JOURNAL OF BACTERIOLOGY, June 1973, p. 956-960
Vol. 114, No. 3

Effect of Cycloheximide on L-Leucine Transport by *Penicillium chrysogenum*: Involvement of Calcium

DOUGLAS R. HUNTER,[1] CAROL L. NORBERG, AND IRWIN H. SEGEL
Department of Biochemistry and Biophysics, University of California, Davis, California 95616

Received for publication 15 March 1973

Cycloheximide (actidione) has an immediate inhibitory effect on amino acid transport by nitrogen-starved or carbon-starved mycelium suspended in phosphate buffer. High concentrations of phosphate alone are slightly inhibitory; cycloheximide appears to potentiate the effect of phosphate. Ca^{2+} reverses the inhibition of transport caused by phosphate plus cycloheximide. Ca^{2+} did not relieve the inhibition of protein synthesis. Cycloheximide promotes a continual uptake of $^{45}Ca^{2+}$ by the mycelium. The cumulative results suggest that (i) membrane-bound Ca^{2+} is involved in amino acid transport, (ii) cycloheximide labilizes the membrane-bound Ca^{2+}, and (iii) phosphate forms a complex with Ca^{2+} making it unavailable for its role in transport. The effect of cycloheximide described above is observed within 1 to 2 min after addition of the antibiotic. This initial inhibition occurs more rapidly with 10^{-3} M cycloheximide than with 10^{-5} M cycloheximide. However, after a longer preincubation time, a curious inverse relationship between cycloheximide concentration and amino acid transport is observed. The mycelium incubated with 10^{-5} M cycloheximide remains strongly inhibited (unless the antibiotic is washed away). The mycelium incubated with 10^{-3} M cycloheximide recovers about 40% of the transport activity lost during the rapid initial phase. We have no obvious explanation for the inverse effect.

Cycloheximide (actidione) is a potent inhibitor of protein synthesis in eukaryotic organisms (14). Earlier work in our laboratory showed that cycloheximide (i) stimulates a decay in the activity of several derepressible transport systems in *Penicillium chrysogenum* over a period of several hours (1, 3, 8), (ii) partially prevents the substrate-induced "transinhibition" of further substrate transport (10), and (iii) inhibits the efflux of α-keto acid from nitrogen-starved mycelium preloaded with a high concentration of any hydrophobic amino acid substrate of the general amino acid transport system (10). It is not clear how these effects are related to the ability of the antibiotic to prevent protein synthesis, but some suggestions have been offered (10). In this paper, we describe a very rapid inhibition of L-leucine-^{14}C transport by cylcoheximide. This effect is observable within 1 min after addition of cycloheximide to the mycelial suspension and is most pronounced in phosphate buffer. In contrast, phenomena mentioned above are relatively long-term effects of cycloheximide and persist even after the antibiotic is washed away. Recently, Evans (6) reported a similar very rapid effect of cycloheximide on several transport systems in *Euglena*. Several groups noted the long-term cycloheximide-induced decay in transport activity (1, 3, 7, 8, 12, 16).

MATERIALS AND METHODS

The experiments described in this paper were carried out with *P. chrysogenum*, wild-type strain PS-75. The cultivation techniques and permease assays were described in detail in earlier papers (2, 3, 9). In general, transport rates were determined from three samples taken at 20-s intervals immediately after adding the labeled substrate. Transport rates are reported on a cell (dry weight) basis. L-Leucine-^{14}C (200 mCi/mmol) and $^{32}P_i$ (carrier-free) were obtained from New England Nuclear Corp. These substrates were diluted with unlabeled carrier to a specific activity of 10^6 counts per min per μmol for the transport assays. $^{45}CaCl_2$ (2×10^6 counts per min per

[1] Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wis. 53706.

μmol) was a gift from R. Baskin from the Department of Zoology, University of California at Davis. Cycloheximide was obtained from Mann Research Lab., Inc. All other chemicals were the purest grade available from commercial sources.

RESULTS

Effect of buffer and pH. When cycloheximide (10^{-5} to 10^{-3} M) is added to a suspension of nitrogen-starved mycelium in phosphate buffer, the specific activity of the general amino acid transport system is immediately reduced (Table 1). The inhibition increases as the pH of the phosphate buffer increases. Relatively little inhibition is observed in 2-(*N*-morpholino)propanesulfonic acid (MOPS) or arsenate buffers. Carbon-starved mycelium (which are as fully derepressed for the general amino acid transport system as nitrogen-starved mycelium [9]) behave similarly. Protein synthesis (measured by the incorporation of the labeled amino acid into the boiling water-insoluble pool) is inhibited >90% under all the above conditions. In preliminary studies, we established that phosphate alone at >0.05 M inhibits L-leucine transport, the pH pattern being the same as that shown in Table 1. Thus, it appears that cycloheximide potentiates or amplifies the effect of phosphate. (Results shown in Table 1 were obtained with 0.02 M phosphate, which by itself is only slightly inhibitory. Furthermore, the results are all relative to the buffer control and, therefore, represent only the additional effect of cycloheximide.)

TABLE 1. *Effect of cycloheximide on* L-*leucine-*^{14}C *transport activity*[a]

Assay buffer[b]	Inhibition of L-leucine-^{14}C transport (%)	
	10^{-5} M Cycloheximide	10^{-3} M Cycloheximide
Potassium phosphate, pH 6	33	45
Potassium phosphate, pH 7	65	81
Sodium phosphate, pH 7	70	73
Potassium phosphate, pH 8	80	93
Potassium MOPS[c], pH 7	21	20
Sodium MOPS, pH 7	17	18
Potassium arsenate, pH 7	0	29

[a] Nitrogen-starved mycelia were preincubated under standard assay conditions in the buffers shown. Cycloheximide was added 1 min before addition of the labeled substrate (2×10^{-5} M). Transport activity was determined from three samples taken at 20-s intervals immediately after addition of the L-leucine-^{14}C.

[b] Concentration, 0.02 M.

[c] MOPS, 2-(*N*-morpholino)propanesulfonic acid.

Effect of cations on cycloheximide inhibition of L-leucine transport. Table 2 shows that Ca^{2+} and Ba^{2+} (and to a lesser extent Mg^{2+} and Sr^{2+}) are able to reverse the inhibition of L-leucine transport by cycloheximide. Essentially identical results were obtained with 10^{-5} M cycloheximide. Ca^{2+} at 10^{-4} M was about half as effective as 10^{-3} M Ca^{2+} in reversing the effect of cycloheximide. The reversal was less pronounced when the phosphate concentration was increased. Ca^{2+} had no effect on the inhibition of protein synthesis. Aniline by itself also partially reverses the inhibition, whereas aniline plus Ca^{2+} almost completely restores the specific transport activity to the original level.

Effect of cycloheximide on Ca^{2+} transport. Results (Tables 1 and 2) suggest that the inhibition of L-leucine transport by phosphate may result from removal of Ca^{2+} from a strategic location within the cell membrane. The synergism exhibited by phosphate and cycloheximide can be explained if cycloheximide labilizes the membrane Ca^{2+}, making it available to form a complex with phosphate. Cycloheximide is not known to be a chelator or ionophore for Ca^{2+}, although the antibiotic does bear a slight structural resemblance to ammonium purpurate (Murexide), a strong Ca^{2+} chelator (5). Experiments designed to demonstrate the cycloheximide-stimulated efflux of $^{45}Ca^{2+}$ from preloaded cells were inconclusive. This is not surprising since only a small fraction of the total cellular Ca^{2+} may be membrane bound. A more conclusive result is shown in Fig. 1. Cycloheximide clearly promotes a continual uptake of $^{45}Ca^{2+}$ by nitrogen-starved mycelium. The uptake could represent either net transport or exchange with an unlabeled pool of cellular Ca^{2+}.

Effect of cycloheximide on L-lysine transport. *P. chrysogenum* possesses a constitutive transport system for basic amino acids (9). This system is fully active in nutrient-sufficient mycelium where the general amino acid transport system (which also accepts lysine and arginine as substrates) is relatively inactive. Preliminary experiments established that (i) 10^{-5} M cycloheximide strongly inhibits L-lysine-^{14}C transport from phosphate buffer by nutrient-sufficient mycelium, and (ii) 10^{-3} M Ca^{2+} almost completely reversed the inhibition by cycloheximide.

Effect of citrate and EDTA. If phosphate acts by removing Ca^{2+} from the cell membrane, then other extracellular Ca^{2+} chelators might work as well. When mycelium was suspended in 0.04 M potassium MOPS buffer, pH 7.0, the addition of 0.02 M sodium citrate alone had

TABLE 2. *Effect of different cations on the cycloheximide inhibition of L-leucine-^{14}C transport activity*[a]

Addition	L-Leucine-^{14}C transport rate (μmol per g per min)	
	Minus cycloheximide	Plus cycloheximide (10^{-3} M)
0.02 M Phosphate	4.1	0.6
+ 1 mM $CaCl_2$	3.6	2.6
+ 1 mM $BaCl_2$	4.0	3.2
+ 1 mM $SrCl_2$	4.2	1.7
+ 1 mM $MgCl_2$	3.7	1.8
+ 1 mM $MnCl_2$	2.8	1.2
+ 1 mM KCl	4.0	0.5
+ 10 mM Aniline	5.2	1.9
+ 10 mM Aniline + 1 mM $CaCl_2$	5.0	3.7
0.05 M Phosphate	4.3	0.8
+ 1 mM $CaCl_2$	4.3	1.3

[a] Nitrogen-starved mycelium were suspended at a density of 2 g/100 ml in phosphate buffer, pH 7. After 15 min of aeration, the labeled substrate was added at an initial concentration of 2×10^{-5} M. Cycloheximide was added 1 min before the labeled substrate. The salts were added 10 s before the labeled substrate. Aniline was added 15 min before the labeled substrate.

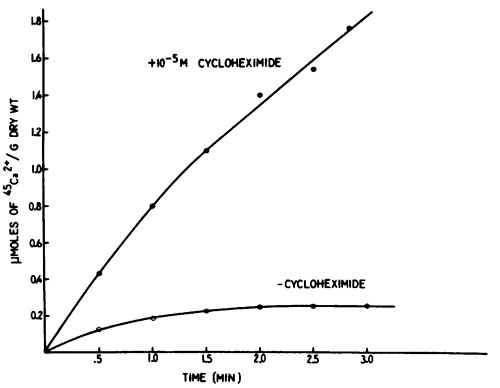

FIG. 1. *Nitrogen-starved mycelium was washed and resuspended in 0.02 M K MOPS buffer, pH 7.0. After 15 min of aeration, $^{45}Ca^{2+}$ was added at an initial concentration of 2×10^{-5} M. Cycloheximide was added 2 min before adding the labeled substrate. Periodically, 5-ml samples were filtered, washed with cold 10^{-3} M unlabeled $CaCl_2$, and counted for radioactivity.*

little effect, but 0.02 M sodium citrate plus 10^{-5} M cycloheximide inhibited 56%. Similarly, 0.02 M ethylenediaminetetraacetic acid (EDTA) alone had no effect, whereas 0.02 M EDTA plus 10^{-5} M cycloheximide inhibited 16%. In the same experiment, 0.02 M potassium phosphate plus 10^{-5} M cycloheximide inhibited 63%. $Citrate^{3-}$ is a better chelator of Ca^{2+} than HPO_4^{2-}, whereas EDTA is a far better chelator than either $citrate^{3-}$ or HPO_4^{2-} (5). However, the relative effectiveness as transport inhibitors would depend on the ability of the chelator to penetrate the membrane to the site of the bound Ca^{2+}. In this respect, it is unlikely that the mycelium possesses a specific binding protein for EDTA. Citrate transport was not measured, but we do know that *P. chrysogenum* (strain PS-75) will not grow in media containing citrate as the sole carbon source.

Time and concentration dependence of the cycloheximide effect. Figure 2 shows the time course of the cycloheximide inhibition of L-leucine transport from phosphate buffer. The experiments with cycloheximide described above were all performed within 1 min after adding the antibiotic. During the first few minutes, the higher cycloheximide concentration causes a faster decline in specific transport activity, which is not unexpected. We have no satisfactory explanation for the inverse relationship between specific transport activity and cycloheximide concentration that is observed after longer incubation times. Evans (6) reported an identical inverse relationship for the effect of cycloheximide on the uptake of 2,4-dinitrophenol, phenylalanine, and glucose by *Euglena*.

If the cycloheximide is washed away before the transport assay is performed, a substantial fraction of the initial rapid inhibition is eliminated (Fig. 3). The continual decrease in specific transport activity from about 2 min on very likely results from turnover of a permease component, i.e., the long-term effect of cycloheximide noted by several workers (7, 10, 12, 16).

DISCUSSION

The effects of cycloheximide, phosphate, and Ca^{2+} on membrane transport were observed separately by other workers. For example, Evans (6) showed that low concentrations of cycloheximide inhibited the transport of L-phenylalanine-^{14}C by *Euglena*. The incubation medium used contained 1.1 mM phosphate and 17.6 mM citrate. Evans (6) suggested that cycloheximide might induce a redistribution of intracellular ions, resulting in a disruption of a pH gradient across the membrane. There is some evidence that a proton or charge gradient is involved in amino acid transport in *P. chrysogenum* (11). Reilly, Furhmann, and Rothstein (13) showed that cycloheximide (0.1–2.0 mM) inhibits K^+ transport by *Saccharomyces carlsbergensis*. The experiments

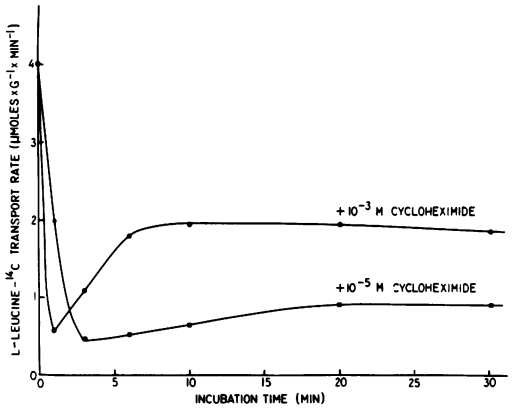

FIG. 2. *A suspension of nitrogen starved mycelium (2 g [wet weight]/100 ml) in 0.02 M phosphate buffer, pH 7, was divided into two parts. Cycloheximide was added to final concentrations of 10^{-5} M or 10^{-3} M. Periodically, samples were removed and assayed immediately for L-leucine-^{14}C transport activity in the same incubation medium. The initial L-leucine-^{14}C concentration was 2×10^{-5} M. The transport assay took 1 min to perform (9).*

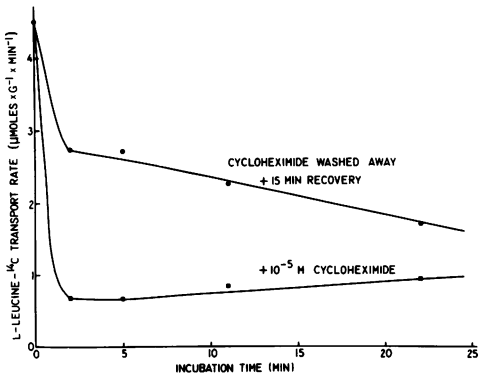

FIG. 3. *Cycloheximide (10^{-5} M) was added to a suspension of nitrogen-starved mycelium in 0.02 M phosphate buffer, pH 7. At the times indicated, two samples were removed. One sample was assayed immediately for L-leucine transport at 2×10^{-5} M L-leucine-^{14}C. The mycelium in the other sample was filtered off, washed, and resuspended in fresh phosphate buffer. After 15 min of aeration, the L-leucine-^{14}C transport activity was assayed.*

were performed in phosphate buffer. Cameron and LeJohn (4) presented strong evidence that Ca^{2+} is involved in amino acid transport by the fungus *Achlya*. These workers reported that cycloheximide had no effect on amino acid transport. However, the uptake assay was performed in a tris(hydroxymethyl)aminomethane acetate buffer that did not contain phosphate or citrate. The cumulative results suggest that membrane-bound Ca^{2+} may be directly involved in transport (e.g., as a contributor to a charge gradient that energizes membrane transport or as an activator of a membrane adenosine triphosphatase). Thus, phosphate, citrate, or any other Ca^{2+} chelator that can penetrate the membrane will act as a transport inhibitor. Somehow, cycloheximide makes the membrane-bound Ca^{2+} more available for chelation. Alternately, Ca^{2+} may be involved in a passive way (e.g., the ion may play a role in stabilizing the cell membrane). The removal of Ca^{2+} may result in ion movements that destroy a proton or charge gradient driving force. The mechanism of the anti-cycloheximide action of aniline is unknown. The unionized species (which is lipid soluble), may diffuse into a proton-rich compartment within the membrane where it becomes trapped as the R-NH_3^+ form. The positive species may then contribute to a charge gradient (replacing the lost Ca^{2+}) or, alternately, act as a proton reservoir (in the event that a proton gradient is involved in energizing amino acid transport in *P. chrysogenum*).

An unusual inverse relationship between cycloheximide concentration and transport inhibition is observed after longer incubation. It appears that at 10^{-3} M cycloheximide, the mycelium can recover partially from the initial inhibition, but at 10^{-5} M cycloheximide no recovery occurs (Fig. 2). At present, we have no satisfactory explanation for this phenomenon. Evans (6) reported a similar inverse effect. Cycloheximide at 3.6 μM significantly inhibited L-phenylalanine-^{14}C transport by *Euglena*, whereas 360 μM cycloheximide had relatively little effect. Both concentrations inhibited protein synthesis.

ACKNOWLEDGMENTS

We thank Shelley Siegel for her assistance in various phases of this research.

This investigation was supported by National Science Foundation grant GB-19243. D.R.H. is a Public Health Service predoctoral trainee under grant GM 119-13 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Bellenger, N., P. Nissen, T. C. Wood, and I. H. Segel. 1968. Specificity and control of choline-O-sulfate transport in filamentous fungi. J. Bacteriol. **96**:1574–1585.
2. Benko, P. V., T. C. Wood, and I. H. Segel. 1970. Multiplicity and regulation of amino acid transport in *Penicillium chrysogenum*. Arch. Biochem. Biophys. **129**:498–508.
3. Benko, P. V., T. C. Wood, and I. H. Segel. 1967. Specificity and regulation of methionine transport in filamentous fungi. Arch. Biochem. Biophys. **122**:783–804.
4. Cameron, L. E., and H. B. LeJohn. 1972. On the involvement of calcium in amino acid transport and growth of the fungus *Achlya*. J. Biol. Chem. **247**:4729–4739.
5. Dawson, R. M. C., D. Elliott, W. H. Elliott, and K. M.

Jones (ed.) 1969. Data for biochemical research, 2nd ed. Oxford University Press.
6. Evans, W. R. 1971. The effect of cycloheximide on membrane transport in *Euglena*. J. Biol. Chem. **246**:6144–6151.
7. Grenson, M., M. Crabeel, J. M. Wiame, and J. Bechet. 1968. Inhibition of protein synthesis and stimulation of permease turnover in yeast. Biochem. Biophys. Res. Commun. **30**:414–419.
8. Hackette, S. L., G. E. Skye, C. Burton, and I. H. Segel. 1970. Characterization of an ammonium transport system in filamentous fungi with methyl-ammonium-^{14}C. J. Biol. Chem. **245**:4241–4250.
9. Hunter, D. R., and I. H. Segel. 1971. Acidic and basic amino acid transport systems in *Penicillium chrysogenum*. Arch. Biochem. Biophys. **144**:168–183.
10. Hunter, D. R., and I. H. Segel. 1973. Control of the general amino acid permease of *Penicillium chrysogenum* by transinhibition and turnover. Arch. Biochem. Biophys. **154**:387–399.
11. Hunter, D. R., and I. H. Segel. 1973. Effect of weak acids on amino acid transport by *Penicillium chrysogenum*: evidence for a proton or charge gradient as the driving force. J. Bacteriol. **113**:1184–1192.
12. Marzluf, G. A. 1972. Control of the synthesis, activity, and turnover of enzymes of sulfur metabolism in *Neurospora crassa*. Arch. Biochem. Biophys. **150**:714–724.
13. Reilly, C., G. Fuhrmann, and A. Rothstein. 1970. The inhibition of K^+ and phosphate uptake in yeast by cycloheximide. Biochim. Biophys. Acta **203**:583–585.
14. Siegel, M. R., and H. D. Sisler. 1963. Inhibition of protein synthesis *in vitro* by cycloheximide. Nature (London) **200**:675–676.
15. Skye, G. E., and I. H. Segel. 1970. Independent regulation of cysteine and cystine transport in *Penicillium chrysogenum*. Arch. Biochem. Biophys. **138**:306–318.
16. Wiley, W. R., and W. H. Matchett. 1968. Tryptophan transport in *Neurospora crassa*. II. Metabolic control. J. Bacteriol. **95**:959–966.