QUANTITATIVE DETERMINATION OF NUCLEIC ACIDS IN SALMONIDAE MILT BY VARIOUS METHODS

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Procedures for the determination of total nucleic acids from ultraviolet absorption and for the separate determination of DNA and RNA by means of color reactions using the same tissue extracts have been developed. The results of the total and separate determination of the nucleic acids and the analysis of milts preserved by various methods are given. The discrepancies between the total amount of nucleic acids determined by the color reactions and by ultraviolet absorption do not exceed ±1.9%.

In connection with the program for the complex utilization of the resources of the seas and oceans, the use of the wastes from the fish-processing industry, including milts, is becoming an urgent problem. The mature milts of the Salmonidae contain a considerable amount of deoxyribonucleic acid (DNA) and are therefore a suitable raw material for its isolation on the industrial scale [1, 2]. In the organization of production, the necessity arises for developing methods of preserving and standardizing the milts, and the selection of methods for isolating DNA suitable for industrial realization.

An important index of milts (salted, frozen, lyophilized) in their standardization is their nucleic acid (NA) content. A. S. Spirin's method [3], based on the absorption of light by a tissue extract in the ultraviolet region of the spectrum at two wavelengths, is usually used for determining NAs in biological materials. However, A. S. Spirin's method enables only the total amount of NAs to be judged.

The task of the present work consisted of the development of a procedure for the separate determination of DNA and RNA and the comparison of results obtained on the same tissue extracts by color reactions and by the method of ultraviolet absorption.

Before the tissue extracts were prepared, acid-soluble impurities were eliminated by heating the sample with a cold 0.2 M solution of perchloric acid. The total amount of NAs in the tissue extracts was determined by A. S. Spirin's method [3]. In all cases, to show the applicability of A. S. Spirin's method, an additional control measurement was made at 260 nm. The optical densities at 260 nm were approximately equal to the optical densities at 270 nm (the deviations amounted to from ±1.9 to ±3.2%) which indicated the presence of a small amount of interfering impurities of nonnucleic acid nature. DNA was determined by the color reaction of deoxyribose with diphenylamine by Dische's method [4], and RNA by the color reaction of ribose with orcinol (Meibaum's modification) [4]. In view of the inadequate specificity of the orcinol reaction, we plotted an additional calibration graph to allow for the intensity of the coloration developed in the reaction of DNA with orcinol. Thus, in the calculation of the amount of RNA, a deduction was made for the intensity given by the DNA.

The statistically treated results obtained are given in Table 1. It can be seen from this Table that the total of NAs determined from color reactions of the carbohydrate components agreed well with the amount of NAs determined in the ultraviolet absorption of the purine and pyrimidine bases. The differences between the indices obtained by these two methods do not exceed ±1.9%.

Thus, tissue extracts obtained by A. S. Spirin's method can be used successfully for the separate determination of DNA and RNA by color reactions. It is always possible to

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TABLE 1. Amounts of NAs in Milts Preserved by Various Methods

Type of milt	NA content				Discrepancy between the methods, %
	total by Spirin's method, %	by color reactions		total by color reactions, %	
		DNA, %	RNA, %		
Lyophilized, batch 1	25.6	19.5	4.6	27.1	±1.5
Lyophilized batch 2	26.5	25.1	3.3	28.4	±1.9
Salted, batch 1	7.5	5.7	1.6	7.3	±0.2
Salted, batch 2	6.0	5.6	0.8	6.4	±0.4
Frozen	10.1	8.5	2.9	11.4	±1.3

check the results obtained by an additional determination of the absorption in the ultraviolet, which does not take long.

It can also be seen from the figures in Table 1 that milts preserved by different methods differ greatly with respect to their NA content, including the DNA content. This was taken into account in the development of methods for isolating DNA and in the prediction of the yield, which amounted to 50-60% of the content determined.

EXPERIMENTAL

Light absorption was measured on a Specord M 40 spectrometer (GDR) using cells with a thickness of the absorbing layer of 1 cm.

Purified preparations of DNA and RNA, the amount of the main substance in which was determined by A. S. Spirin's method [3], were used as standards for plotting calibration graphs. The diphenylamine and orcinol reagents were prepared as described in [4].

Preparation of Tissue Extracts. Weighed samples of milts (0.05-0.8 g of lyophilized materials and 0.20-0.25 g of salted or frozen materials) were rapidly ground with 10 ml of cold 0.2 M perchloric acid solution in a cooled mortar, and the homogenate was immediately centrifuged and the supernatant liquid was decanted off. The residue was transferred with 30-40 ml of 0.5 M perchloric acid solution to a 50-ml measuring flask and was extracted in the boiling water bath for 20 min, after which it was cooled to room temperature and 0.5 M perchloric acid solution was added to the mark. The mixture was then filtered into another flask.

Determination of the Total Amount of NAs. Part of the filtrate was diluted tenfold with 0.5 M perchloric acid solution and the optical densities of the diluted filtrate at 270 and 290 nm were measured in a spectrophotometer relative to 0.5 M perchloric acid.

The total amount of nucleic acids (as a percentage) in the sample analyzed was calculated from the formula

$$\frac{(D_{270} - D_{290}) \cdot 10.3 \cdot v \cdot n \cdot 100}{0.19 \cdot m \cdot 10^{-6}},$$

where $\frac{(D_{270} - D_{290}) \cdot 10.3}{0.19}$ is the concentration of NAs according to the formula proposed by A. S. Spirin, μg/ml;

m is the weight of the sample, g;

v is the capacity of the measuring flask for the preparation of the extract, ml;

n is the dilution factor for the filtrate from the extract; and

10^{-6} is the factor for passing from μg to g.

Determination of the DNA Content. To prepare a standard solution of DNA (with a concentration of 0.5 mg/ml), 50 ml (calculated at 100% material) of a standard preparation, weighed with an error not exceeding ±0.0002 g, was transferred into a 100 ml measuring flask, 60-70 ml of 0.5 M perchloric acid was added, and the mixture was kept in the boiling

water bath for 20 min, i.e., the same conditions of treatment were observed as for the sample. After this, it was cooled to room temperature and was made up to the mark with 0.5 M perchloric acid.

Working solutions for plotting the calibration graph were prepared in the following way. Each of a number of 25-ml measuring flasks was charged with 2.5-25.0 ml of the standard solution which was then made up to the mark with 0.5 M perchloric acid. The concentrations in the working solutions were 0.05-0.50 mg/ml. Then, from each working solution, 2.0 ml was transferred to a test-tube and 4.0 ml of Dische's reagent was added. The tubes were placed in the boiling water bath for 10 min and were then cooled to room temperature and the optical densities were measured relative to a control at 595 nm.

To determine the DNA in a sample, 2.0 ml of an undiluted filtrate of the tissue extract was transferred into a test-tube and 4.0 ml of Dische's reagent was added, the subsequent procedure being as in the plotting of the calibration graph.

Determination of the RNA Content. To prepare a standard solution of RNA with a concentration of 0.5 mg/ml, 25 mg (calculated as 100% material) of a standard preparation charged into a 50-ml measuring flask, 30-40 ml of 0.5 M perchloric acid solution was added, the mixture was kept at 100°C for 20 min and was cooled to room temperature, and it was made up to the mark with 0.5 M perchloric acid solution. The concentrations of RNA in the working solutions were 0.01-0.06 mg/ml.

Then from each working solution 2.0 ml was transferred to a test-tube, 2.0 ml of the orcinol reagent was added, the mixture was kept in the boiling water bath for 40 min and was cooled to room temperature and the optical density at 670 nm was measured relative to a control situation.

To determine the RNA in a sample, to each of a number of test-tubes was added 2.0 ml of twofold-diluted filtrate of the tissue extract and then 2.0 ml of the orcinol reagent, and the subsequent procedure was the same as in the plotting of the calibration graph.

An additional calibration graph was plotted for working solutions of DNA with concentrations of 0.05-0.50 mg/ml with the orcinol reagent. From the previously determined concentration of DNA in the filtrate (with allowance for the dilution in the determination of the RNA), the optical density corresponding to the twofold diluted concentration of DNA with orcinol in the sample was found from the calibration graph. From the difference in these optical densities and the total optical density of the twofold diluted filtrate of the tissue extract with orcinol the concentration of RNA in the twofold diluted filtrate of the tissue extract was found from an RNA calibration graph.

The amount of NAs (as a percentage) determined from the color reactions was calculated from the formula

$$x = \frac{C \cdot v \cdot n \cdot 100}{m \cdot 1000},$$

where C is the concentration of NAs found from the corresponding calibration graph, mg/ml;

n is the dilution factor of the filtrate in the performance of the color reactions;

m is the weight of the sample, g;

v is the volume of the filtrate from the tissue extract, ml; and 1000 is the factor for converting to mg.

SUMMARY

The possibility has been shown and methods are given for the separate determination of deoxyribonucleic acid from the color reaction with diphenylamine and of ribonucleic acid with orcinol and also a determination of the total amount of nucleic acids from ultraviolet absorption in one and the same tissue extracts obtained from Salmonidae milts preserved by different methods.

The discrepancies between the total amounts of nucleic acids determined from the color reactions and the ultraviolet absorption do not exceed ±1.9%.

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IMMOBILIZATION OF SNAKE VENOM ON EPOXY-ACTIVATED SILOKHROMS

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The production of immobilized phospholipase A_2 preparations by the covalent addition of the proteins of viper venom to Silokhroms containing epoxide groups is described. The dependence of the amount of protein bound to the support on its concentration and on the amount of epoxide groups in the sorbent has been investigated. The phospholipase activity of the preparations obtained did not depend on the amount of immobilized enzyme but was determined by diffusional factors.

Phospholipases A_2 (EC 3.1.1.4) – enzymes that catalyze the splitting out of the fatty acid from the second position of sn-glycero-3-phospholipids – are widely used in the chemical modification of various types of synthetic and natural phospholipids and in the study of their structures. In a number of cases the immobilization of the enzyme on various supports enables stable enzyme preparations for repeated use to be obtained. As a rule, the method of covalent attachment of proteins to the sorbents is used for the immobilization of lipolytic enzymes. Thus, the production of preparations of covalently bound phospholipases A_2 from cobra venom [1-3] and from the venom of the Far Eastern mamushi [4] and of cottonseed lipase [5] has been described. The inclusion of enzymes in polymers during their polymerization has been used to obtain preparations of C.cylindracea lipase [6] and of phospholipases A and D [7]. The properties of mitochondrial phospholipase A_2 bound adsorptively with an affinity phospholipid-containing sorbent have been studied [8].

The immobilization of lipolytic enzymes is accompanied by a considerable decrease in enzymatic activity. The choice of support and method of attachment ensuring the least inactivation of the protein molecule is the main task in the production of an immobilized enzyme. As supports for the attachment of phospholipases and lipases are used activated agarose [1, 3] and porous glass [2], organosilicon sorbents [4], and a water soluble copolymer of N-vinylpyrrolidone with glycide acrylate [5].

It appeared desirable to investigate the possibility of using a readily available modified silica – Silokhrom – containing epoxide groups (epoxy-Silokhrom) for the covalent attachment of phospholipases. Sorbents with epoxy groups (epoxy-Sepharose, water-soluble polymers) have previously been used successfully in the immobilization of various types of proteins [5, 9, 10] and, in particular, in the production of heat-stable preparations of cottonseed lipase [5].

Snake venoms possess a high phospholipase activity. This permits the use of the whole venom and not the purified enzyme for obtaining immobilized phospholipase preparations with a good activity. In the present paper we describe a method of immobilizing viper venom.

For the retention of the activity of the enzymes on their attachment to a support it is very important that the functional groupings present in the active center do not

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