AZIDE AS INHIBITOR OF PROTEIN SYNTHESIS IN YEAST PROTOPLASTS

W.J.C. AMESZ and B.A.M. van der ZEIJST
Van 't Hoff Laboratorium, Sterrenbos 19, Utrecht, The Netherlands

Received 15 June 1972

1. Introduction

Azide is known to uncouple oxidative phosphorylation in anaerobically growing yeast [1]. Azide also inhibits the protein and RNA synthesis in yeast [2]. It is not known, however, at which level azide acts as an inhibitor of protein synthesis.

In this report, we show that *in vivo* sodium azide (NaN_3) has a dual effect: one on the initiation and one on a later step of protein synthesis. The inhibition of the initiation prevails at concentrations of 10^{-3} M NaN_3 and lower. In a later study [8], it will be reported that the application of NaN_3 is useful for the large-scale preparation of derived ribosomal subunits from yeast.

2. Methods

Saccharomyces carlsbergensis (strain 74 N.C.Y.C., England) was grown and protoplasts were made as described before [3].

In vivo protein synthesis was measured as follows: protoplasts were incubated in a medium containing per liter 50 mmoles sodium potassium phosphate pH 6.2, 1 mmole $MgCl_2$, 120 g mannitol, 3 g glucose, 0.01 mmole [^{14}C] leucine (312 mCi/mmole), 0.09 mmole ^{12}C-leucine and 0.1 mmole of the other 19 amino acids. The amount of protoplasts protein was 0.25 mg per ml. Hot trichloroacetic acid precipitable radioactivity in samples from the incubation mixture was measured according to the method of Mans and Novelli [4] in a Nuclear Chicago Mark II scintillation counter with an efficiency of 65%.

For analysis of polysome distribution protoplasts were incubated in the medium described above except that [^{14}C] leucine was omitted and the concentration of ^{12}C-leucine was 0.1 mmole per liter. Cycloheximide was added to a final concentration of 10^{-3} M to stop protein synthesis immediately and to prevent "run off" of the ribosomes during the subsequent procedure [8]. The protoplasts were collected by centrifugation for 5 min at 3,000 *g* and lysed in a medium containing per liter 50 mmoles Tris-HCl pH 7.6, 10 mmoles $MgCl_2$ and 50 mmoles KCl.

The lysate was layered onto an isokinetic sucrose gradient (with 15% w/w of sucrose at the top and with the same ionic composition as the lysate throughout the gradient) and centrifuged for 180 min in the SW 25.1 rotor in the Beckman L2-65K centrifuge at 4°. The absorbancy profile of the gradient was recorded continuously with an LKB Uvicord and a logarithmic recorder.

The amount of radioactive N-terminal amino acid was determined according to the method of Sanger [5]. After reaction of FDNB* with the N-terminal amino acid, the excess FDNB was removed by ether extraction. The DNP-protein* was hydrolysed in 6.7 N HCl for 24 hours at 110°. The DNP-amino acids were extracted with ether. The extract was evacuated and the remaining radioactivity was counted in a Bray solution. In all experiments freshly prepared NaN_3 solutions were used.

3. Results and discussion

As can be seen from fig.1 protein synthesis is completely inhibited 3 min after the addition of NaN_3

*Abbreviations
FDNB: 1-fluoro-2,4 dinitrobenzene.
DNP-: N-2,4 dinitrophenyl-1- derivatives.

to a final concentration of 10^{-3} M. At lower concentrations of NaN_3 the final inhibition (between 0 and 100%) is also reached after 3 min. In other experiments, not shown here, this time varied no more than 0.5 min.

The polysome–ribosome distribution changes markedly during the 4 min period after the addition of NaN_3, as shown in fig. 2. There is a shift from the heavy polysomes to the smaller polysomes and 80 S ribosomes. The amount of 80 S ribosomes is increased from 9.5% at zero time to 42% after 2.5 min and 70% after 4 min. Presumably, the inhibition by NaN_3 of a later step of protein synthesis prevents the "run off" of the remaining 30% of the polysomes (see below).

The 80 S ribosomes formed during the azide treatment can be dissociated completely by 0.5 M KCl, which is possible with free ribosomes but not with complexed ribosomes [6, 7]. In fact, in our laboratory, this method is used for the isolation of subunits freed from mRNA and peptidyl-tRNA, which are very active in subcellular protein synthesis [8].

The experiments illustrated in fig. 3 confirm that NaN_3 at a concentration of 10^{-3} M affects preferentially the initiation of protein synthesis. In this experiment [^{14}C]alanine has been selected as a marker of the N-terminus, because alanine is the N-terminal amino acid in 50% of the polypeptide chains in *S. carlsbergensis* [9]. Thus, with [^{14}C]alanine in combination with the DNP-method, the incorporation in N-terminal positions and the total incorporation could be compared. Thus, it was found that the incorporation of N-terminal alanine is stopped completely within 20 sec after the addition of NaN_3, whereas the total incorporation continues for 2.5 min. This observation is in close agreement with the time required for the synthesis of an average size protein in yeast [10].

In order to obtain a preferential inhibition of the initiation, the choice of the NaN_3 concentration is rather critical. When the experiment described in fig. 2 was repeated with 5×10^{-3} M NaN_3, the shift in the 80 S ribosomes was from 10% at zero time to 30% after 2.5 and 4 min. Consequently, at higher concentrations of NaN_3 one or more other steps of protein synthesis are completely inhibited as well (results not illustrated). Which step in the series of initiation reactions is blocked by NaN_3 is not yet known. The inhibition by sodium azide is not identical with the inhibition of NaF in reticulocytes [11, 12]. NaF blocks a step between the binding of initiator tRNA and mRNA to the 40 S subunit and

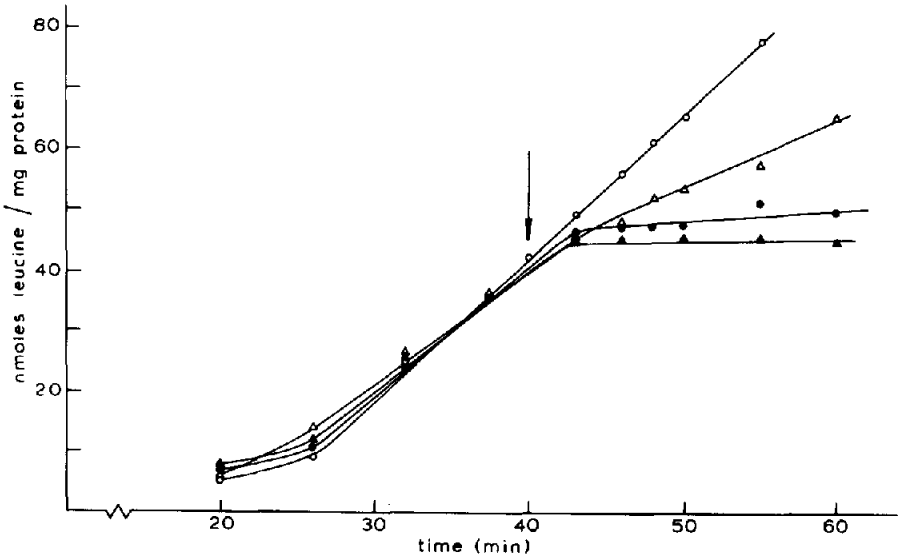

Fig. 1. Effect of NaN_3 on the rate of protein synthesis in yeast protoplasts. Protein synthesis in protoplasts was measured as described in the Methods. Sodium azide was added at 40 min to a final concentration of 1) (▲–▲–▲) 10^{-3} M; 2) (●–●–●) 8×10^{-4} M; 3) (Δ–Δ–Δ) 6×10^{-4} M; 4) (o–o–o) no addition of NaN_3.

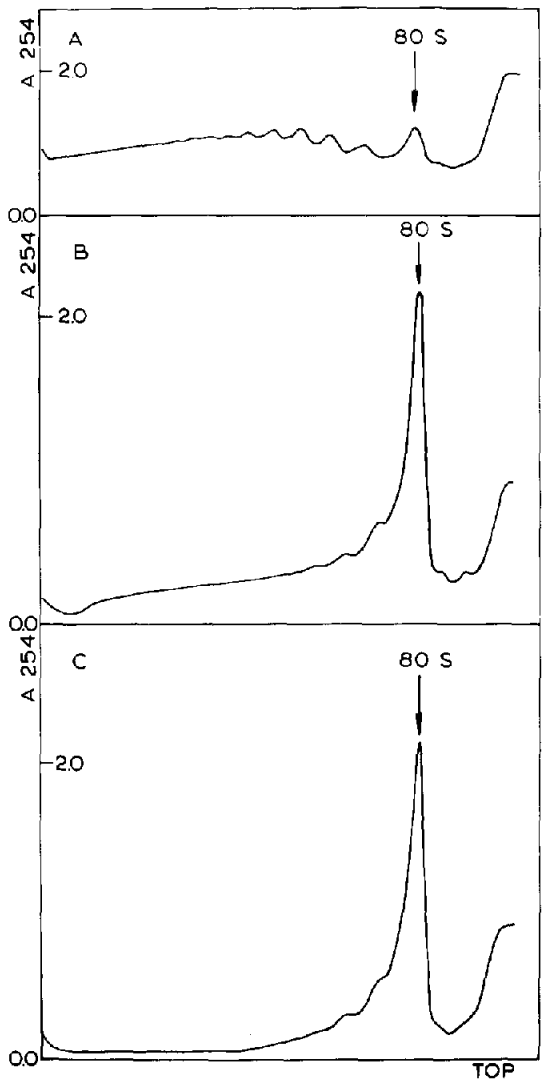

Fig. 2. Effect of NaN_3 on polysome distribution. The polysome distribution was determined as described in the Methods at different times after the addition of NaN_3 to a final concentration of 10^{-3} M. A) at zero time; B) after 2.5 min and C) after 4 min.

the binding of the first aminoacyl-tRNA to the ribosome [13, 14], presumably by preventing the addition of the 60 S subunit [15]. Sodium fluoride also inhibits the initiation of protein synthesis in an endogenous cell free system as well as in a poly U dependent cell free system [16, 17]. Sodium azide, however, has no inhibitory effect on the poly U dependent polyphenylalanine synthesis in a cell free system

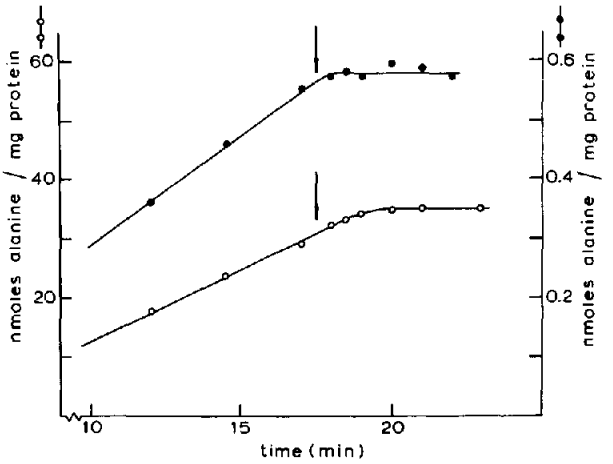

Fig. 3. Effect of NaN_3 on the total protein synthesis and on the incorporation of N-terminal amino acids. N-terminal alanine incorporation and total alanine incorporation was measured as described in the text. NaN_3 was added after 17.5 min to a final concentration of 10^{-3} M. [^{14}C] alanine was present at a concentration of 10^{-4} M. (o–o–o) Total alanine incorporation; (•–•–•) incorporation of N-terminal alanine.

derived from yeast (results not shown).

In summary, it is concluded that NaN_3 leads to an instant inhibition of protein synthesis in protoplasts of *S. carlsbergensis*, the inhibition being complete at concentrations of 10^{-3} M of NaN_3 or higher. Moreover, increasing concentrations of the inhibitor cause increasing inhibition of a later step of the protein synthesis, presumably the elongation.

At the critical concentration of 10^{-3} M NaN_3, however, the effect on the initiation prevails largely (figs. 2 and 3); thus, the application of NaN_3 is a useful method for the preparation of active derived ribosomal subunits [8].

Acknowledgement

We thank H.J. van Lönkhuyzen for technical assistance.

References

[1] L. Kováč and A. Istenesová, Biochim. Biophys. Acta 82 (1964) 162.
[2] L. Jarett and R.W. Hendler, Biochemistry 6 (1967) 1693.

[3] R. Hartlief and V.V. Koningsberger, Biochim. Biophys. Acta 166 (1968) 512.
[4] R.J. Mans and G.D. Novelli, Arch. Biochem. Biophys. 94 (1961) 48.
[5] F. Sanger, Biochem. J. 39 (1945) 507.
[6] T.E. Martin and L.H. Hartwell, J. Biol. Chem. 245 (1970) 1504.
[7] G.R. Lawford, Biochem Biophys. Res. Commun. 37 (1969) 143.
[8] B.A.M. van der Zeijst, A.J. Kool and H.P.J. Bloemers, European J. Biochem. (1972) in press.
[9] S.S. Sarimo and M.J. Pine, J. Bacteriol. 98 (1969) 368.
[10] L.H. Hartwell and C.S. McLaughlin, Proc. Natl. Acad. Sci. U.S. 62 (1969) 468.
[11] B. Colombo, L. Felicetti and C. Baglioni, Biochem. Biophys. Res Commun. 18 (1965) 389.
[12] P. A. Marks. E.R. Burka, F.M. Conconi, W. Perl and R.A. Rifkind, Proc. Natl. Acad.Sci. U.S. 53 (1965) 1437.
[13] T. Obrig, J. Irvin, W. Culp and B. Hardesty, European J. Biochem. 21 (1971) 31.
[14] W. Culp, J. Morrisey and B. Hardesty, Biochem. Biophys. Res Commun. 40 (1970) 777.
[15] W. Hoerz and K.S. McCarthy, Proc. Natl. Acad. Sci. U.S. 63 (1969) 1206.
[16] S. Lin, R.D. Mosteller and B. Hardesty, J. Mol. Biol. 21 (1966) 51.
[17] J.M. Ravel, R.C. Mosteller and B. Hardesty, Proc. Natl. Acad. Sci. U.S. 56 (1966) 701.