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Synthesis of Malformin by an Enzyme Preparation from *Aspergillus niger*

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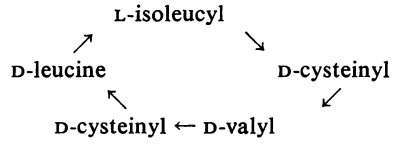
ABSTRACT

YUKIOKA, M. (University of Hawaii, Honolulu), AND T. WINNICK. Synthesis of malformin by an enzyme preparation from *Aspergillus niger*. J. Bacteriol. **91**:2237–2244. 1966.—An enzyme fraction derived from disrupted *Aspergillus* cells was able to utilize each of the component labeled amino acids of malformin for the synthesis of this cyclic pentapeptide. The process was stimulated by adenosine triphosphate, K^+, and Mg^{++}, and was optimal at approximately *p*H 8.5. It was not affected by inhibitors of protein synthesis (ribonuclease, chloramphenicol, puromycin). There is evidence that cysteine, rather than cystine, was incorporated into peptide linkage, so that the disulfide bridge of malformin was formed subsequently. Although only the D isomers of cysteine and leucine occur in the malformin molecule, the L, as well as the D form of these amino acids, was readily utilized by the enzyme preparation. As in the case of several other microbial peptide systems, it appears that the D enantiomorph can arise from the L isomer at an intermediate stage of polypeptide synthesis.

A series of elegant studies by Curtis and associates have elucidated the structure and biological properties of malformin of *Aspergillus niger* (1). The major A component of this interesting cyclic pentapeptide has the structure:



The biologically active form of the molecule has a disulfide bridge between the two half-cystine residues.

In a preliminary study of the biogenesis of malformin (Yukioka and Winnick, Biochim. Biophys. Acta, *in press*), it was observed that washed mycelium of *Aspergillus* was able to incorporate suitable labeled amino acids efficiently into the peptide molecule, as well as into protein. Procedures were described for the identification of the isolated radioactive malformin. The present paper is concerned with the characterization of this biosynthetic process with an enzyme (protein) fraction, derived from disrupted *A. niger* cells.

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MATERIALS AND METHODS

Radioactive amino acids. The following compounds were supplied by New England Nuclear Corp., Boston, Mass., with the indicated specific activities (millicuries per millimole): DL-cystine-*1*-C^{14}, 6; DL-valine-*1*-C^{14}, 10; L-leucine-*1*-C^{14}, 24; D-leucine-*1*-C^{14}, 25; DL-leucine-*1*-C^{14}, 23; L-isoleucine-*1*-C^{14}, 30; DL-phenylalanine-*1*-C^{14}, 6; L-alanine-*1*-C^{14}, 6; uniformly labeled L-proline-C^{14}, 200; DL-valine-*3,4*-H^3, 137. L-Tryptophan-*1*-C^{14} and L-glutamic acid-*1*-C^{14}, each with 7 mc/mmole, were obtained from Calbiochem.

Biochemicals. Chloramphenicol was purchased from Parke, Davis & Co., Detroit, Mich., and puromycin from Nutritional Biochemicals Corp., Cleveland, Ohio. Crystalline pancreatic ribonuclease was supplied by Sigma Chemical Co., St. Louis, Mo.

Preparation of DL-*cysteine-1-*C^{14}. A 50-μc amount of DL-cystine-*1*-C^{14} was treated with 2 mg of sodium borohydride in 5 ml of water for 3 hr at 37 C. Excess borohydride was destroyed by the addition of 5 ml of acetone. The solution was evaporated to dryness, and the residue was dissolved in a small volume of water. A sample of this solution was analyzed by paper chromatography, and the radioactive component was shown to have the correct R_F of 0.44 for cysteine, with butanol-propanol–0.1 N HCl (1:1:1) as solvent. (The R_F for cystine was 0.15.)

A. niger culture. Strain 58-883 of the organism was cultivated at 25 C in rotatory shaking culture as previously described (Yukioka and Winnick, *in press*).

Preparation of enzyme fraction. Mycelium was separated by filtration from 800 ml of harvested

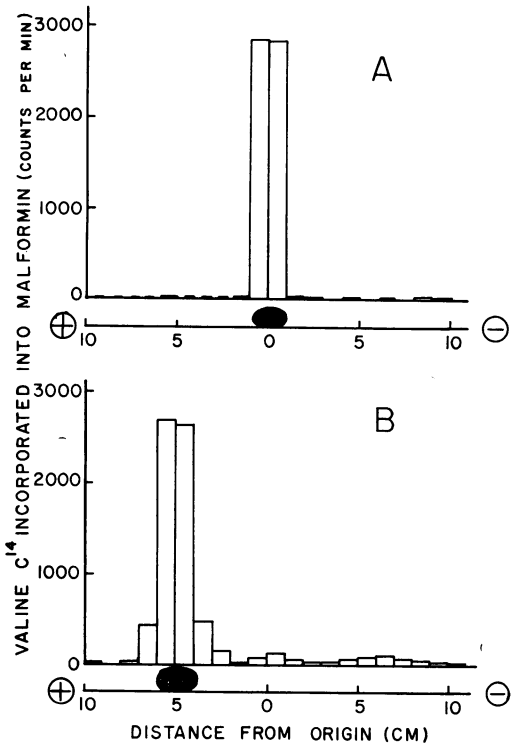

FIG. 1. *Paper electrophoresis of radioactive malformin: (A) before, and (B) after oxidation with performic acid. The solid ovals show the positions of the —S—S— and the —SO_3H forms of the polypeptide, revealed by colorimetric tests. A current of 1,200 v was applied for 3 hr. The "buffer" employed was 50% acetic acid.*

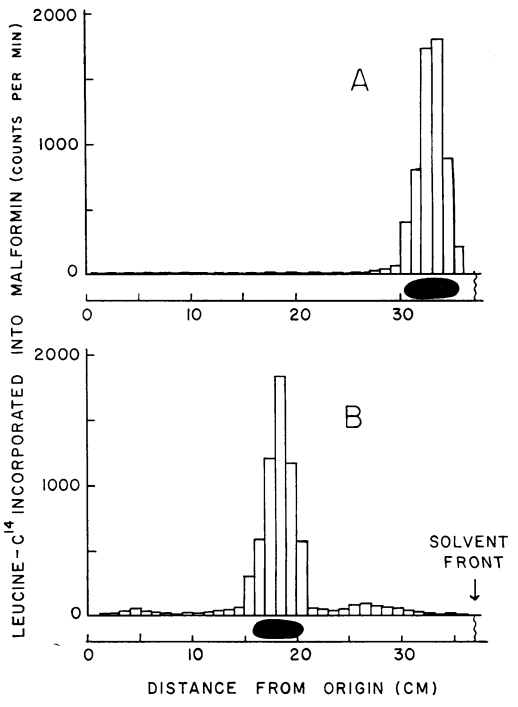

FIG. 2. *Paper chromatography of radioactive malformin: (A) before, and (B) after performic oxidation. The solvent was isoamyl alcohol-pyridine-water* (1:1:1).

Aspergillus culture, and was washed with 2 liters of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-Cl buffer (*p*H 7.0). The subsequent steps were performed at approximately 2 C. The mycelium was suspended in 500 ml of the above buffer, and passed through a French press at a pressure of 1,500 psi. (American Instrument Co., Inc., Silver Spring, Md.) The resulting extract was centrifuged for 15 min at 30,000 × *g*. The precipitate was discarded. The supernatant solution was saturated with solid $(NH_4)_2SO_4$, while maintaining a *p*H of 7.0 by the addition of 6 N NH_4OH.

The protein precipitate was collected by centrifugation (about 15 min at 10,000 × *g*). It was then dissolved in 100 ml of 0.1 M Tris-Cl buffer (*p*H 8.5), and dialyzed overnight in a cellophane tube (2-cm diameter) against two 2-liter volumes of the same buffer solution. The protein went completely into solution during this process, and no detectable residue remained. This solution, containing 3 to 4 mg of protein per ml, served as the enzyme system for malformin synthesis.

Assay procedure. The standard incubation mixture contained the following in a total volume of 2 ml: 1 ml of enzyme solution, 5 μmoles of phosphoenolpyruvate (PEP), 20 μg of pyruvic kinase, 150 μmoles of Tris (*p*H 8.5), 200 μmoles of KCl, 20 μmoles of magnesium acetate, 5 μmoles of ATP, 1 μmole of DL-leucine, 1 μmole of L-isoleucine, 2 μmoles of DL-cysteine, and 5 μc of DL-valine-H^3. In some experiment, C^{14}-labeled isoleucine, leucine, and other amino acids were used in place of radioactive valine at indicated levels of radioactivity. In these cases, the corresponding nonisotopic amino acid was omitted from the reaction mixture, and 1 μmole of L-valine was included.

After a reaction period of 5 hr at 37 C, the *p*H of the system was lowered to 3.0 by the addition of 0.2 ml of 1 N HCl. A 5-ml amount of ethyl alcohol was added, and the mixture was allowed to stand for 2 to 12 hr at room temperature. After brief centrifugation (at about 5,000 × *g*), the supernatant fluid was removed, and the precipitated protein was suspended in 5 ml of ethyl alcohol and recentrifuged. The combined supernatant fluids were evaporated to dryness at 40 to 50 C with the aid of an air stream. The residue was dissolved in 5 ml of water and applied to a column (0.9 by 3.0 cm) packed with a charcoal (Merck NF 18351)-Celite (The Eagle-Picher Co., Cincinnati, Ohio) mixture in proportions of 1:9 by weight. Subsequently, the column was washed with 20 ml of water (under gravity), and the effluent was discarded. The malformin fraction was completely eluted by 5

TABLE 1. *Importance of individual constituents of incubation mixture*

Component omitted from standard reaction mixture	Valine-H^3 incorporated into malformin†
	count/min
None (control)	10,800
KCl	3,900
Mg^{++}	4,700
Nonisotopic amino acid mixture	600
ATP	7,500
PEP, pyruvic kinase	7,200
ATP, PEP, pyruvic kinase	1,600
ATP; GTP-substituted*	6,200
ATP; UTP-substituted*	6,300
ATP; CTP-substituted*	5,400

* Five μmoles of the indicated nucleoside triphosphate was used in place of ATP.

† All results were corrected for a zero-time value of 200 counts per min.

ml of acetone. The eluate was concentrated to a volume of about 1 ml. The resulting aqueous solution was extracted with 4 ml of ethyl acetate. The organic phase was washed with 1 ml of water and evaporated to dryness. The residue was taken up in about 0.2 ml of ethyl alcohol and transferred to a vial containing scintillation mixture. The radioactivity was then measured in a Packard liquid scintillation counter. In experiments with C^{14}, a Nuclear-Chicago gas-flow Geiger counter was employed.

Chromatography of labeled malformin. To confirm the identity of the radioactive substance isolated in the above procedure, certain preparations were subjected to further purification, both before and after oxidation with performic acid (Yukioka and Winnick, *in press*). Figure 1 gives the results of paper electrophoretic analysis. The radioactive material migrated toward the anode (consistent with the expected conversion of a disulfide bridge to sulfonic acid groups), with the same mobility as authentic oxidized malformin A. By contrast, the untreated substance (without electric charge) failed to migrate.

Similar results were obtained by paper chromatography (Fig. 2). The oxidized radioactive compound had the same R_F as an oxidized malformin standard, about 0.50, whereas the untreated material showed a higher R_F of approximately 0.90, corresponding to dithiol-malformin A.

RESULTS

Essentiality of various components for malformin biosynthesis. The standard value of 10,800 counts per min in Table 1 represents the utilization of approximately 0.4% of the labeled amino acid employed (based on 2.5 μc of tritium-labeled L-valine) for malformin synthesis. It may be seen that the omission of KCl or Mg ions led to a decrease of more than 50% in activity. When DL-cysteine, L-isoleucine, and D-leucine were omitted, the radioactivity recovered in malformin was only about 5% of the control value.

The omission of either adenosine triphosphate (ATP) or the ATP-generating system had relatively little effect. However, when both energy sources were excluded, a marked reduction in malformin synthesis was observed. It may be seen that none of the other nucleoside triphosphates was able to substitute for ATP. It was also found that the addition of a mixture of guanosine triphosphate (GTP), cytosine triphosphate (CTP), and uridine triphosphate (UTP) to the standard system resulted in no increase in activity (data not included in Table 1).

Importance of ATP and metal ions. Figure 3 shows that the optimal level of ATP was about 2 μM, with an apparent inhibiting effect at higher concentrations. The considerable biosynthetic activity in the absence of added ATP reflects pyruvic kinase action (Table 1). Magnesium ions were most effective at a concentration of approximately 10^{-2} M (Fig. 4). In the case of K^+, a higher concentration, in the vicinity of 0.1 M, was needed for optimal activity.

Influence of some general conditions on malformin synthesis. The rate of incorporation of labeled valine into malformin as a function of reaction time is shown in Fig. 5. It may be seen that the rate was most rapid initially, and then decreased gradually during periods of up to 5 hr.

The direct proportionality between the extent of malformin synthesis and the quantity of enzyme employed is shown in Fig. 6. The optimal *p*H of enzyme activity was in the alkaline region, at about 8.5 (Fig. 7).

Some information on the stability of the enzyme preparation is given in Fig. 8. It may be seen that

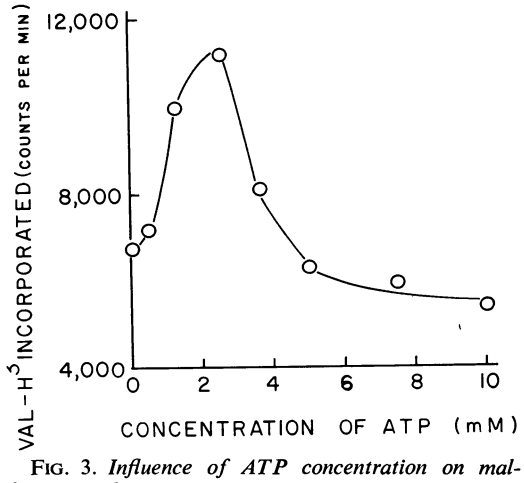

FIG. 3. *Influence of ATP concentration on malformin synthesis.*

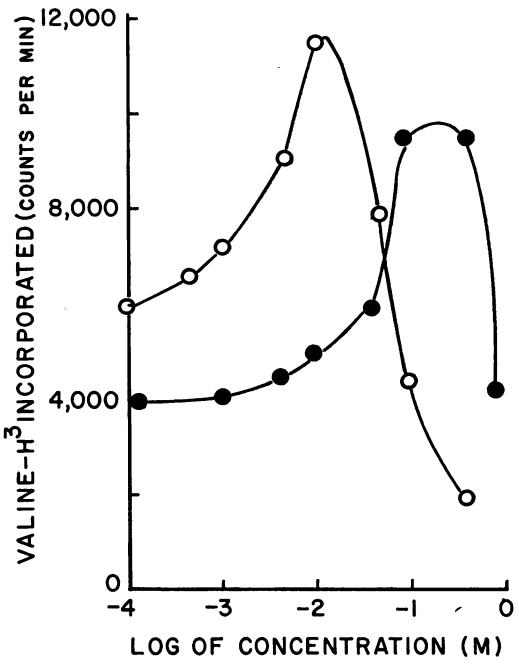

FIG. 4. *Effect of magnesium and potassium ion concentrations on malformin formation. Symbols:* ○, Mg^{++}; ●, K^{+}.

the protein precipitated by $(NH_4)_2SO_4$ had about 85% of its initial biosynthetic activity after storage at −20 C for 7 days. The same preparation, freed from salt by dialysis, was slightly less stable, and retained 70% of its original activity when similarly stored.

Specificity of amino acid incorporation into malformin. As in studies with washed cells (Yukioka and Winnick, *in press*), it was found that the soluble enzyme system of *Aspergillus* utilized the four component amino acids of the malformin molecule to approximately the same extent, and that the uptake of several other amino acids was insignificant (Table 2). These results strongly suggest that the process measured was indeed the formation of the cyclic peptide.

Tests with inhibitors of protein synthesis. It was of interest to try such classic antibiotics as chloramphenicol and puromycin, as well as ribonuclease, to ascertain whether these substances influenced the biosynthesis of malformin (Table 3). It may be seen that none of these agents exerted appreciable effect, even at very high concentrations.

Comparison of the relative utilization of cystine and cysteine for malformin synthesis. The experiments in Table 4 were designed to provide a clue

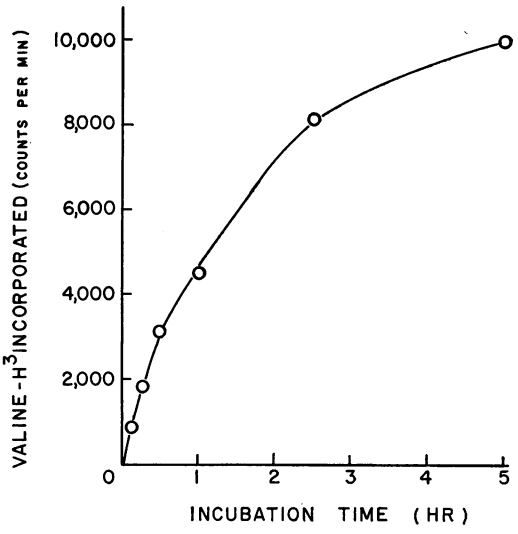

FIG. 5. *Time course of radiovaline incorporation into malformin.*

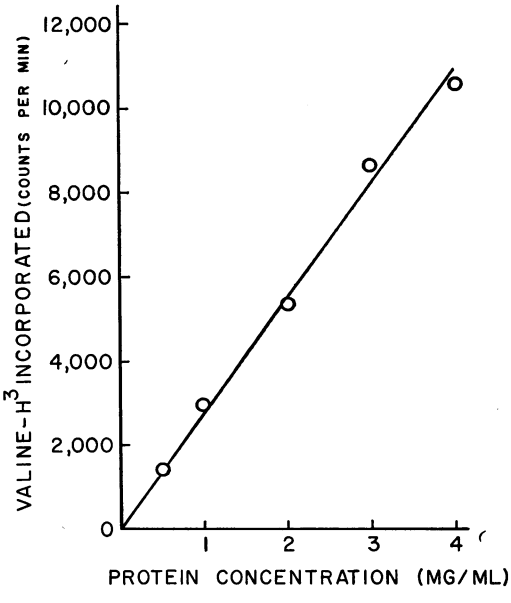

FIG. 6. *Relation of protein (enzyme) concentration to rate of malformin synthesis.*

to the mode of incorporation of the sulfur-containing amino acid residues into the malformin structure. Under the usual conditions of assay, it was found that cysteine was utilized several times more efficiently than was the oxidized dithiol form of the amino acid. By contrast, in the presence of a suitable reducing agent (KCN or β-mercaptoethanol), both forms of the labeled

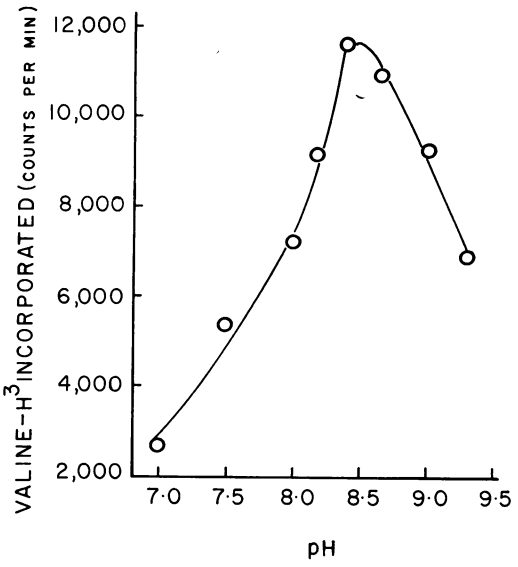

FIG. 7. *Effect of varying pH on malformin synthesis.*

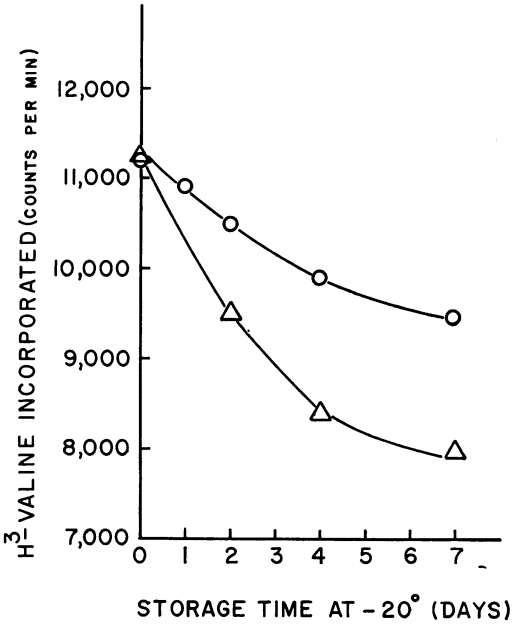

FIG. 8. *Effect of storage at low temperature on the activity of enzyme preparations. Symbols:* ○, *$(NH_4)_2SO_4$ precipitate;* △, *dialyzed protein.*

compound were about equally effective. When the sulfhydryl reagent, iodoacetamide, was present in the reactive mixture, neither cysteine nor cystine was well incorporated. Since sulfhydryl compounds are quite susceptible to oxidation in the presence of oxygen (particularly at alkaline

TABLE 2. *Utilization of various C^{14}-amino acids for malformin synthesis**

Amino acid employed	Radioactivity found in malformin
	count/min
L-Isoleucine	2,870
DL-Leucine	1,670
DL-Valine	1,320
DL-Cystine	2,090
DL-Phenylalanine†	90
L-Proline	50
L-Alanine	30
L-Tryptophan	20
L-Glutamine	50

* Except where otherwise indicated, 1 μc of isotopic compound was used in each experiment.
† A 2-μc amount of labeled amino acid was employed.

TABLE 3. *Attempted inhibition of malformin synthesis by antibiotics and ribonuclease*

Agent tested	Concn in standard assay system	Valine-H^3 incorporated into malformin
	μg/ml	*count/min*
None (control)		16,700
Chloramphenicol	10	17,500
	100	16,500
	1,000	16,600
Puromycin	10	17,000
	100	15,300
	1,000	19,800
Ribonuclease	50	16,000
	500	16,500

*p*H), the experiments were repeated with tubes flushed with nitrogen gas, prior to incubation. However, the results were quite similar to those with aerobic conditions.

Comparison of D- *and* L-*amino acid utilization.* The presence of a D-leucyl and two D-cysteinyl residues in the malformin molecule raises the interesting question of the mode of incorporation of these unusual amino acids.

In preliminary experiments with whole cells of *Aspergillus* (Yukioka and Winnick, *in press*), both stereoisomeric forms of leucine were utilized for malformin synthesis. The same observation was made with the cell-free enzyme system. In Fig. 9A, the rate of incorporation of isotopic D-leucine was strongly depressed by a relatively large excess of nonlabeled L-leucine. By contrast, an excess of D-leucine-C^{12} had relatively

TABLE 4. *Effect of certain reagents, and of anaerobic versus aerobic conditions, on the availability of radioactive cystine and cysteine for malformin synthesis*

Conditions of incubation	Reagent added (10 μmoles /ml)	Isotope incorporated into malformin (count/min)	
		With DL-cystine-C^{14}*	With DL-cysteine-C^{14}*
Air atmosphere	None (control)	170	930
	Mercapto-ethanol	1,350	1,520
	KCN	1,180	1,350
	Iodoacetamide	160	160
N_2 atmosphere	None (control)	140	1,360
	Mercapto-ethanol	1,490	1,600
	Iodoacetamide	140	120

* In each assay, 0.5 μc of labeled amino acid was used.

little effect on the uptake of L-leucine-C^{14} (Fig. 9B).

The separate enantiomorphs of cysteine were not readily obtainable in isotopic form, and hence experiments were performed with the racemic C^{14}-amino acid. Somewhat unexpectedly, it was found that an excess of either D- or L-cysteine-C^{12} severely reduced the utilization of DL-cysteine-C^{14} for malformin synthesis (Fig. 10).

Another approach was to test the effect of varying concentrations of nonisotopic D- and L-cysteine on the incorporation of another component amino acid of the cyclic polypeptide. Figure 11 shows that the utilization of labeled valine was stimulated markedly, and to the same extent, by addition of either D- or L-cysteine-C^{12}.

DISCUSSION

The data in Table 1 indicate the essentiality of ATP as the energy source for peptide bond synthesis. Other common nucleoside triphosphates were not effective. Also, the presence of all four of the constituent amino acids of malformin was required in order for isotopic valine to be utilized. This experiment, together with the demonstrated specific character of the incorporation process (Table 2), supports the view that de novo synthesis of malformin occurred.

Certain of the optimal conditions for malformin synthesis (Fig. 3, 4, and 5) appear quite similar to those for the formation of the tripeptide, glutathione (from γ-glutamyl cysteine and glycine), by a soluble liver enzyme (13). For both processes, ATP concentration was optimal at approxi-

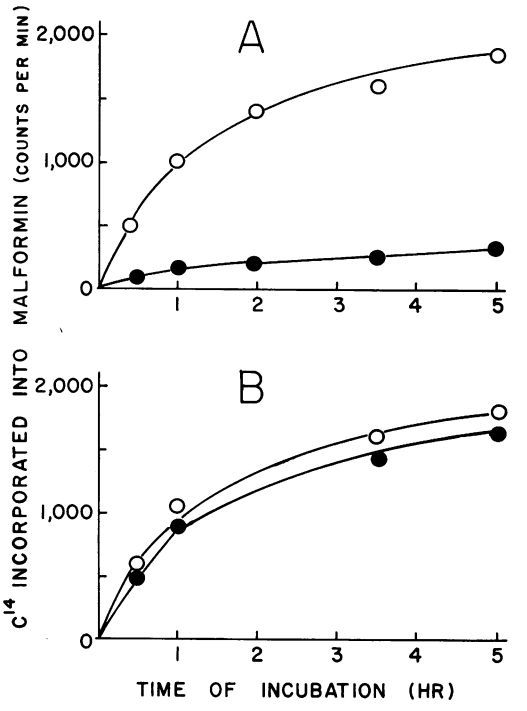

FIG. 9. *Effect of excess nonisotopic* D- *or* L-*leucine on the rate of utilization of the opposite form o the radioactive amino acid. A:* ○, *1 μc of* D-*leucine-C^{14},* ●, *1 μc of* D-*leucine-C^{14} plus 20 μmoles of* L-*leucine-C^{12}. B:* ○, *1 μc of* L-*leucine-C^{14};* ●, *1 μc of* L-*leucine-C^{14} plus 20 μmoles of* D-*leucine-C^{12}.

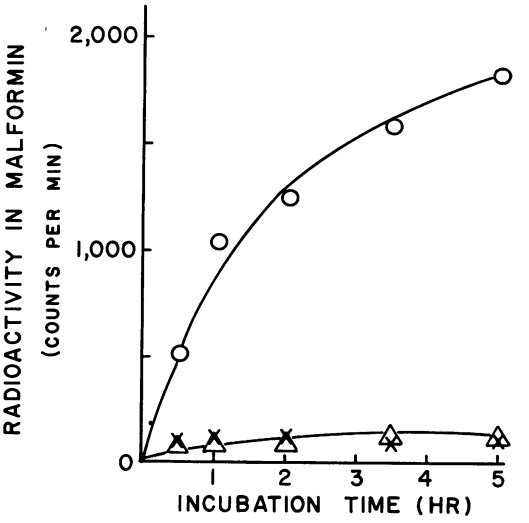

FIG. 10. *Effect of excess nonlabeled* D- *and* L-*cysteine on the rate of utilization of radioactive* DL-*cysteine (1 μc) for malformin synthesis. Symbols:* ○, *control;* △, *20 μmoles of* D-*cysteine-C^{12};* ×, *20 μmoles of* L-*cysteine-C^{12}.

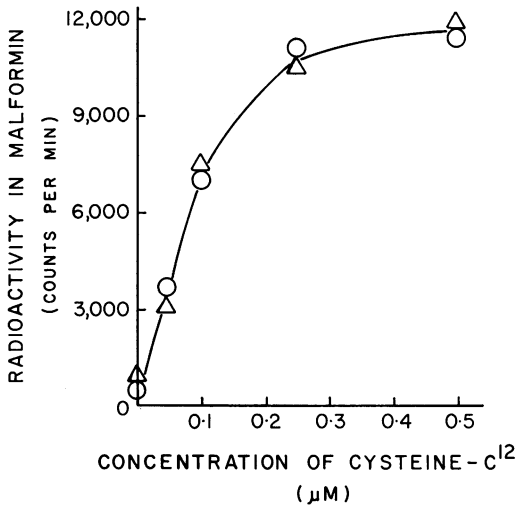

FIG. 11. *Stimulation by nonisotopic* D- *and* L-*cysteine of the incorporation of radiovaline into malformin; 5 μc of* DL-*valine-H³ was used in each assay. Varying concentrations of:* D-*cysteine-C¹²* (●), *and* L-*cysteine-C¹²* (△).

mately 0.002 M (and inhibitory above this level), whereas Mg^{++} and K^{+} were most stimulatory at 0.01 and 0.1 M, respectively. Likewise, the optimal *p*H was about 8.5 for the synthesis of both malformin and glutathione.

The experiments with labeled cysteine and cystine (Table 4) suggest strongly that the sulfhydryl, rather than the disulfide, form of the amino acid is incorporated into peptide linkage, and that the bridge between the two half-cystines arises at a later stage in the biogenesis of the malformin molecule. However, more definitive work is required to clarify this mechanism.

The present enzyme preparation appeared to be completely free from particles. The solution lost no activity upon centrifugation for 4 hr at 100,000 × *g*. This observation, together with the insensitivity of malformin synthesis to puromycin, chloramphenicol, and ribonuclease (Table 3), suggest that the pathway of peptide formation is unlike that of protein biosynthesis. A similar conclusion has been reached in studies on polypeptides of diverse microbial origin: polymyxin B (10), bacitracin (4), actinomycin (6), tyrocidine (8), and gramicidin S (3, 14). On the other hand, Hall et al. (5) isolated from *Bacillus brevis* a specific messenger ribonucleic acid which codes for gramicidin S synthesis in the presence of ribosomes and soluble components. In the case of malformin, experiments in progress suggest that the synthesis may proceed via a series of steps involving several discrete enzymes.

Various studies, in which whole cells, protoplasts, and cell-free preparations were used, have been reported on the mechanism of integration of D-amino acid residues into antibiotic polypeptides (generally cyclic structures): D-ornithine of bacitracin (4, 12); D-leucine of circulin (7); D-valine of penicillin (2), actinomycin (11), and gramicidin (9); and D-phenylalanine of tyrocidine (9). In a number of cases, both D and L forms of the amino acid could be utilized. However, several investigators have suggested that the physiological pathway involves the incorporation of the L enantiomorph into peptide linkage, followed by its inversion to the D isomer.

A rather similar conclusion may be reached from the present experiments with malformin, in which L-, as well as D-leucine-C^{14}, gives rise to labeling in the final product (Fig. 9). Furthermore, the effective competition of L-leucine-C^{12} with D-leucine-C^{14}, together with the inability of excess D-leucine-C^{12} to inhibit the utilization of L-leucine-C^{14}, could be interpreted in terms of a greater affinity of a relevant enzyme for the L isomer of this amino acid. It seems unlikely that a transformation of free D- to L-leucine occurred (by a racemase action), since this would have resulted in a dilution of L-leucine-C^{14} by L-leucine-C^{12} (derived from excess D-leucine-C^{12}), with a consequent lowering of malformin synthesis.

The observation (Fig. 11) that either D- or L-cysteine promoted the incorporation of radioactive valine into malformin implies that both enantiomorphs of cysteine could be utilized, as in the leucine experiments. However, the strong inhibition of DL-cysteine-C^{14} uptake, exerted by an excess of either D- or L-cysteine-C^{12} (Fig. 10) is somewhat at variance with the leucine experiments; hence, there is no indication that one isomeric form of cysteine is preferentially active in the formation of the cyclic peptide.

The foregoing experiments are obviously inadequate for an understanding of the genesis of D-amino acid residues in malformin. The investigation of intermediate stages in the pathway, as well as the cyclization step, should be illuminating. It is hoped that future studies will elucidate the detailed mechanism of biogenesis of this unique peptide.

ACKNOWLEDGMENTS

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