

INHIBITION OF NADP DEPENDENT OXIDOREDUCTASES BY THE 6-AMINONICOTINAMIDE ANALOGUE OF NADP

Elisabeth KÖHLER, Hans-Jürgen BARRACH and Diether NEUBERT

*Pharmakologisches Institut der Freien Universität Berlin, Abteilung Embryonal-Pharmakologie,
Berlin-Dahlem, Germany*

Received 18 November 1969

1. Introduction

6-Aminonicotinamide (6-AN) can replace the nicotinamide moiety of pyridine nucleotides both *in vitro* and *in vivo* in the reaction catalysed by the enzyme NAD(P) glycohydrolase (E.C.3.3.3.6) or in the course of a *de novo* synthesis of NAD or NADP; the resulting antimetabolites (6-ANAD or 6-ANADP) may inhibit pyridine nucleotide linked reactions [1–5]. After injection of 6-AN into pregnant experimental animals, embryotoxic effects can be demonstrated [6–8]. In adult animals, the application of this antimetabolite leads to typical lesions of the central nervous system, including irreversible paralysis [4,9]. An increase in the concentration of 6-phosphogluconate (6-PG) in rat brains *in vivo* following the injection of 6-AN [12, 13] suggests an impairment at the 6-phosphogluconate dehydrogenase level.

Earlier studies have shown an impaired function of the oxidative part of the pentose phosphate pathway (PPP) in embryos of experimental animals treated with 6-AN and an inhibition of the incorporation of carbon atoms from glucose into the ribose moiety of nucleic acids via the NADP dependent steps [8]. We have tried to localize the block in the PPP.

In this paper, we present the results of kinetic studies on the effect of 6-aminonicotinamide adenine dinucleotide phosphate (6-ANADP).

2. Materials and methods

2.1. Kinetic studies

Hyaloplasmic fractions from various rat tissue were used for the kinetic studies. After homogenizing the tissue in 0.32 M sucrose + 1 mM Mg^{2+} , pH 7.4 and

Table 1
Effect of 6-ANADP on some NADP-dependent enzyme reactions of various rat tissues.

Tissue	Enzyme	V_{max}^a	K_m NADP(H ₂) (μM)	K_i 6-ANADP (μM)
Embryo, day 13	G-6-PD	32	14	70
	6-PGD	30	11	0.2
	GSSG reductase	9	6	46
Liver	G-6-PD	35	20	110
	6-PGD	73	13	0.2
Mammary gland	6-PGD	249	13	0.1

All numbers give the mean values of several experiments.

^a $\mu moles$ NADP(H₂) converted $\times min^{-1} \times mg$ protein⁻¹.

Table 2

Formation of $^{14}\text{CO}_2$ and incorporation into DNA and lipids from $1\text{-}^{14}\text{C}$ - and $6\text{-}^{14}\text{C}$ -glucose by intact rat embryos *in vitro*
(Mean \pm S.D., $n = 10$).

	CO_2 (dpm $\times \mu\text{g DNA}^{-1}$)		DNA (dpm $\times \mu\text{g DNA}^{-1}$)		Lipids (dpm $\times \mu\text{g DNA}^{-1}$)			
	C ₁	C ₆	C ₁	C ₆	C ₁ /C ₆	C ₁	C ₆	C ₁ /C ₆
Control	8.80 ± 2.30	< 0.02	0.95 ± 0.16	0.89 ± 0.06	1.07	1.80 ± 0.20	1.60 ± 0.10	1.11
6-AN-treated	$< 0.02^a$	< 0.02	1.03 ± 0.05	0.73 ± 0.07^a	1.41	0.92 ± 0.20^a	0.85 ± 0.09	1.08

^a $p < 0.005$

15 mg kg^{-1} 6-AN were given i.p. to the mothers 4 hr before the experiment. Rat embryos (day 13) were incubated at 37°C for 3 min in a saline medium containing $10 \mu\text{Ci}$ glucose ($3.3 \mu\text{Ci}/\mu\text{mole}$).

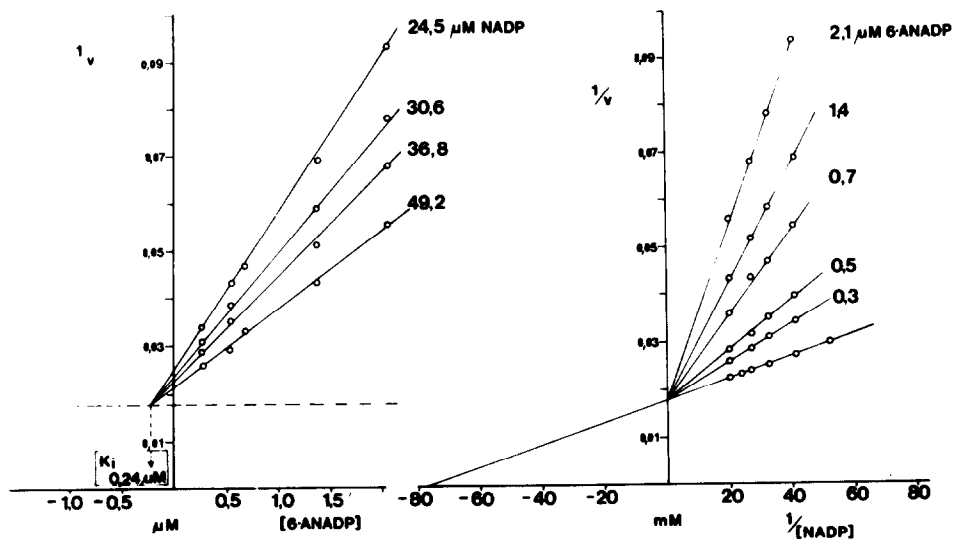


Fig. 1. Inhibition of 6-PGD from rat embryos by 6-ANADP. $v = \mu\text{moles NADP reduced} \times \text{min}^{-1} \times \text{mg protein}^{-1}$. $K_m = 13 \mu\text{M}$. $K_i = 0.24 \mu\text{M}$. Assay mixture: 4 mM Mg^{2+} , 10 mM nicotinamide, 20 mM 6-phosphogluconate, trisodium and 100 mM tris-HCl pH 7.5.

centrifuging for 90 min at $100,000 g$ in a Spinco ultracentrifuge, the supernatant was passed through Sephadex G-25 columns to remove substances of low molecular weight. Enzymic activities were measured spectrophotometrically at 340 nm or 366 nm . Recording spectrophotometers from Leitz-Unicam or Eppendorf were used. All experiments were performed at 25°C . V_{max} , K_m and K_i values were derived both graphically and mathematically using a Wang 370 computing system and the statistical estimations of

Wilkinson [10] from at least 4 independent sets of data. DNA was measured the method of Burton [14].

2.2. Inhibitor

The 6-AN analogue of NADP was prepared using hog brain NAD(P) glycohydrolase (E.C.3.3.3.6). The analogue was isolated and purified by chromatography [3]. The product had a purity of at least 95%.

2.3. Metabolism of ^{14}C -glucose

Pregnant rats were treated with 5–15 mg/kg 6-AN i.p. 4–40 hr prior to the experiment. Embryos (day 13) were removed and incubated for 3 min at 37°C with $1\text{-}^{14}\text{C}$, $2\text{-}^{14}\text{C}$ or $6\text{-}^{14}\text{C}$ -glucose ($10\text{ }\mu\text{Ci}/3\text{ }\mu\text{moles}$) in 1 ml of a medium containing 150 mM NaCl, 50 mM KCl, 3 mM Mg^{2+} and 60 mM phosphate buffer pH 7.5. $^{14}\text{CO}_2$ was absorbed with NaOH and incorporation of ^{14}C into lipids, nucleic acids and proteins was measured, respectively, by successive extraction of the washed (5 times) perchloric acid (0.2 N) precipitate with alcohol-ether (3:1, 3 times) and 0.3 N NaOH (37°C , 1 hr) followed by acidification with 0.2 N perchloric acid (75°C , 15 min, twice).

3. Results and discussion

So far, no indication that 6-ANADP or 6-ANAD can be reduced by any enzymatic reaction has been obtained, but earlier studies have shown the analogues to be competitive inhibitors of some NAD(P)-linked oxidoreductases [2,4].

3.1. Kinetic studies with 6-ANADP

Results obtained with enzymes from embryonic tissues are shown in fig. 1 and table 1. Glucose-6-phosphate dehydrogenase (G-6-PD, E.C. 1.1.1.49) is inhibited competitively; the K_i found with the enzyme from mammalian embryos ($70\text{ }\mu\text{M}$) is somewhat higher than that published [2] for the corresponding enzyme from yeast ($20\text{--}30\text{ }\mu\text{M}$). It is interesting to note that 6-ANADP the analogue in the oxidized form, also inhibits the conversion catalysed by glutathione reductase (E.C.1.6.4.2), an enzyme reaction proceeding in the direction NADPH_2 to NADP. Similarly to G-6-PD, this enzyme is also moderately inhibited, the K_i being $46\text{ }\mu\text{M}$.

In contrast to enzymic studies so far, 6-phosphogluconate dehydrogenase (6-PGD, E.C.1.1.1.44) from mammalian embryos is inhibited by concentrations of the analogue that are several orders of magnitude lower. As shown in fig. 1, the K_i for 6-ANADP in this reaction is $0.2\text{ }\mu\text{M}$. Because of substrate inhibition, conclusive data could be obtained only in a narrow range of NADP concentrations but from a variety of plots ($1/v$ versus $1/s$, s/v versus s , $1/v$ versus i and i/α versus s) the data available suggest competitive

inhibition. To our knowledge, this is the strongest inhibition of an oxidoreductase by an analogue of pyridine nucleotides so far published. The malic enzyme reaction [4], which also includes a decarboxylation step was previously found to be most sensitive to 6-ANADP.

To compare the sensitivity of the embryonic enzyme with those of enzymes from adult tissues, the K_i for 6-ANADP on 6-PGD in a variety of tissues (table 1) was measured but no real differences were found. These results were confirmed recently with an enzyme from rat brain [15].

3.2. Studies on the metabolism of ^{14}C -glucose

To elucidate further the effect of the NADP-analogue on the function of the pentose phosphate pathway (PPP) and its coupling to nucleic acid synthesis, the metabolism of glucose was studied in intact mammalian embryos. In embryonic tissue, ribose-5-phosphate is an essential requirement, since nucleic acid synthesis proceeds at a very high rate and its content may increase 7 fold during 24-hr period [11]. The results of one experiment with $1\text{-}^{14}\text{C}$ and $6\text{-}^{14}\text{C}$ -glucose *in vitro*, showing the effect of 6-AN treatment *in vivo*, are given in table 2. The data show clearly that under our experimental conditions, the reactions of the PPP are studied almost exclusively and that CO_2 formation via respiration does not contribute to a measurable extent. Furthermore, as judged from studies with $2\text{-}^{14}\text{C}$ -glucose, "recycling" may be neglected. 5 hr after the injection of 15 mg/kg 6-AN to the mother animal, no formation of $^{14}\text{CO}_2$ from $1\text{-}^{14}\text{C}$ -glucose could be detected in the embryo, indicating a complete inhibition of the oxidative part of the PPP. This confirms our earlier findings [8].

Although 6-AN treatment leads to a complete block in the oxidative part of PPP, apparently at the level of 6-PGD, the embryotoxic ribose effect is not caused by prevention of ribose formation via 6-PG. No significant inhibition of the overall incorporation of glucose-fragments into the ribose moiety of DNA and RNA by low doses of 6-AN at short time intervals was detectable in these experiments and in many experiments a significant increase in the rate of labelling was seen.

Earlier experiments performed in collaboration with Dr. Brand (Max-Planck-Institut für Ernährungsforschung, Dortmund) and to be reported elsewhere in detail, have shown that the oxidative part of the

PPP plays a minor role in early mammalian embryos compared with the non-oxidative part. As calculated from the data given here, at least 85% of the radioactivity found in the DNA was incorporated via the non-oxidative part; thus even complete block of the oxidative part of the PPP would not significantly effect ribose formation.

Following a complete block in the oxidative part of the PPP, the ratio of the incorporation of 1-¹⁴C to 6-¹⁴C-glucose would be expected to increase and approach the theoretical value of 1.5. Under suitable conditions this effect can be demonstrated (table 2). In contrast to the incorporation of glucose into the ribose moiety of DNA and RNA, the labelling of lipids significantly decreased under the same experimental conditions (table 2). Since the rate of aerobic lactate formation did not change, an inhibition of the glycolytic pathway is unlikely. An impaired production of reduced NADP may therefore be of greater importance.

The rate of incorporation of ¹⁴C-glucose into RNA, lipids, DNA and proteins decreased if the dose of 6-AN was increased or the metabolism was studied at longer time intervals (more than 7 hr) following the injection of inhibitor. The ratio of incorporation of 1-¹⁴C-glucose to 6-¹⁴C-glucose also decreased, suggesting additional effects of the analogue, possibly a disturbance in the isomerisation of triose phosphates. It is undecided whether such effects are caused directly by 6-ANADP or are a secondary result of 6-PG accumulation as suggested by Herken and coworkers [12,13].

6-ANADP is an extremely potent competitive inhibitor of 6-PGD; concentrations that do not effect G-6-PD, may completely block 6-PGD. Addition of this compound allows measurement of G-6-PD in the presence of excess 6-P GD.

(The results of a detailed analysis of the inhibition of these two enzymes from different sources will be published elsewhere). The analogue may also provide a valuable tool for studying the significance of the oxidative part of the PPP and the coupling of this metabolic pathway to other reactions *in vivo* and *in vitro* in a variety of tissues.

Acknowledgements

We are indebted to Mr. Römer for preparing the 6-ANADP and to Miss Teske for skillful assistance. The work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 29, Embryonal-Pharmakologie.

References

- [1] L.S.Dietrich, I.M.Friedland and L.A.Kaplan, J. Biol. Chem. 233 (1958) 964.
- [2] H.Coper and D.Neubert, Biochim. Biophys. Acta 89 (1964) 23.
- [3] A.Brunnemann, H.Coper and D.Neubert, Arch. Exptl. Pathol. Pharmacol. 246 (1964) 437.
- [4] D.Neubert and H.Coper, Biochem. Z. 341 (1965) 485.
- [5] H.Coper, H.Hadass and H.Lison, Arch. Exptl. Pathol. Pharmacol. 255 (1966) 97.
- [6] M.L.Murphy, in: Teratology, principles and techniques, eds. J.G.Wilson and J.Warkany. (University Chicago Press, 1965) p. 145.
- [7] J.G.Chamberlain, Anat. Record 156 (1966) 31.
- [8] E.Köhler and D.Neubert, Arch. Exptl. Pathol. Pharmacol. 260 (1968) 154.
- [9] H.Coper and H.Herken, Deut. Med. Wochschr. 88 (1963) 2025.
- [10] G.N.Wilkinson, Biochem. J. 80 (1961) 324.
- [11] D.Neubert and E.Köhler, 4 Internat. Congr. Pharmacol., Basel (1969).
- [12] H.Herken and K.Lange, Arch. Exptl. Pathol. Pharmacol. 263 (1969) 496.
- [13] H.Herken, K.Lange and H.Kölbe, Biochem. Biophys. Res. Commun. 36 (1969) 93.
- [14] K.Burton, Biochem. J. 62 (1956) 315.
- [15] H.Herken, personal communication.