ably because it fails to cross the blood-brain barrier. With appropriate chemical modification, DALA could, conceivably, give rise to a totally synthetic opiate peptide analgesic which would be active after systemic administration.

It has long been known (*19*) that introduction of unnatural, D-amino acids into peptides can convey resistance to degradative enzymes. This principle has recently been applied to produce a potent analog of luteinizing hormone releasing hormone (LHRH) by substitution of Gly^6 with D-Ala^6 (*20*), resulting in resistance to enzymatic degradation (*21*). Presumably, the methyl group of the D-alanine side chain of DALA is positioned so that opiate receptor recognition is relatively unhindered while enzymatic access to the critical Tyr^1-Gly^2 bond is blocked. In support of this explanation, a number of additional enzyme-resistant DALA analogs have been synthesized which contain position-2 substitutions by other D-amino acids, L-proline and sarcosine (*22*).

Note added in proof: Also, a recent report of chemical analysis of enzymatic breakdown products of enkephalin (*23*) suggests that cleavage of the Tyr^1-Gly^2 amide bond is the initial deactivation step.

CANDACE B. PERT
AGU PERT

Section on Biochemistry,
Adult Psychiatry Branch,
National Institute of Mental Health,
Bethesda, Maryland 20014

JAW-KANG CHANG
BOSCO T. W. FONG

Bioproducts Department,
Beckman Instruments, Inc.,
Palo Alto, California 94304

References and Notes

1. C. B. Pert and S. H. Snyder, *Science* **179**, 1011 (1973); L. Terenius, *Acta Pharmacol. Toxicol.* **33**, 377 (1973); E. Simon, J. Hiller, I. Edelman, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1947 (1973).
2. S. H. Snyder, *Nature (London)* **257**, 185 (1975); C. B. Pert, in *Cell Membrane Receptors for Viruses, Antigens and Antibodies, Polypeptide Hormones and Small Molecules*, R. F. Beers and E. G. Bassett, Eds. (Raven, New York, 1976), p. 435.
3. J. Hughes, *Brain Res.* **88**, 295 (1975); G. W. Pasternak, R. Goodman, S. H. Snyder, *Life Sci.* **16**, 13 (1975); L. Terenius and A. Wahlstrom, *Acta Physiol. Scand.* **35**, 55 (1975).
4. B. M. Cox, K. E. Opheim, H. Teschemacher, A. Goldstein, *Life Sci.* **16**, 25 (1975); R. Guillemin, N. Ling, R. Burgus, *C. R. Acad. Sci. Ser. D* **282**, 523 (1976); C. H. Li and D. Chung, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1145 (1976).
5. L. Terenius and A. Wahlstrom, *Life Sci.* **16**, 7 (1975).
6. C. B. Pert, A. Pert, J. Tallman, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2226 (1976).
8. J. Hughes, T. W. Smith, J. W. Kosterlitz, L. A. Fothergill, B. A. Morgan, H. R. Morris, *Nature (London)* **258**, 577 (1975).
9. R. Simantov and S. H. Snyder, *Life Sci.* **18**, 781 (1976).
10. J. D. Beluzzi, N. Grant, V. Garsky, D. Sarantakis, C. D. Wise, L. Stein, *Nature (London)* **260**, 604 (1976).
11. J. K. Chang, B. T. W. Fong, A. Pert, C. B. Pert, *Life Sci.* **18**, 1473 (1976).
12. C. B. Pert and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2243 (1973). The abbreviations for the amino acid residues in these peptides are: Tyr, tyrosine; Gly, glycine; Phe, phenylalanine; Met, methionine; Leu, leucine; also, K_D, dissociation constant.
13. F. E. D'Amour and D. L. Smith, *J. Pharmacol. Exp. Ther.* **72**, 74 (1941).
14. W. O. Evans, *Psychopharmacologia* **2**, 318 (1961).
15. A. Herz, K. Albus, J. Metys, P. Shubert, H. Teschemacher, *Neuropharmacology* **9**, 539 (1970); K. Tsou and C. S. Jang, *Sci. Sin.* **7**, 1099 (1964); Y. F. Jacquet and A. Lajtha, *Science* **182**, 490 (1973).
16. A. Pert and T. L. Yaksh, *Brain Res.* **80**, 135 (1974).
17. J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis* (Freeman, San Francisco, 1969). The relative amino acid contents were as follows: Met-enkephalin, Gly 1.96, Met 1.01, Tyr 1.00; [D-Ala^2]-Met-enkephalinamide, Met 1.00, Gly 1.03, Ala 0.95, Tyr 0.98, Phe 1.04, NH_3, 0.98; and [L-Ala^2]-Met-enkephalinamide, Gly 1.03, Ala 1.00, Met 0.97, Tyr 0.98, Phe 1.03, NH_3, 0.99. Thin-layer chromatography was performed in three solvent systems: (i) chloroform, methanol, ammonium hydroxide (60 : 30 : 5), silica gel; (ii) *n*-butyl alcohol, acetic acid, water (4 : 1 : 5), upper layer, cellulose; and (iii) *n*-butyl alcohol, pyridine, acetic acid, water (15 : 10 : 3 : 12), silica gel [high-voltage electrophoresis (4000 volts, 1 hour, pyridine acetate buffer, *p*H 5.6, Whatman No. 1 paper) in which all of the peptides moved toward the cathode, with Met-enkephalin being the internal standard]. The results of the above analyses were: For Met-enkephalin, R_F in solvent (i) was 0.52, in solvent (ii) it was 0.81, and in solvent (iii) it was 0.70; the relative electrophoretic mobility was 1.00. For [D-Ala^2]-Met-enkephalinamide, the R_F in solvent (i) was 0.85, in solvent (ii) it was 0.82, and in solvent (iii) it was 0.77; the electrophoretic mobility was 2.64. For [L-Ala^2]-Met-enkephalinamide the R_F in solvent (i) was 0.85, in solvent (ii) it was 0.78, and in solvent (iii) it was 0.74; the electrophoretic mobility was 2.61.
18. C. B. Pert and S. H. Snyder, *Mol. Pharmacol.* **10**, 868 (1974); ———, E. L. May, *J. Pharmacol. Exp. Ther.* **196**, 316 (1976); R. Wilson *et al.*, *J. Med. Chem.* **18**, 240 (1975).
19. T. Bersin, in *Handbuch der enzymologie*, F. F. Nord and R. Weidenhagen, Eds. (Akademische Verlagsgesellschaft, Leipzig, 1940), p. 599.
20. M. W. Monahan, M. Amoss, H. A. Anderson, W. Vale, *Biochemistry* **12**, 4619 (1973).
21. N. Marks and F. Stein, *Biochem. Biophys. Res. Commun.* **61**, 1458 (1974).
22. C. B. Pert, D. L. Bowie, B. T. W. Fong, J. K. Chang, in *Opiates and Opioid Peptides, Proceedings of the Fifth Meeting of the International Narcotics Research Conference, Aberdeen, Scotland*, H. Kosterlitz, S. Archer, E. J. Simon, A. Goldstein, Eds. (Elsevier, Amsterdam, in press).
23. J. M. Hambrook, B. A. Morgan, M. J. Rance, C. F. C. Smith, *Nature (London)* **262**, 783 (1976).
24. We thank D. L. Bowie for biochemical technical assistance and L. Berthold and B. E. Jewett for surgical assistance. Address correspondence to C.B.P., Building 10, Room 2N315, National Institute of Mental Health, Bethesda, Maryland 20014.

14 June 1976; revised 10 August 1976

Ascorbic Acid as a Factor Controlling the Development of Cyanide-Insensitive Respiration

Abstract. *Lycorine, an inhibitor of ascorbic acid biosynthesis, prevents the elicitation of KCN-insensitive respiration in aerated potato tuber slices. Ascorbic acid administration prevents the lycorine effect. Because KCN-insensitive respiration, which takes place during the aerobic incubation of potato tuber slices, specifically depends on the synthesis of new proteins, we suggest that ascorbic acid could be required to carry out a synthetic process.*

While the role of many vitamins in cell metabolism is now recognized at the molecular level, a considerable amount of uncertainty still exists with regard to that of vitamin C (ascorbic acid). Our work suggests an alternative approach to the definition of the role of ascorbic acid (AA) in cell metabolism.

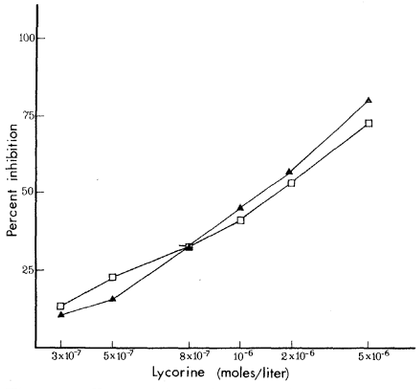

Fig. 1. Effect of lycorine on biosynthesis of ascorbic acid (AA) (□) and on the development of KCN-insensitive respiration (▲). The AA biosynthesis was evaluated as described (*1*, *15*).

Lycorine, an alkaloid extracted from Amarillidaceae, is a quite specific inhibitor of AA biosynthesis (*1*). The physiological effects induced by lycorine in both plants (*2*) and animals (*3*) are probably due to this peculiar action. Therefore lycorine appears to be a good tool to determine what metabolic reactions in the cell are directly related to AA variations. We present data showing that the development of cyanide-insensitive respiration is a process closely controlled by AA.

Potato tuber slices aerobically maintained show a strong increment of oxygen uptake within a day after cutting, and the respiration becomes relatively insensitive to carbon monoxide and to 1 m*M* cyanide (*4*). The rate of respiration in "fresh" slices (that is, slices used 30 minutes after cutting) is roughly 20 to 30 μl of O_2 per hour per gram of fresh tissue and rises in "activated" slices (*5*) (those used after a day's aeration) to 100 to 120

Table 1. Changes in the respiratory rate during the aerobic incubation of potato tuber slices. Respiratory rates were monitored following the reduction of tetrazolium salts. Because of the presence of phenol oxidase, this technique prevents interference from AA (if added). TTC and NBT (*12*), which are good electron acceptors (*13, 14*), were used. With this procedure, similar to Warburg manometric technique, a fivefold increase of respiration was found. Cylinders of potato (*Solanum tuberosum* L.) tissue, 0.9 cm in diameter, were removed with a cork borer and sliced on a sliding microtome. The slices, 1 mm thick, were washed repeatedly in tap water and either used immediately ("fresh" slices) or maintained in water at 20°C for 24 hours, with air bubbled into the medium ("activated" slices). TTC and NBT reduction were assayed in small petri dishes maintained in vacuum for 60 minutes (with fresh slices) and 30 minutes (with activated slices). Each dish contained 15 slices and 15 ml of a solution of 0.04*M* phosphate buffer (*p*H 7.0), 1 percent TTC, or 0.25 percent NBT. The $TTCH_2$ and $NBTH_2$ were estimated according to Marrè and Arrigoni (*13*) and Lester and Smith (*14*), respectively. Weights are fresh weights.

O_2 (μl/g per hour)	$TTCH_2$ (μg/g per hour)	$NBTH_2$ (μg/g per hour)
	Fresh slices	
23*	48	118
	Activated slices	
110*	264	620

*Data obtained by using Warburg manometric technique.

μl of O_2 per hour per gram (Table 1). In fresh slices, respiration is almost completely inhibited by cyanide and in activated slices is only 25 to 35 percent inhibited. Hence, the term "cyanide-insensitive respiration" has been used.

During the period of aeration, the slices, while developing increased respiration, actively synthesize AA (*1, 6*). Adding lycorine inhibits AA biosynthesis (*1*) and, at the same time, prevents the development of respiration (Table 2). At $5 \times 10^{-6}M$, the increase of respiration is almost completely inhibited. The similarity between the degree of inhibition of AA biosynthesis and that of the respiratory rise (Fig. 1) suggests that AA is required to develop KCN-insensitive respiration. Further support to this conclusion was obtained from the demonstration that the administration of AA prevents lycorine inhibition in slices of stored tubers. In fact, the 60 percent inhibition of respiration induced by 2 μM lycorine is wholly prevented when 1 m*M* AA is also added to the slices during the activation period.

Still other data support this assumption. When slices from newly harvested potatoes are used, respiration rate increases 11-fold, whereas it increases 5.3-fold in stored potato slices (Table 3); adding 2 μM lycorine inhibits only 12 percent in the former and almost 60 percent in the latter.

Such diverse behavior of the two groups of slices can be accounted for by their different AA plus dehydroascorbic acid (DHA) contents. Slices from newly harvested potatoes contain much more AA than stored ones (Table 3), which is the reason for their low sensitivity to lycorine and for the strong rise in respiration.

The close relation between AA content and the development of cyanide-insensitive respiration enables us to suggest that AA could be required for synthesis of proteins related to KCN-insensitive respiration. Click and Hackett (*5*) showed that the rise of respiration in potato slices depends on newly synthesized RNA and proteins; in fact, both actinomycin D and puromycin prevent the development of cyanide-insensitive respiration. Because lycorine also inhibits the increase of respiration while AA prevents this effect, it may be suggested that AA is somehow involved in the synthesis of the proteins linked to the development of cyanide-insensitive respiration. In accordance with this, we reported earlier (*7*) that lycorine inhibits incorporation of [^{14}C]leucine in vivo, but that its effect on protein synthesis was indirect in that isolated polysomes were found to be lycorine-insensitive. Further, these data support the view that the effects of lycorine in both plants and animals are mediated by the AA system (*1, 8*).

KCN-insensitive respiration is not a peculiar process of the plant cell, but occurs in animal cells as well (*9*). Human granulocytes generate the superoxide necessary for phagocytic killing through a cyanide-insensitive respiration (*10*). Phagocytosis by granulocytes decreases when the AA content in the blood is low (*11*). We have some data showing that lycorine inhibits phagocytosis in rats. We therefore suggest that AA plays a role in the biosynthesis of the proteins (probably through proline hydroxylation) related to KCN-insensitive respiration in both plants and animals.

Table 2. Effect of lycorine on development of respiration in slices from stored potato tubers (maintained at 10°C). Potato slices were kept in water or in lycorine for 24 hours. At the end of this period TTC reduction was evaluated. Lycorine did not affect the rate of respiration directly in either "fresh" or "activated" slices. In fact, when lycorine was added to the reaction medium during tetrazolium salts reduction in vacuum, no difference was observed in the amount of $TTCH_2$ formed. Weights are fresh weights.

Lycorine (*M*)	$TTCH_2$ (μg/g per 30 minutes)	Percent inhibition
	Fresh slices	
None	23	None
	Activated slices	
Water only	122	None
3×10^{-7}	109	11
5×10^{-7}	96	21
10^{-6}	67	45
2×10^{-6}	52	57
5×10^{-6}	25	80

Table 3. Relation between the AA plus DHA content and the development of KCN-insensitive respiration in potato tuber slices. The AA was assayed by the 2,4-dinitrophenylhydrazine reaction (*1, 15*); weights are fresh weights.

AA + DHA content (μg/g)	$TTCH_2$ (μg/g per 30 minutes)		Increment of respiration
	Fresh slices	Activated slices	
	Stored potatoes		
85	26.2	138	5.3-fold
	Newly harvested potatoes		
335	30.2	334	11-fold

ORESTE ARRIGONI
ROSALIA ARRIGONI-LISO
GIUSEPPE CALABRESE

Institute of Botany,
University of Bari,
70126 Bari, Italy

References and Notes

1. O. Arrigoni, R. Arrigoni-Liso, G. Calabrese, *Nature (London)* **256**, 513 (1975).
2. P. De Leo, G. Dalessandro, A. De Santis, O. Arrigoni, *Plant Cell Physiol.* **14**, 481 (1973).
3. T. Mineshita, K. Yamaguchi, K. Takeda, K. Kotera, *Annu. Rep. Shionogi Res. Lab.* **6**, 119 and 131 (1956).
4. K. V. Thimann, C. S. Yocum, D. P. Hackett, *Arch. Biochem. Biophys.* **53**, 239 (1954); D. P. Hackett, D. W. Haas, S. H. Griffiths, D. J. Niederpruem, *Plant Physiol.* **35**, 8 (1960).
5. R. E. Click and D. P. Hackett, *Proc. Natl. Acad. Sci. U.S.A.* **50**, 243 (1963).
6. F. S. Smith, *Plant Physiol.* **27**, 736 (1952).
7. P. De Leo, G. Dalessandro, A. De Santis, O. Arrigoni, *Plant Cell Physiol.* **14**, 487 (1973).
8. R. Liso and G. Calabrese, *Phycologia* **14**, 9 (1975).
9. I. Fridovich, in *Horizons in Biochemistry and Biophysics*, E. Quagliarello, F. Palmieri, T. P. Singer, Eds. (Addison-Wesley, Reading, Mass., 1974), vol. 1, pp. 1–37.
10. B. M. Babior, R. S. Kipnes, J. T. Curnutte, *J. Clin. Invest.* **52**, 741 (1973).
11. G. A. Goldsmith, *Ann. N.Y. Acad. Sci.* **92**, 230 (1961); P. Barkhan and K. R. Howard, *Biochem. J.* **70**, 163 (1958).
12. TTC, 2,3,5-triphenyltetrazolium chloride; NBT, 2,2-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)-ditetrazolium chloride.
13. E. Marrè and O. Arrigoni, *Nuovo G. Bot. Ital.* **61**, 21 (1954).
14. R. Lester and A. Smith, *Biochim. Biophys. Acta* **47**, 475 (1961).
15. J. H. Roe and M. J. Oesterling, *J. Biol. Chem.* **152**, 511 (1944).
16. Supported by the Consiglio Nazionale delle Ricerche, Italy.

31 October 1975; revised 27 April 1976