ON THE FORMATION OF A NOVEL ADENYLIC COMPOUND BY ENZYMATIC EXTRACTS OF LIVER NUCLEI.

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In a previous paper (Chambon, Weill and Mandel, 1963) we reported the existence in animal cell nuclei of an enzymatic system which catalyzed the incorporation of the adenylic moiety of ATP into an acid-insoluble product and which was at least 1000 fold stimulated by nicotinamide mononucleotide (NMN).

We have now observed that NMN and ATP are incorporated in equimolar amounts and have obtained evidence that DPN is the immediate precursor. The acid-insoluble product appears to be a polymer of adenosine diphosphoribose (ADPR). The product of alkaline hydrolysis is not 2'3'-AMP but 5'-AMP, as demonstrated by the action of snake venom 5'-nucleotidase (phosphate detachment).

MATERIALS AND METHODS

Particulate fractions of nuclear extracts from hen liver were prepared according to the method previously described (Busch, Chambon, Mandel and Weill, 1962). The incubation medium (0.25 ml) contained in µmoles : glycine-NaOH buffer, pH 8.6, 25.0 ; Mg^{++}, 7.5 ; 2-mercaptoethanolamine, 0.25 ; either 0.25 µmole of ATP plus 0.25 µmole of NMN (one of them labelled ADPR) ; enzymatic extract (1-2 mg protein).

After 20 minutes at 37°C, the incubation mixture was treated with 2.5 µmoles of pyrophosphate pH 7.0 and the reaction stopped with trichloracetic acid (TCA). The precipitate was collected by centrifugation, washed with TCA, dissolved in M sodium-ammonium phosphate, reprecipitated with TCA and counted in a Packard Tricarb scintillation counter.

In some experiments, in order to prevent possible degradation of the product by the TCA treatment, we cooled the reaction mixture to 0°C, added pyrophosphate and precipitated the product with twenty volumes of saturated ammonium sulphate at pH 8. The precipitate was collected by centrifugation for 20 minutes at 70,000 g, suspended twice in 0.25 ml of 0.05 M tris-phosphate buffer pH 7.4, reprecipitated with ammonium sulphate and centrifuged. Finally the precipitate was counted after two washings with 0.05 M glycine buffer pH 8.6. Both methods give the same result.

ATP-8-C^{14} adenine was purchased from Schwarz BioResearch. We prepared ATP-α-P^{32}, NMN-P^{32}, NMN-ribose-C^{14}, NMN-nicotinamide-C^{14}, DPN-nicotinamide-C^{14}, DPN-P^{32} and ADPR-P^{32} (equally labelled in both phosphate residues) and DPN-ribose-C^{14}. The ribose of the NMN moiety had a specific activity about twice that of the AMP ribose.

Results are expressed in mµmoles of precursor incorporated per mg of protein in 20 minutes at 37°C.

RESULTS and DISCUSSION

Table I shows that the adenylic moiety of ATP as well as the ribose and the phosphate of NMN are incorporated into the acid-insoluble product. This suggests that DPN might be an intermediate. Several experiments confirmed this hypothesis : DPN-P^{32} or

DPN-ribose-C^{14} are incorporated in equal amounts (Table I) ; addition of cold DPN decreases the incorporation of ATP and NMN (Table II) ; DPN is a better precursor than ATP plus NMN (Table I) ;

TABLE I

Precursors	mμmoles incorporated (+)
ATP-C^{14} or ATP-α-P^{32}	0.01
ATP-C^{14} or ATP-α-P^{32} + NMN	15.30
NMN-P^{32} + ATP	16.30
NMN-ribose-C^{14} + ATP	17.25
NMN-nicotinamide-C^{14} + ATP	0.37
DPN-P^{32}	33.60
DPN-ribose-C^{14}	33.50
DPN-nicotinamide-C^{14}	0.27

(+) See under materials and methods

and finally the addition of beef spleen DPNase to the incubation medium reduces considerably the incorporation of labelled ATP or labelled DPN (Table II).

Neither the nicotinamide moiety of DPN nor that of NMN is incorporated (Table I). These facts suggest the incorporation of ADPR since the constituents of this molecule are found in equimolar amounts in the acid-insoluble precipitate.

Moreover, incubation of the reaction product with DPNase (Table II) does not render soluble any radioactivity. To exclude the possibility of the formation of DPNH with subsequent loss of the dihydronicotinamide during the acid precipitation, we isolated the product with ammonium sulphate instead of TCA and still found no nicotinamide in it. ADPR is not a precursor since ADPR-P^{32} is not incorporated (Table II) and the addition of cold ADPR to the incubation medium does not inhibit the incorporation of DPN-P^{32} (Table II). DPN seems thus to be an obligatory intermediate.

TABLE II

Precursors	Incubation conditions	mμmoles incorporated (+)
DPN-P^{32}	Normal	34.4
"	+ beef spleen DPNase added before incubation	1.4
"	+ beef spleen DPNase added after incubation	34.5
ATP-α-P^{32} + NMN	Normal	15.35
" "	+ beef spleen DPNase added during incubation	2.64
" "	+ beef spleen DPNase added after incubation	20.00
" "	+ 0.5 μmole cold DPN in the incubation	2.30
ADPR-P^{32}	Normal	0.40
DPN-P^{32}	+ 0.25 μmole cold ADPR in the incubation	38.00

(+) See under materials and methods.

Several lines of evidence argue against a simple adsorption of ADPR on an acid-insoluble product. ADPR-P^{32} itself is not attached. The acid-insoluble radioactivity is not removed by repeated washing with cold ADPR, by extended dialysis or electrodialysis, or by incubation in 8 M urea for 30 minutes at 37°C.

The formation of the acid-insoluble product from DPN is abolished by heating the extract for 2 minutes at 100°C ; it is decreased by 86 per cent by $2 \cdot 10^{-3}$ M para-chloromercuribenzoate and is stimulated by Mg^{++} ions. The incorporation is inhibited by DNase and can be restored by DNA or certain polyanions such as heparin or dextran sulphate but not by RNA, poly A or poly U.

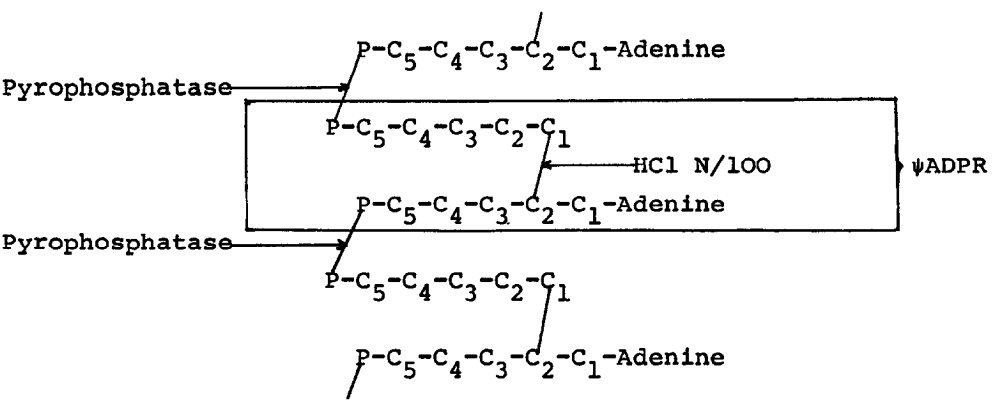
Thus our extract contains at least two enzymatic systems, one probably identical with that already described (Kornberg, 1950 - Atkinson, Jackson and Morton, 1961) which synthesizes DPN from ATP and NMN, the other one transforming DPN into an acid-

insoluble product which seems to contain ADPR. In order to exclude the possibility that the latter enzyme system was identical with DPNase, experiments were done with both beef spleen DPNase and hog brain DPNase which showed no incorporation of radioactivity from the precursors we used.

After treatment with phenol-SDS the isolated product of the reaction was excluded by Sephadex G 25 or G 50, and by ultracentrifugation its sedimentation coefficient S_{20}= 2.73.

Hydrolysis by snake venom pyrophosphatase gave a compound with a ratio adenine/ribose/phosphate : 1/2/2 that is an isomer of ADPR (ψADPR), and the two phosphate groups may be removed by alkaline phosphatase, yielding a ribosyladenosine. Hydrolysis of ψADPR by dilute mineral acid liberated ribosyl-5-phosphate and 5'-AMP which indicates a linkage between C_1 of the ribose-5-phosphate and C_2 or C_3 of the 5'-AMP. Methylation of the ribosyladenosine followed by acid hydrolysis (work done in collaboration with F. Petek) produced N_{10} methyladenine, 2,3,5-trimethyl-ribose and 3,5-dimethyl-ribose.

These results therefore suggest a linkage between C_1 and C_2 and that the structure of the polymer is :



The enzyme appears to be a transglycosidase which catalyses the polymerisation of DPN molecules by the formation of a lin-

kage between the ribose molecules with the simultaneous removal of the nicotinamide moiety.

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