extracted from different species (Shewan, 1953, 1955).

The removal of 'salt', ammonium ion and trimethylammonium ion by electro dialysis (Consden *et al.* 1947) is unsatisfactory in so far as ribose was both liberated from bound forms and eliminated by reaction with amino compounds not removed at the cathode (cf. Smith, Stevens & Jepson, 1956, for other examples of degradation occurring during electrolytic desalting).

Suitable mixtures of sulphonated polystyrene resins in hydrogen form with either strongly basic resins in carbonate form or weakly basic resin in hydroxyl form are equally effective for manipulation in batches in the removal of interfering salt and amino compounds, but the former precludes shaking in closed vessels. The observed hydrolysis of adenosine by sulphonated polystyrene resin is not a source of significant error under the conditions of analysis of extracts of codling and herring muscles, but the author is not aware of previous reports of this action and it could lead to artifacts in the indiscriminate use of such resin forms with other biological extracts and fluids. While this work was in progress a report by Strange *et al.* (1955) appeared describing the use of Amberlite IR-120 (H⁺)-IR-4B (OH⁻) to remove the amino acids of casein hydrolysate from bacteriological media for the reductometric analysis of glucose. The present study shows that a similar procedure does not remove taurine (a common constituent of tissue extracts) and that, unlike reductometry, chromatographic analysis is not affected by this compound.

Analyses of muscle extracts have shown that free ribose is absent from the muscle of living

Fig. 1. Liberation of ribose in sterile codling muscle at 0°. Trawled codling were killed by a blow on the head. Samples of sterile muscle were dissected from the anterior-dorsal part of the fillet and stored in sealed Petri dishes. CSE extracts were deionized with Amberlites IR-120 (H⁺)-IR-4B (OH⁻) mixture and lyophilized. Ribose was estimated chromatographically. Points are means of five samples $\pm$ s.d.

Table 1. *Free glucose in the skeletal muscle of trawler codling*

Codling were killed by a blow on the head. Muscle was dissected immediately and CSE extract equivalent to 2 g. of tissue was shaken with 2 g. of Amberlite IR-120 (H⁺)-Amberlite IR-4B (OH⁻) (1:1, w/w). Deionized extracts were lyophilized, chromatographed in butanol-acetic acid and glucose was estimated absorptiometrically. Five estimates per sample were made. Results are expressed as mg./100 g. of tissue $\pm$ s.d.

Fish no.				
1	2	3	4	5
12.4 $\pm$ 0.5	17.8 $\pm$ 0.8	32.9 $\pm$ 0.8	7.3 $\pm$ 0.4	19.1 $\pm$ 0.8
6	7	8	9	10
21.5 $\pm$ 1.0	13.9 $\pm$ 0.6	29.4 $\pm$ 1.5	9.5 $\pm$ 0.4	13.3 $\pm$ 0.7

Table 2. *Recovery of glucose and ribose from deionized CSE extracts of herring muscle after chromatography*

Extract with and without added sugars was treated with IR-120 (H⁺)-IR-4B (OH⁻) resin mixture (see Methods and Table 1). Results are means of three determinations and expressed as mg./100 g. of tissue equivalent.* Fish were kept for 14–20 hr. after death at 0°. Percentage recoveries are given in parentheses.

Glucose		Ribose	
No. addition	20 mg. added	No. addition	20 mg. added
18.6	38.4 (99%)	5.8	25.1 (97%)

* Results with other resin mixtures were very similar.

codling and is liberated by autolytic systems after death, in agreement with the work of Tarr (1955) on other species. Codling in different degrees of exhaustion had levels of glucose in muscle of 8–32 mg./100 g. of tissue. Sugar analyses of herring were similar.

Details of changes in the sugar content of muscle during commercial chill storage and processing will be described elsewhere.

SUMMARY

1. Ribose in extracts of herring and codling muscles reacts readily with nitrogenous constituents during application to chromatograms. Glucose is stable.

2. Electrolytic desalting interferes with the estimation of ribose.

3. Sulphonated-polystyrene resins in hydrogen form hydrolyse adenosine at room temperature but the error introduced thereby in muscle analyses is insignificant.

4. Removal of salt and amino nitrogen by suitable mixed ion-exchange resins followed by lyophilizing and quantitative chromatography gave an estimate of glucose and ribose in codling and herring accurate to $\pm 6\%$.

The work described in this paper was carried out as part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

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The Oligosaccharides of Human Milk

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(Received 18 September 1957)

The recognition of L-fucose as a constituent sugar in oligosaccharides obtained from human milk was reported by Kuhn (1952) as resulting from the work of one of his associates, A. Gauhe (see also György, Kuhn, Norris, Rose & Zilliken, 1952). Since that time Kuhn and his colleagues have characterized four fucose-containing compounds from this source: a trisaccharide called fucosido-lactose (Kuhn, Baer & Gauhe, 1955, 1956a), two pentasaccharides called lacto-N-fucopentaose I and lacto-N-fucopentaose II, and a hexasaccharide, lacto-N-difucohexaose (Kuhn, Baer & Gauhe, 1956b). The last three compounds are all fucose

derivatives of a non-fucose compound, β -D-galactosido-(1 $\rightarrow$ 3)- β -acetylglucosamino-(1 $\rightarrow$ 3)-lactose, referred to by Kuhn and his associates as lacto-N-tetraose (Kuhn & Baer, 1956).

Montreuil (1957) has also reported the occurrence of lacto-N-tetraose and a series of fucose-containing oligosaccharides in human milk which, in addition to those identified in Kuhn's laboratory, include a second fucosido-lactose compound and at least five other substances in which fucose is present. These he has characterized by the molecular proportions of their constituent monosaccharides—galactose, glucose, fucose and acetylglucosamine.