Acta Medica Okayama

Volume 21, *Issue* 5 1967 *Article* 2

OCTOBER 1967

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Abstract

a) A modified procedure of the WIDNELL and TATA8 method yields rat liver nuclei manifesting a high degree of purity and activity. b) These nuclei contain a nucleoside-dependent phosphorylating activity that is readily released and apparently unrelated to either glycolysis or respiration. c) The main incorporation of the 32Pi is into ribose-I-phosphate; nucleoside phosphorylase activity satisfactorily accounts for the observed purine nucleoside stimulation of the nuclear phosphorus metabolism.

*PMID: 4232094 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 21 207—211 (1967)

NUCLEOSIDE-DEPENDENT SYNTHESIS OF ORGANIC PHOSPHOROUS COMPOUNDS BY RAT LIVER NUCLEI*

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Received for publication, August 19, 1967

Since the initial discovery of OSAWA, ALLFREY and MIRSKY[1] that calf thymus nuclei *in vitro* synthesize ATP from endogenous ADP and AMP in an oxygen-dependent process, the mechanism of this ATP formation has been actively sought. Also the distribution of this activity in other nuclei as in rat liver has also been investigated. Although it is clear that ATP formation in rat thymus is indeed oxygen-dependent, it is also clear from the studies of MCEWEN, ALLFREY and MIRSKY[2] and BETEL and KLOUWEN[3] that the mechanism of this process differs from oxidative phosphorylation in mitochondria. By contrast to thymus nuclei, investigations of ATP formation by rat liver nuclei have met with considerable difficulty. One problem has been the isolation of fresh intact rat liver nuclei in a high state of purity. Hence, earlier results suggesting ATP synthesis were subject to the criticism that they reflected extranuclear contamination.

PENNIALL, LIU and SAUNDARS[4] and PENNIALL, SAUNDERS and LIU[5] have reported an active incorporation of ^{32}P-labeled inorganic phosphate ($^{32}P_i$) into acid-soluble phosphorus compounds. The synthesis of these acid-soluble phosphorus compounds is oxygen-dependent and incorporation was observed to be enhanced in the presence of adenosine and guanosine[6]. BETEL and KLOUWEN[3] have observed aerobic synthesis of ATP by aged rat thymus nuclei to be stimulated by inosine and adenosine. This nucleoside stimulation of nuclear phosphorylation has also been confirmed by BASHIRELAHI and BALLAM[7] who specifically reported that the ATP synthesis in rat thymus nuclei was enhanced under these conditions. In view of this, it seemed worthwhile to investigate further the occurrence of this enzyme system in isolated and highly purified rat liver nuclei in order to determine optimum conditions for incorporation, and to identify the acid-soluble organic phosphorus compounds synthesized.

* This research was supported by Cancer Research Funds of the University of California.

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MATERIALS AND METHODS

After an extensive evaluation of existing methods for isolation of rat liver nuclei, the method of WIDNELL and TATA[8] involving sucrose density gradient fractionation was adopted. In the final step of this procedure the nuclear pellet, designated N_1, forms a clear supernatant (S_1) below. This nuclear fraction was further purified by centrifugation through a 10-fold volume of 0.25 M sucrose to yield another supernatant (S_2) and upon recentrifugation under these conditions a final nuclear (N_2) fraction was obtained. The N_2 fraction is composed of highly purified intact nuclei based upon the following criteria: a) electron microscopy reveals virtually no contamination by other particles; b) RNA: DNA ratio decreased 10 per cent as compared to nuclei isolated by the method of WIDENELL and TATA; c) glucose-6-phosphatase and NAD pyrophosphorylase of 177 and 105 mμmoles/mg nuclear protein/120 min respectively. Glucose-6-phosphatase activity was about half and NAD pyrophosphorylase was 30 per cent higher than found by WIDNELL and TATA for the N_1 fraction.

For the estimation of $^{32}P_i$ incorporation into organic phosphorus compounds, nuclei were incubated in 0.25 M sucrose, 5.0 mM Tris-HCl buffer (pH 7.1), 3.0 mM $MgCl_2$, 1.0 μc carrier-free $^{32}P_i$, and 2.0 mM inosine (when present) in a final volume of 2.5 ml. The reaction was stopped by adding 5.0 ml of 0.6 M Na_2SO_3 in 0.9 M $HClO_4$, and then carrier phosphate (1.0 mM as sodium salt) was added. Organic phosphorus compounds were separated from inorganic phosphate by the column chromatographic method of HAGIHARA and LARDY[9] and then radioactivity of the organic phosphorus compounds was determined.

Separation of individual phosphorus compounds was made using Dowex-1-formate according to the column chromatographic method of SIEKEVITZ and POTTER[10] as modified by TERADA[11]. Ribose-1-phosphate was identified by paper chromatography according to MORTIMER[12]. Protein was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL[13]. Chemicals were obtained commercially; nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The phophorylation activities of the N_1, N_2, S_1 and S_2 fractions were examined by testing for the formation of ^{32}P-organic phosphorus compounds. Typical results are given in Table 1 for controls and fractions supplemented with inosine. By comparing the inosine effect on S_1 with that on S_2 one would predict a similar degree of stimulation if the effect was due to extranuclear enzymes. However, the result shows that the inosine stimulation of nuclear phosphorylation is not due to a cell sap component but rather to a loosely bound

Table 1 Inosine Stimulation of $^{32}P_i$ Incorporation into Acid-soluble Organic Phosphorus Compounds by Rat Liver Nuclei

Fraction*	Total incorporation (cpm)	Specific activity (cpm/mg protein)	Relative inosine effect
N_1	3,920	512	
N_1+Inosine	10,460	1,366	2.7×
S_1	3,380	4,500	
S_1+Inosine	4,980	6,640	1.5×
N_2	1,890	242	
N_2+Inosine	2,700	346	1.4×
S_2	645	2,790	
S_2+Inosine	7,350	31,100	10.3×

* The reaction mixture was incubated aerobically with shaking at 25°C for 30 min as described in the methods employing 0.6 ml of each fraction.

component of the nuclear fraction which is significantly released into the S_2 fraction upon washing the N_1 fraction. This interpretation is corroborated by the finding that after washing N_1 twice the inosine stimulation is still retained by the N_2 fraction. The time course of inosine stimulation of $^{32}P_i$ incorporation into organic phosphorus compounds of N_2 proceeds in a linear fashion over a 60-min assay interval.

A number of inhibitors were tested on the inosine-dependent phosphorylation. The patterns observed did not offer clear results which might suggest a role for glycolysis or of an electron transport type of system in the measured activities. For example, the inhibition of activity was only 19 per cent for iodoacetate and antimycin A, 11 per cent for 2, 4-dinitrophenol when tested at concentrations which check glycolysis or respiration.

Since it had already been reported[6] that guanosine stimulates nuclear phosphorylation, parallel experiments were made to compare effects of inosine and guanosine. Figure 1 illustrates the type of elution pattern obtained for the labeled components of the acid-soluble extract. In this experiment, a 30-sec incubation in a nucleoside-free reaction medium was examined. Incubation of the N_2 fraction under aerobic conditions even for a 30-sec interval is sufficient to demonstrate incorporation of $^{32}P_i$ into organic phosphorus compounds; e. g. it is clear that significant radioactivity has already found its way into the ATP and ribose-1-phosphate fractions and some radioactivity is indicated in the ADP fractions and in certan unidentified peaks. Figure 2 compares the relative amounts of the identified substances for controls, and for the nucleoside-containing reaction mixtures. Both nucleosides may act similarly since both stimulate the ribose-1-phosphate formation. It is also evident from Fig. 2 that the decay in

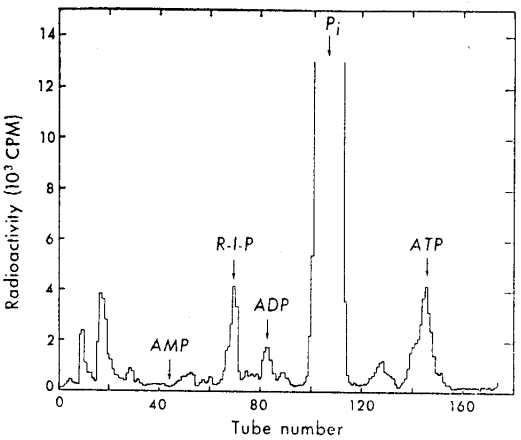

Fig. 1 Organic phosphorus compounds identified in the acid-soluble extract of the rat liver nuclear fraction.

The reaction mixture contained 48 mg protein of the N_2 fraction and other conditions as in Table I, in a final volume of 12 ml. Incubation was for a 30-sec period at 0°C in the absence of nucleoside and the reaction was stopped by adding ice-cold $HClO_4$ to a final concentration of 0.5 N. AMP, ADP and ATP were added to the extract to facilitate identification.

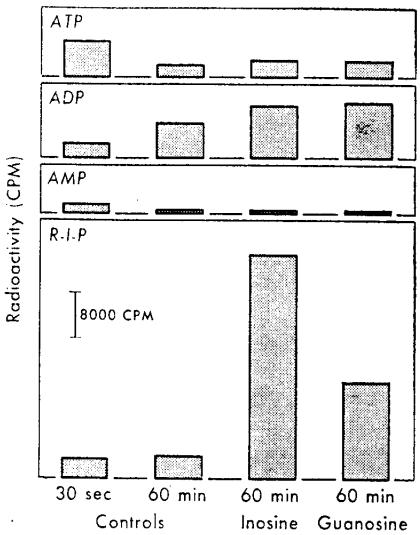

Fig. 2 $^{32}P_i$ incorporation into adenine nucleotides and ribose-1-phosphate by the nuclear (N_2) fraction.

Conditions as in Fig. 1 and Table 1. Controls were incubated for 30 sec at 0°C and for 60 min at 25°C. Experimentals were incubated for 60 min at 25°C in the presence of nucleosides (either 20 μmoles of inosine or 2 μmoles of guanosine per 12 ml of reaction mixture). The relative amounts of labeled compounds were estimated from the area of the elution peaks.

the ^{32}P-ATP peak that occurs during incubation is paralleled by an increase in the ^{32}P-ADP component, suggesting an interconversion of ADP and ATP.

COMMENT

Inosine is at a "cross-road" for pathways of nucleotide metabolism and so it would be expected to promote nuclear phosphorylation. In other experiments the N_2 fraction has been observed to demonstrate an active inosine-dependent hypoxanthine formation. This data concerning net inosine stimulation of $^{32}P_i$ incorporation into the acid-soluble fraction by rat liver nuclei show a similar effect for net increase of ATP synthesis as was reported for rat thymus nuclei by BETEL and KLOUWEN[9]. Fractionation of the acid-soluble organic phosphorus compounds shows that ^{32}P-ATP is synthesized by incubation at 0°C for 30 sec, and shows a similar elution pattern of nucleotides to that found by OSAWA *et al*[1] in rapidly isolated calf thymus nuclei. After continued incubation, however, the

^{32}P-ATP-level decreases, an effect which might reflect ATPase and adenylate-kinase activities and/or ATP regulation of other unknown pathways as is suggested by the simultaneous rise in the ^{32}P-ADP peak. In the presence of inosine or guanosine the ^{32}P-ADP levels are slightly higher than in the control incubated under identical conditions except for the addition of nucleoside This may indicate that the tested nucleosides stimulate ADP formation by an undetermined pathway. The main effect of added nucleosides is in promoting $^{32}P_i$ incorporation. This might be attributed to a (purine) nucleoside phosphorylase since the main labeled peak was identified as ribose-1-phosphate. Hence, a significant portion of the nuclear phosphorylating activity does not appear as nucleoside phosphorylation and is channelled into other pathways having ribose-1-phosphate as intermediate. Therefore, the action of added nucleoside in rat liver nuclei differs from that observed in thymus nuclei.

SUMMARY

a) A modified procedure of the WIDNELL and TATA[8] method yields rat liver nuclei manifesting a high degree of purity and activity. b) These nuclei contain a nucleoside-dependent phosphorylating activity that is readily released and apparently unrelated to either glycolysis or respiration. c) The main incorporation of the $^{32}P_i$ is into ribose-1-phosphate; nucleoside phosphorylase activity satisfactorily accounts for the observed purine nucleoside stimulation of the nuclear phosphorus metabolism.

ACKNOWLEDGEMENT

The author expresses his thanks to Prof. LESTER PACKER for helpful suggestions, and to MIGUEL LLINAS for assistance. The award of a Research Travel Grant from the Japanese Ministry of Education is gratefully acknowledged.

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