p. 285, Interscience, New York, 1957.
(9) Guadagni, D. G., Sorber, D. G., Wilbur, J. S., *Food Technol.* **3,** 359 (1949).
(10) Haas, V. A., Stadtman, E. R., *Ind. Eng. Chem.* **41,** 983 (1949).
(11) Harper, J. S., Tappel, A. L., *Advan. Food Res.* **7,** 171 (1957).
(12) Henriques, V., Sörensen, S. P. L., *Z. Physiol. Chem.* **64,** 120 (1909).
(13) Hestrin, S., Feingold, D. S., Schramm, M., "Methods in Enzymology I," S. P. Colowick and N. O. Kaplan, eds., p. 251, Academic Press, New York, 1955.
(14) Hodge, J. E., J. AGR. FOOD CHEM. **1,** 928 (1953).
(15) Huang, I-Yih, Draudt, H. N., *Food Technol.* **18,** 124 (1964).
(16) Ingles, D. L., *Australian J. Chem.* **13,** 404 (1960).
(17) Ingles, D. L., Reynolds, T. M., *Ibid.*, **11,** 575 (1958).
(18) *Ibid.*, **12,** 483 (1959).
(19) Jacobs, M. B., "The Chemical Analysis of Foods and Food Products," pp. 156, 728, Van Nostrand, Princeton, N. J., 1958.
(20) Joslyn, M. A., *J. Assoc. Offic. Agr. Chemists* **33,** 504 (1950).
(21) Joslyn, M. A., Bedford, C. L., Marsh, G. L., *Ind. Eng. Chem.* **30,** 1068 (1938).
(22) Joslyn, M. A., Marsh, G. L., *Ibid.*, **27,** 186 (1935).
(23) Koppanyi, T., Vivino, A. E., Veitch, R. P., Jr., *Science* **101,** 541 (1945).
(24) Maier, V. P., Schiller, F. H., *J. Food Sci.* **26,** 322 (1961).
(25) Mohammad, A., Fraenkel-Conrat, H., Olcott, H. S., *Arch. Biochem. Biophys.* **24,** 157 (1949).
(26) Nelson, N., *J. Biol. Chem.* **153,** 375 (1944).
(27) Nichols, P. F., Mrak, E. M., Bethel, R., *Food Res.* **4,** 67 (1939).
(28) Nichols, P. F., Reed, H. M., *Western Canner Packer* **23,** 11 (1931).
(29) Reynolds, T. M., *Advan. Food Res.* **12,** 1 (1963).
(30) Richert, P. H., J. AGR. FOOD CHEM. **1,** 610 (1953).
(31) Salwin, H., "Freeze Drying of Foods," F. R. Fisher, ed., p. 58, National Academy of Sciences–National Research Council, 1962.
(32) Somogyi, M., *J. Biol. Chem.* **195,** 19 (1952).
(33) Stadtman, E. R., *Advan. Food Res.* **1,** 325 (1948).
(34) Stadtman, E. R., Barker, A. H., Mrak, E. M., Mackinney, G., *Ind. Eng. Chem.* **38,** 99 (1946).
(35) Stone, W., *Biochem. J.* **31,** 508 (1937).
(36) Weast, C. A., Mackinney, G., *Ind. Eng. Chem.* **33,** 1408 (1941).
(37) Wong, F. F., Dietrich, W. C., Harris, J. G., Lindquist, F. E., *Food Technol.* **10,** 96 (1956).

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NUCLEOTIDE MEASUREMENT

Rapid Measurement of Inosine Monophosphate and Total Adenosine Nucleotides in Fish Tissue

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A method is described for the rapid determination of inosine monophosphate (IMP) and total adenosine nucleotides in fish tissue. Perchloric acid extracts of the tissue are made, and the nucleotides are absorbed and separated from nucleosides and purine bases on a Dowex 1-X4-(Cl) resin. After elution from the resin with H_2SO_4, total adenosine and inosine nucleotides are determined by measuring the absorbancy of the effluent at 250 mμ. Adenosine nucleotides in the effluent are then determined chemically and subtracted from the total nucleotides to yield the IMP content of the extract. The method is useful over varying concentrations of adenosine and inosine nucleotides, and values are in close agreement with those obtained by using classical ion exchange systems.

The measurement of inosine monophosphate (IMP) and related nucleotides in fish tissue is important because it furnishes information regarding the freshness of the fish (*7, 10*) and also because IMP is an important flavor constituent of the tissue (*4, 11*). IMP is derived as the hydrolytic deamination product of adenosine triphosphate (ATP), and amounts generally ranging from 4 to 7 μmoles per gram (*7, 10, 15*) accumulate in fish tissue soon after the death of the fish by the following pathway: ATP → ADP → AMP → IMP. Although the rate of total nucleotide breakdown varies between species (*6, 10*), nearly all of the nucleotides found post mortem in commercially important fish are those of adenosine and inosine. Small amounts of guanosine, cytidine, and uridine-based nucleotides have been reported in rested freshly killed cod (*8*). In some species, the breakdown of IMP is almost complete after 8 to 10 days of storage (*10*), whereas in other species, such as halibut, there are still substantial quantities of both adenosine nucleotides and IMP even after 20 days of storage (*14*).

Although the extent of nucleotide dephosphorylation can be rapidly estimated by several methods (*9, 13, 15*), accurate measurements of IMP are generally made by chromatographic ion-exchange separations (*5, 16*).

The work presented here shows that IMP and the adenosine nucleotides in fish tissue can be rapidly and accurately measured after a simple separation of the total nucleotides from their corresponding nucleoside and purine bases. The method should be applicable to the determination of total adenosine nucleotides in animal tissue other than fish.

Method

Preparation of Tissue Extracts. Extracts were prepared by grinding 40 grams of fish tissue with 80 ml. of chilled 3% $HClO_4$ for 1 minute in a blender. The homogenate was filtered, and 20 ml. were immediately neutralized with 10% KOH to pH 6.5 to 6.8. The neutralized extract was stored for approximately 30 minutes at 0° C. to permit crystallization of the $KClO_4$.

Separation of Nucleotides. Dowex 1-X4-(Cl) was recycled with 10% NH_4OH and 4*N* HCl. Twenty milliliters of the neutralized extract were passed over a 1 × 2 cm. resin bed to separate the nucleotides from nucleosides, purines, and free sugars (*13*). Distilled water (30 to 35 cc.) was passed over the column until the effluent was free from ultraviolet-absorbing material. The column was then eluted with 25 ml. of 1*N* H_2SO_4, followed by 5 ml. of 6*N* H_2SO_4. This removed 98 to 100%

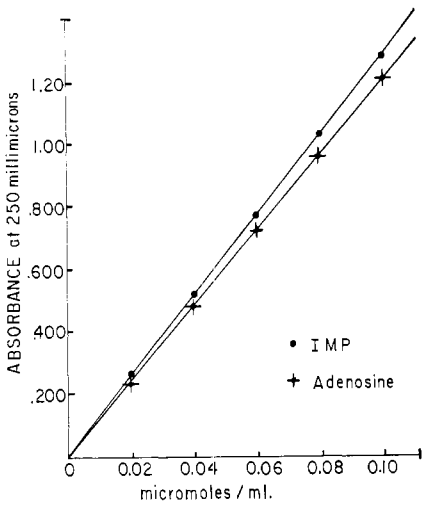

Figure 1. Standard absorption curve for adenosine nucleotides and inosine monophosphate measured at 250 mμ

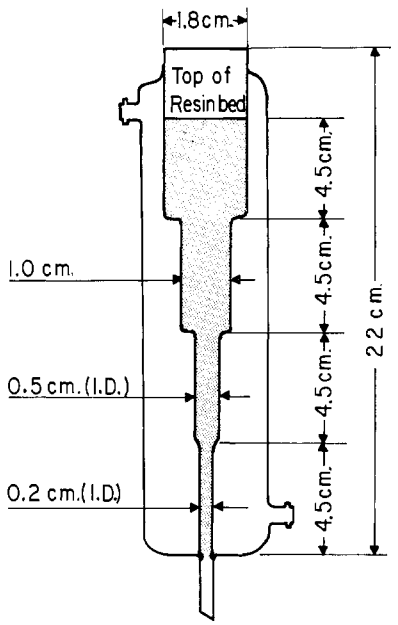

Figure 2. Jacketed multibore column used for nucleotide separation

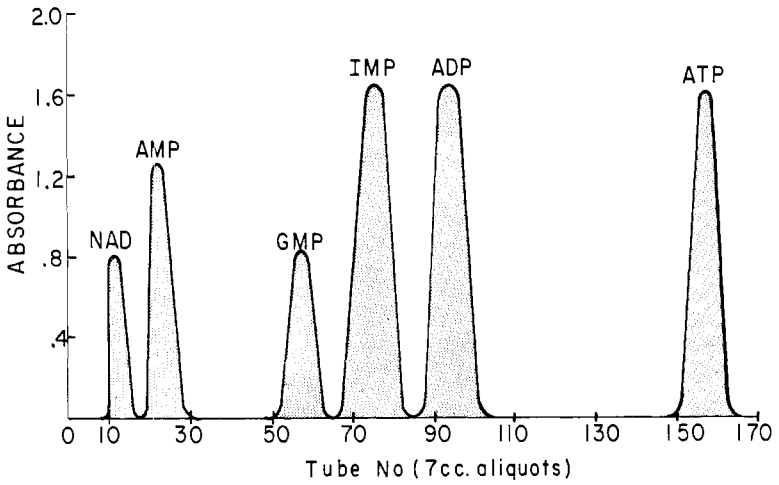

Figure 3. Elution pattern of nucleotides using multibore column operated at 2 ml. per minute at 6° C.

of the ultraviolet-absorbing materials.

Measurement of Total Nucleotides. An appropriate dilution was made of the column effluent (usually 1 to 10) with distilled water, and the absorbance of the solution was taken at 250 mμ. Apparent IMP was then calculated from a standardized curve prepared by dissolving IMP in 0.1*N* H_2SO_4 (Figure 1). At this wave length and acid concentration, the adenosine nucleotides (*12*) and IMP have approximately the same molecular extinction coefficient. All absorbance measurements were made with a Beckman DB spectrophotometer.

Measurement of Adenosine Nucleotides. The amount of adenosine nucleotides in the column effluent can be accurately determined by the method described by Davis and Morris (*2*).

Pipet 2 ml. of the column effluent into a test tube, and add 0.20 ml. of 18*N* H_2SO_4 and 0.10 ml. of 0.20*M* KBr. Shake and then add 0.30 ml. of 1*N* $KMnO_4$. After 5 minutes, add 6% H_2O_2 dropwise to decolorize the excess permanganate. Adjust the volume to 3.0 ml., and after 15 minutes, read the color at 330 mμ against a reagent blank. Prepare standard by dissolving adenine in 1.8*N* H_2SO_4 containing 0.05*M* NaCl. Find the amount of IMP by subtracting the number of moles of adenine found from the moles of apparent IMP.

When the relative concentrations of both IMP and adenosine nucleotides were estimated by the use of simultaneous equations (*7*), results were poorer than when adenine was determined chemically, particularly when small quantities of adenosine nucleotides occurred in the presence of large amounts of inosine nucleotide.

Measurement of Nucleotides by Column Chromatography. For comparative purposes, IMP was determined by column chromatography, using the nonlinear formate gradient proposed by Jones (*6*). Better separations of adenosine monophosphate (AMP) from nicotinamide adenine diphosphate (NAD) and of IMP from adenosine diphosphate (ADP) could be obtained if a multibore column (Figure 2), such as the one proposed by Fischer and Kabara (*3*), was used in place of the straight-bore column. The elution pattern of the nucleotides using this column are shown in Figure 3. Nucleotides obtained by this system were pooled and analyzed spectrophotometrically against an appropriate blank.

Results and Discussion

Recovery of Adenosine and Inosine Nucleotides from Dowex 1(Cl) Column. Column performance and recovery of nucleotides were determined by adding individual nucleotides and mixtures of nucleotides to the column and then determining recovery after elution. By eluting the column, in the proposed stepwise procedure with H_2SO_4 solutions, complete recovery was obtained with 30 ml. of the acid solution. The acid concentration in 30 ml. of effluent is 1.8*N* and needs no correction for the adenine determination.

Table I shows typical individual nucleotide recoveries after the elution step. Mixtures of varying concentrations of adenosine monophosphate (AMP) and IMP were added to the column, and the effluents were analyzed by the proposed method. The recovery data (Table II) show that the method operates

Table I. Recovery of Nucleotides from Dowex 1 (Cl^-) Resin Eluted with 25 Ml. of 1*N* H_2SO_4 and 5 Ml. of 6*N* H_2SO_4

	Nucleotide, μmoles		
Nucleotide on Column	Present	Found in 30 ml. of column effluent	Recovery, %
ATP	16.2	16.4	102
ADP	10.1	10.1	100
AMP	12.9	12.9	100
IMP	27.5	27.0	96

Table II. Analysis of Varying Proportions of IMP and AMP Mixtures Recovered from Dowex 1 (Cl^-) Resin

Nucleotide on Column, μmoles		Nucleotide in 30 Ml. of Effluent, μmoles		Nucleotide Recovery, %	
AMP	IMP	AMP	IMP	AMP	IMP
11.3	2.5	10.6	2.5	94	100
7.0	6.2	6.7	5.7	96	92
2.8	10.0	2.7	9.5	97	95

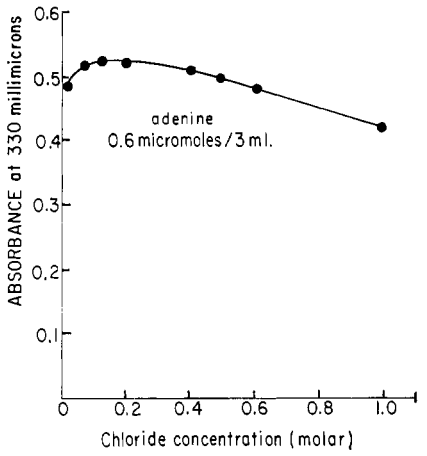

Figure 4. Effect of increasing chloride concentration on chemical determination of adenine

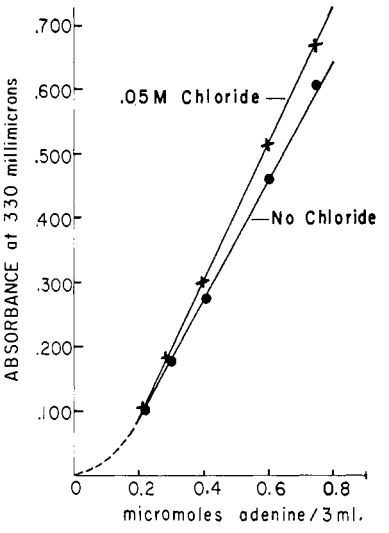

Figure 5. Effect of 0.05M chloride on chemical determination of adenine

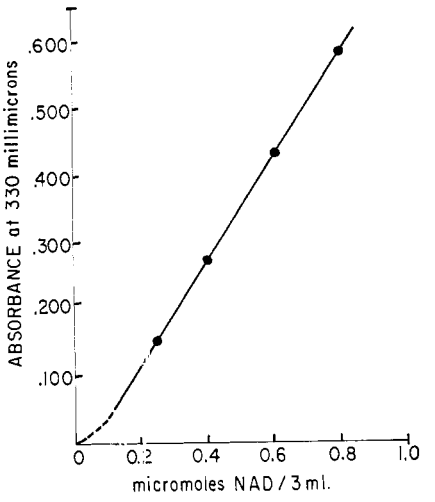

Figure 6. Absorption curve for NAD

Determined by method of Davis and Morris

Table III. Determination of IMP in Fish

Species of Fish	Storage at 33° F., days	IMP, μmoles/G. Rapid method	Column chromatography	Adenine, chemical method
Lingcod (*Ophiodon elongadus*)	2	4.8	5.1	0.69
Rockfish (*Sebastodos flavadis*)	3	3.8	3.7	0.65
Rainbow trout (*Salmi gairdnerii*)	0.16	3.4	3.2	7.2
Petrale sole (*Eopsetta jordani*)	10	<0.1	<0.1	0.50
With 3.0 μmoles/g. added IMP	10	2.8	...	0.50
With 1.0 μmole/g. added AMP	10	<0.1	<0.1	1.50

over the widely varying concentrations of the two nucleotides.

Analysis of Fish Tissue. Different species of fish held for varying lengths of time were analyzed for IMP and total adenosine nucleotide (Table III). IMP values obtained with the proposed method when compared against values obtained by column chromatography are in close agreement. The rainbow trout (Table III) contained the following nucleotides (micromoles per gram): 0.10 AMP, 0.95 ADP, 5.41 ATP, 0.65 NAD, and 3.2 IMP. The 7.1 μmoles per gram of adenosine nucleotides found by column chromatography agrees with the 7.2 found chemically.

Interference. The most serious interference in the determination of adenine was that of chloride, so standard adenine curves were prepared in 0.05*M* chloride, which approximates the normality of the 30 ml. of effluent after removal of the nucleotides with H_2SO_4. The effect of increasing the chloride concentration when adenosine was determined by using the chemical method is shown in Figure 4. Figure 5 shows the effect on the absorbance measurement when adenine is determined in the presence of 0.05*M* chloride. Although there is a displacement in absorbancy, Beer's law is followed over the operating portion of the curve.

Nucleotides were not separated from the purine bases and nucleosides using the formate form of the resin because the liberated formic acid strongly absorbs at 250 mμ. Formate ion also interfered with the adenine method and undoubtedly reacts with the $KMnO_4$ used in the method. Potassium and perchlorate ion do not interfere.

Reaction with NAD. The adenine in NAD was found to react, and a linear relation was found between the concentration of NAD and absorbancy when NAD was determined using the adenine method (Figure 6).

Literature Cited

(1) Bendall, J. R., Davey, C. L., *Biochem. Biophys. Acta* **26,** 93 (1957).
(2) Davis, J. R., Morris, R. N., *Anal. Biochem.* **5,** 64 (1963).
(3) Fischer, G. A., Kabara, J. J., *Ibid.*, **9,** 303 (1964).
(4) Hashimoto, Y., FAO Symposium on Significance of Fundamental Research in Utilization of Fish, Husum, Germany, May 26–30, Paper WP/11/6, 1964.
(5) Hurlburt, R. B., Schmitz, H., Brumm, A. F., Potter, V. R., *J. Biol. Chem.* **209,** 23 (1954).
(6) Jones, N. R., *Analyst* **85,** 111 (1960).
(7) Jones, N. R., Proceedings of XIth International Congress on Refrigeration, Munich IV-S, 1963.
(8) Jones, N. R., Murray, J., *Biochem. J.* **77,** 567 (1960).
(9) Jones, N. R., Murray, J., *J. Sci. Food Agr.* **15,** 684 (1964).
(10) Kassemsarn, B., Perez, B. S., Murray, J., Jones, N. R., *J. Food Sci.* **28,** 28 (1963).
(11) Kuninaka, A., Kibi, M., Sakaguchi, K., *Food Technol.* **18,** 29 (1964).
(12) Pabst Laboratories, Circ. **02-10** (1956).
(13) Shewan, J. M., Jones, N. R., *J. Sci. Food Agr.* **8,** 491 (1957).
(14) Spinelli, J., unpublished data.
(15) Spinelli, J., Eklund, M., Miyauchi, D., *J. Food Sci.* **29,** 710 (1964).
(16) Tarr, H. A., Leroux, M., *Can. J. Biochem. Phys.* **40,** 574 (1962).

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