HYDROXYLAMINE, AN INHIBITOR OF PEPTIDE CHAIN INITIATION

Adam KEPES and Simone BEGUIN
Service de Biochimie Cellulaire, Institut Pasteur, Paris.

Received December 7, 1964

Hydroxylamine has been reported to exert a bacteriostatic effect (1) (2) even at concentrations as low as 10^{-4}M in E. coli (3). Hydroxylamine at 10^{-3}M stops the synthesis of DNA, RNA and protein (2) but with 10^{-4}M hydroxylamine preferential inhibition of protein synthesis is observed (4). Although the rate of overall protein synthesis in a fully inhibited culture may be raised up to 50% of the control (by addition of arginine), the synthesis of active β-galactosidase whether inducible or constitutive, remains zero (4). Hydroxylamine, a highly reactive molecule, has undoubtedly more than one site of action, but the preferential inhibition of protein synthesis at low concentrations could not be tracked down to a block in any of the major metabolic pathways (4).

As the sequence of events in the inducible synthesis of β-galactosidase from the synthesis of messenger RNA to the release of finished enzyme is accessible to kinetic analysis (5), this technique has been used in an attempt to locate the step preferentially inhibited by hydroxylamine.

The technique is based on the finding that the synthesis of the specific messenger RNA which codes for β-galactosidase can be stopped by removal of the inducer (by filtration and washing or simply by dilution) as well as by a certain inhibitors of RNA synthesis (proflavine) or by incorporation of a base analog (5 fluoro uracil). The limited amount of enzyme which appears during the next few minutes is due to the translation of preformed messenger. This can be inhibited by chlo-

ramphenicol, 5 methyl tryptophan or puromycine but not by inhibitors of RNA synthesis. Thus two classes of inhibitors of active β-galactosidase synthesis can be distinguished, those acting upon the synthesis of m RNA and those acting upon the synthesis of the peptide chain. Synthesis of functionally normal m RNA during inhibition of peptide synthesis can also be demonstrated since the removal of the inhibitor after (or at the same time as) the removal of the inducer leads to the synthesis of a limited amount of enzyme.

Figure 1 shows that β-galactosidase specific messenger can be synthesized in the presence of 10^{-4}M hydroxylamine and is eventually translated into active enzyme if hydroxylamine is diluted 50 times (or washed out), but not if hydroxylamine is present during the whole process.

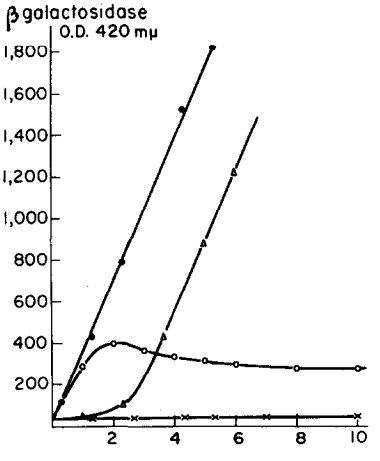

Figure 1. - Exponentially growing E. coli strain 3000 in medium 63 with 4 g/l glycerol is induced by addition of 2.10^{-4}M isopropyl β-d-thiogalactoside (IPTG) at zero time. Samples are diluted in 50 volumes of medium 63 glucose at times shown in abscissa and incubated for another 20 minutes —●—. Samples diluted in 50 volumes of medium containing 50 μg/ml chloramphenicol —Δ—. A parallel culture received 10^{-4}M hydroxylamine two minutes before inducer and samples were diluted in 50 volumes of 63 glucose —○— or 63 chloramphenicol —×—. β-galactosidase measured with ONPG as substrate.

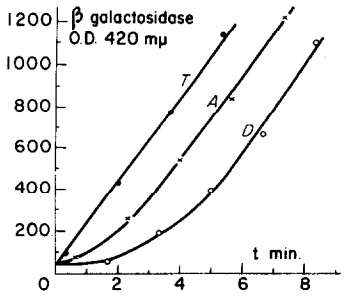

Figure 2. - Strain, culture, induction as in fig.1. Samples diluted in 50 volumes of : 63 glucose : T ; 63 glucose with 10^{-4}M hydroxylamine : A ; 63 chloramphenicol : D.
Enzyme assay as in fig.1.

As shown in figure 2 active enzyme can also be synthesized in the presence of 10^{-4}M hydroxylamine from messenger RNA synthesized before the addition of the inhibitor. The amount of enzyme synthesized under these conditions is about half of what would be synthesized from the same amount of messenger in the absence of hydroxylamine and is equivalent to about 1.5 minutes of maximal synthesis.

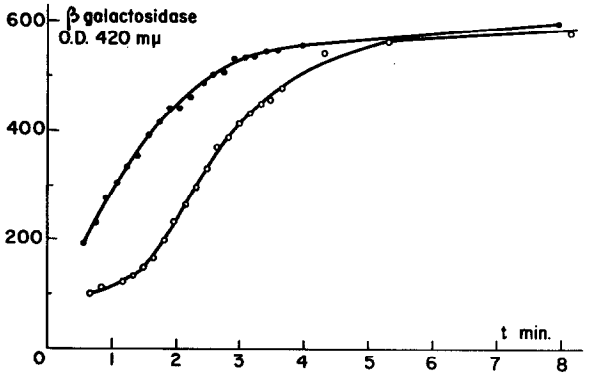

Figure 3. - Strain, culture and induction as in fig.1. The induced culture is diluted in 50 volumes of 63 glucose at 20 seconds and further incubated. Samples of the diluted culture are transferred to 10^{-4}M hydroxylamine at times shown in abscissa and incubation is continued 20 minutes : —●— ; samples transferred to 50 μg/ml chloramphenicol : —○—.
Enzyme assay as in fig.1.

This partial effect of hydroxylamine upon peptide synthesis does not account for the fact illustrated in fig. 1 namely that in the permanent presence of hydroxylamine no enzyme synthesis whatsoever is observed. Figure 3 further illustrates the point that enzyme synthesis is not due to "leaky" inhibition. Here the effect of hydroxylamine is tested during the wave of enzyme synthesis which follows a short pulse of induction when the rate of synthesis is not linear but rather exponentially decreasing. The inhibition due to hydroxylamine is nearly 80% when added 40 seconds after the start of the pulse induction, and becomes nearly zero when added at 3 minutes, whereas the time shift of 1.5 minutes remains constant.

Thus hydroxylamine does not behave as an inhibitor of messenger RNA synthesis, nor as an inhibitor of peptide chain synthesis, but it exerts a complete inhibition of β-galactosidase synthesis after a delay of 1.5 minutes. Such a delay can be due to either of two causes a) 1.5 minutes are necessary for some preparatory step (such as penetration and/or chemical processes) before inhibition occurs, or b) hydroxylamine inhibits immediately and completely some specific step in β-galactosidase synthesis which occurs 1.5 minutes before the release of active enzyme, while it allows all enzyme molecules processed beyond this step to be eventually finished.

Previous analysis has shown that about 1.5 minutes are required for the synthesis of the complete peptide backbone of one β-galactosidase subunit. Thus the step inhibited by hydroxylamine might be situated at the initiation of this process (e.g. attachment of a ribosome to the messenger or formation of the very first peptide bonds or some other biochemical reaction connected with the former). According to the above estimate of the time required for peptide synthesis, the active enzyme which appears after the blocking of messenger synthesis should be considered as made up of two approximately equal fractions,

the first of which corresponds to the termination of peptide chains of various lengths, while the second represents the synthesis of entirely new chains. If hydroxylamine inhibits specifically and preferentially the initiation of new chains, a suitable labeling of β-galactosidase should show that "de novo" synthesis is completely inhibited whereas termination of initiated chains is substantially uninhibited.

The possibility of performing such labeling was provided by the observation that replacement of tyrosine by 3-fluoro tyrosine (6) or replacement of phenylalanine by β-2-thienyl-alanine (7) lead to enzymatically fully active β-galactosidase with decreased heat stability.

If a culture is induced in the presence of the analog, heat labile β-galactosidase will be synthesized. If now such a culture is diluted into a medium containing an excess of the corresponding natural aminoacid, and incubation is continued until full decay of messenger RNA has occurred, the enzyme synthesized from the time of the dilution will represent in part the termination of already initiated chains which should therefore contain the analog and be heat labile, while another part should correspond to molecules synthesized "de novo", which should contain only natural aminoacids and therefore should be heat stable. If the dilution is made in a hydroxylamine containing medium, the latter, heat stable fraction should be missing, provided the assumption that hydroxylamine inhibits the initiation of new peptide chains only, is correct.

Figure 4 shows the result of an experiment performed with 3-fluoro tyrosine. The analog added 2 minutes before induction does not decrease significantly the rate of synthesis of specific messenger during the first few minutes (compare curves A and B). The synthesis of enzyme is somewhat slowed down (D) (compare with lowest curve figure 2) and the enzyme is entirely heat labile (d).

For the samples represented by curves B and C the dilution of in-

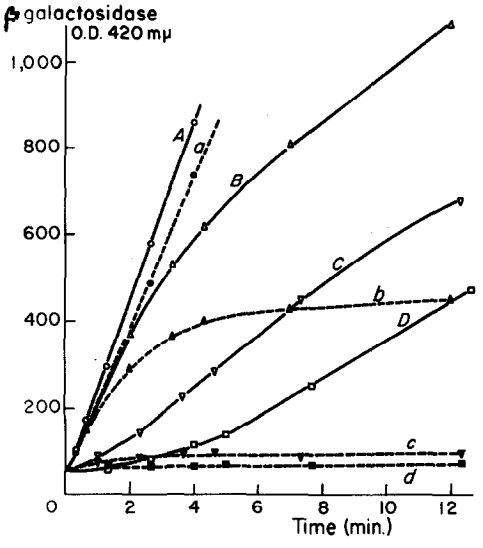

Figure 4. - Strain, culture and induction as in fig.1. Samples diluted in 50 volumes of 63 glucose : A and a. Same culture with 2.10^{-4}M 3-fluoro tyrosine added two minutes before inducer. Samples diluted in 50 volumes of 63 glucose L-tyrosine 5.10^{-4}M : B and b, in 50 volumes of 63 glucose, tyrosine, hydroxylamine 10^{-4}M : C and c, in 50 volumes of 63 chloramphenicol 50 μg/ml : D and d.

A, B, C, D : enzyme assay as in fig.1 ; a, b, c, d : heating 15 minutes at 55°C with 1 mg/ml β-mercaptoethanol before assay.

---

ducer was made into a medium containing excess L-tyrosine so that enzyme synthesized "de novo" should be thermostable. If no hydroxylamine is present in the dilution medium, about 2/3 of the enzyme synthesized after dilution (B-D) are thermostable (b-d). If hydroxylamine is present in the dilution medium, the enzyme (C-D) contains no significant thermostable fraction (c-d), whereas the thermolabile fraction is virtually unchanged (B-b = C-c). Similar experiments with β-2-thienylalanine as the analog gave similar results, except that the thermostable fraction obtained in the presence of hydroxylamine was a little larger, due probably to the fact that enzyme molecules containing but a few analog residues have a heat stability not very different from normal enzyme. It has been established in independent experiments, that hydroxylamine does

not prevent the exchange of a β-2-thienylalanine pool with exogenous phenylalanine.

Beyond the mode of action of hydroxylamine, the above experiments draw attention to a hitherto unrevealed step in protein synthesis occuring between the synthesis of m RNA and the growth of peptide chain but distinct from these two well identified processes. The demonstration of this step through its selective inhibition by low concentrations of hydroxylamine should lead to further inquiry into its chemical nature.

ACKNOWLEDGMENTS

This work has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research, the National Institutes of Health, the "Commissariat à l'Energie Atomique", and the "Délégation Générale à la Recherche Scientifique".

REFERENCES

(1). Borek, E., Gyrossowicz, N. and Waelsh, H. Arch. Biochem. Biophys., 31, 279 (1951).
(2). Rosenkranz, H.S. and Bendich, A.S. Biochim. Biophys. Acta 87, 40, (1964).
(3). Beguin, S. and Kepes, A. C.R. Acad. Sc. Paris, 258, 2427, (1964).
(4). Beguin, S. and Kepes, A. C.R. Acad. Sc. Paris, 258, 2690, (1964).
(5). Kepes, A. Biochim. Biophys. Acta, 76, 293, (1963).
(6). Munier, R.L. and Sarrazin, G. C.R. Acad. Sc. Paris, 259, 937, (1964).
(7). Janacek, J. and Rickenberg, H.V. (in the press).