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CHEMICAL STUDIES ON MALFORMIN—IV.*

CONFORMATIONAL STUDIES OF MALFORMIN A

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Abstract—The conformation of malformin A and thiolmalformin A is altered in various solvents. When thiolmalformin A was heated in neutral or weak alkaline buffer solutions biological activity was lost. Activity was restored by heating the aqueous solution at a lower pH or by extraction with butanol. A higher pH was necessary to induce conformational changes in malformin A. After heating in alkaline solution a conformational isomer of malformin A formed an insoluble precipitate. Both malformin A and its conformational isomer assumed another conformation in trifluoroacetic acid.

INTRODUCTION

THE STRUCTURE, cyclo-L-isoleucyl-D-cysteinyl-L-valyl-D-cysteinyl-D-leucyl, was assigned to malformin A,[1] a cyclopentapeptide isolated from culture filtrate of *Aspergillus niger*.[2] Malformin A induces curvatures and malformations of bean plants[3] and severe curvatures of corn roots.[4] Although thiolmalformin A has one-tenth of the biological activity of malformin A, synthetic thiolmalformin A was inactive.[5] The possibility that the biological activity of natural thiolmalformin A was due to contamination with malformin A was excluded, because it was prepared from inactive benzylmalformin A. The optical rotation of benzylmalformin A ($[\alpha]_D^{24} = +62{\cdot}7°$, trifluoroacetic acid) differed from that of synthetic benzylmalformin A ($[\alpha]_D^{24} = +40{\cdot}2°$, trifluoroacetic acid). The structure of malformin A was re-examined and the structure proposed earlier was judged to be correct.[6] In addition, the presence of an S—S bond was established and a stereoisomer, isomalformin A, was isolated. It was concluded that the difference in biological activity of thiolmalformin A and the synthetic compound could be ascribed to differences in conformation.

Studies concerning the conformation of synthetic and natural polypeptides have been numerous. Interconvertible conformations of insulin, each with different biological activity, have been reported.[7,8] Straight chain nonapeptides of γ-methyl esters of glutamic acid, blocked at the amino end by a benzyloxycarbonyl group, can exist in two interconvertible

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forms, i.e., dioxane-soluble and insoluble.[9,10] Although possible conformations of oligomeric cyclopeptides, i.e., DL-phenylalanyl-glycyl-glycyl-DL-phenylalanyl-glycyl-glycyl[11] and gramacidin S,[12] have been discussed, isolation of different conformations has not been reported. Our report concerns various stable conformations of thiolmalformin A and malformin A having different biological activities and optical rotations.

RESULTS AND DISCUSSION

Thiolmalformin A

Because the solubility of thiolmalformin in most organic and aqueous solvents is extremely low, information concerning conformational changes was obtained by noting changes in biological activity, i.e., the ability to induce curvatures of corn roots by the filter-paper method.[13] Measurement of optical rotation was also limited in our studies because the only solvent which dissolves thiolmalformin readily is trifluoroacetic acid, a random coil solvent.

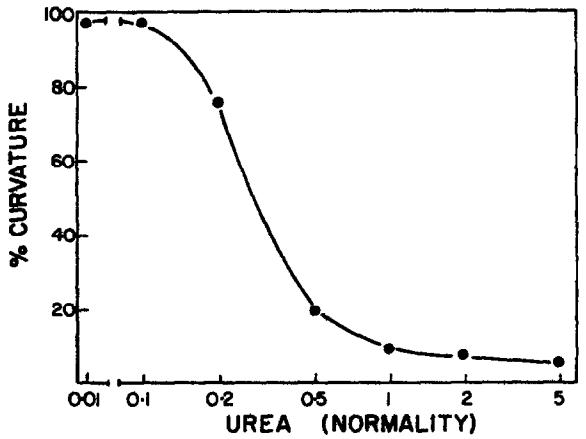

FIG. 1. Effect of heating at 100° for 2 hr in various concentrations of urea on biological activity of thiolmalformin A.

We attempted to induce conformational changes of thiolmalformin using urea or arginine. No decrease in biological activity was observed in 5 N urea or 1 N arginine·HCl after 5 days at room temperature. However, when thiolmalformin was heated at 100° for 2 hr in solutions of urea, biological activity was lost (Fig. 1). Arginine was ineffective under these conditions. Several explanations may account for these results. (1) Because urea liberates ammonia in hot aqueous solutions, air oxidation of thiol groups resulted in the formation of intra- or intermolecular disulfide bonds. The pH of thiolmalformin solutions heated in 0·2 N urea at 100° for 1 hr was 9. (2) Thiolmalformin was converted to an inactive conformation by urea through cleavage of hydrogen bonds. Heating was necessary for a conformational change, probably because the structure of oligomeric cyclopeptides is more rigid than that of straight-chain peptides. (3) A conformational change resulted from liberation of ammonia from urea,

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and the alkalinity, rather than the presence of a protein-denaturing reagent, was responsible for the change.

Quantitative determination of thiol groups by iodometry could not test the first assumption, because of the low solubility of thiolmalformin in aqueous solutions. As a model, L-cysteine was heated for 1 hr at 100° in 0·1 and 0·01 N NAOH and the extent of oxidation determined. L-cysteine was oxidized about 20 per cent in 0·1 NaOH and about 10 per cent in 0·01 N NaOH. In addition, similar experiments performed with thiolmalformin and urea under nitrogen also resulted in loss of biological activity, and polarographic studies indicated that S—S bonds were formed only in trace amounts after thiolmalformin was heated in 0·1 N K_2HPO_4 (pH 9·1) at 100° for 2 hr. We concluded that oxidation of thiol groups was not responsible for the loss in biological activity of thiolmalformin.

The second assumption, that urea acted as a denaturing agent through disruption of intramolecular hydrogen bonds, was considered unlikely because of the failure of arginine to

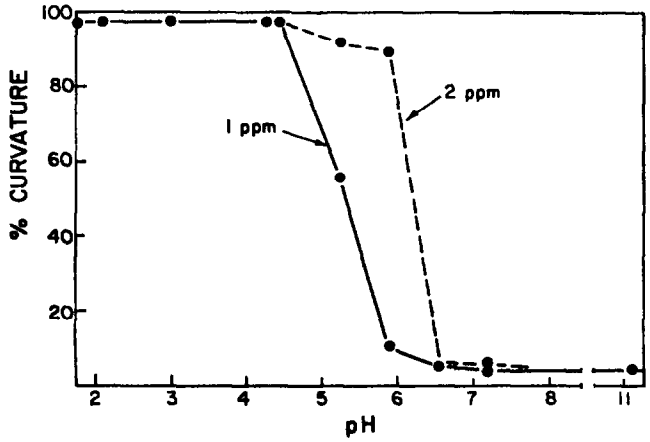
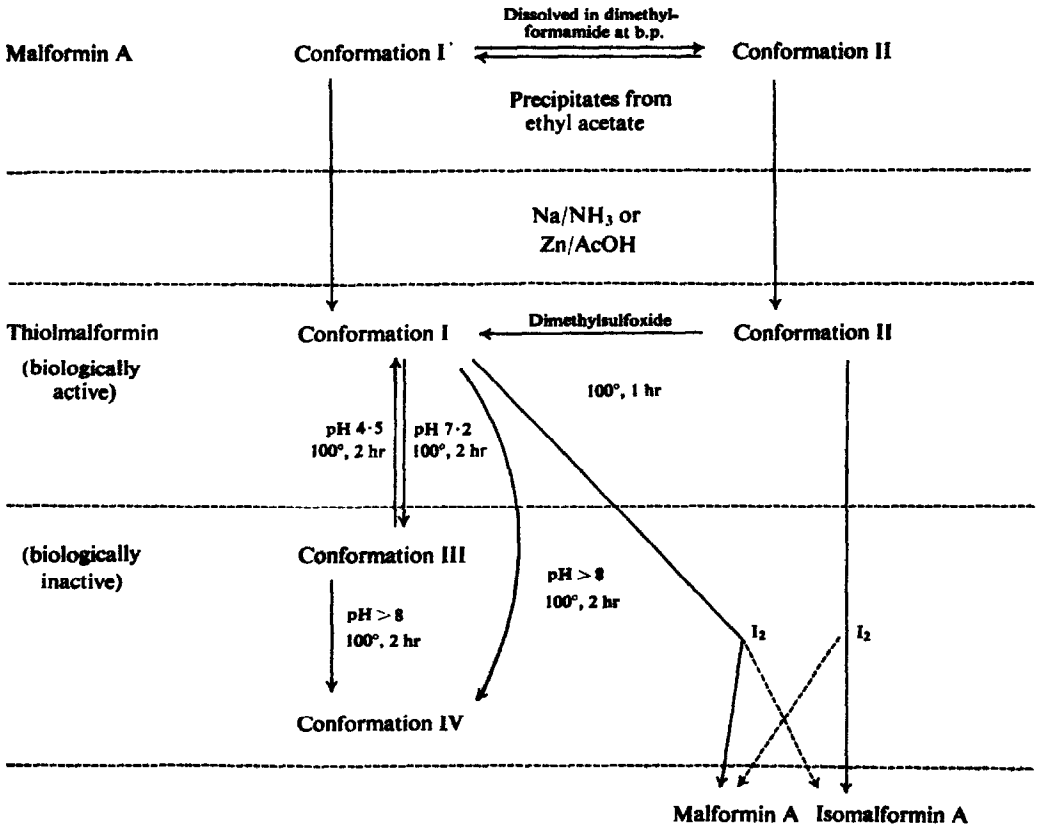


FIG. 2. Effect of heating at 100° for 2 hr in buffers at various values of pH on biological activity of thiolmalformin A.

act in the same manner. In addition, high concentrations of sodium chloride did not alter the biological activity of thiolmalformin.

Regarding the third assumption, we are unaware of studies indicating that a trace of alkali, such as ammonia liberated in hot urea solutions, induces conformational changes of oligopeptides. To test this, thiolmalformin was heated in buffer solutions of various pH. The ability of thiolmalformin to induce curvatures of corn roots was used as a criterion for a change in conformation. The biological activity of thiolmalformin was lost after heating at 100° for 2 hr in citrate or phosphate buffer with a pH of 6.5 or higher (Fig. 2). At pH 6·5 the velocity of air oxidation of thiol groups should be negligible. Recovery of the biological activity of thiolmalformin, inactivated by heating at pH 7·2, would constitute strong evidence that the molecule had undergone a conformational change. When a pH 7·2 inactivated sample was heated at pH 4·5 to 5·0 in citrate buffer, 85 per cent of the biological activity was recovered. Thiolmalformin inactivated at a pH greater than 8 could not be reactivated by this method (scheme I). Because the activity of thiolmalformin inactivated by heating at pH 7·2 (conformation III, scheme I) could be recovered by heating at pH 4·5 and the activity of thiolmalformin inactivated at pH's greater than 8 (conformation IV, scheme I) could not, we

distinguished between the two forms. When thiolmalformin was inactivated by heating in buffer solution of pH 7 to 9 the activity was recovered by extraction with butanol. We believe a reversible conformational change in aqueous solution occurred. Thiolmalformin inactivated by heating in 1 % Na_2CO_3 recovered about 20 per cent activity after heating in dimethylsulfoxide. This does not necessarily indicate the presence of another stable conformation, because oxidation of thiol groups cannot be avoided in alkaline solutions. We attempted to isolate biologically-inactive thiolmalformin (conformation IV, scheme I). However, the



SCHEME I

shift in conformation appeared to occur continuously, depending on the pH, and each isolate had a different $[\alpha]_D$ value.

The following suggests that biologically-active thiolmalformin is not limited to one conformation: a sample of thiolmalformin which had one-half or less the usual biological activity of thiolmalformin was obtained by reduction of malformin A which had been boiled in dimethylformamide for 10 min. When the *less* active thiolmalformin (conformation II, scheme I) was heated in dimethylsulfoxide, its activity increased to that usually encountered (conformation I, scheme I), i.e., optimum activity for corn-root curvatures about 1 ppm. The difference in biological activity of the two kinds of thiol malformin was not great, but a more pronounced difference was noted in their behavior to iodine oxidation. When the more

active form of thiolmalformin (conformation I, scheme I) was oxidized with iodine, the main product was malformin A and smaller amounts of isomalformin A were obtained.[6] When the less active form (conformation II, scheme I) was oxidized similarly, the main product was isomalformin A. The original conformation of malformin A (conformation I, scheme I) is apparently maintained during reduction to thiolmalformin in liquid ammonia. We were unable to convert the more active thiolmalformin (conformation I, scheme I) to less active thiolmalformin (conformation II, scheme I) by direct treatment with dimethylformamide. We believe that conformation II and III are less stable than I and IV and may represent intermediate stages in the shift from I to IV.

In organic solvents, no inactive forms of thiolmalformin were obtained. A decrease in biological activity of thiolmalformin in some aqueous-organic solvent systems was noted and, generally, the greater the ratio of organic solvent, the less was the loss of activity. Attempts to produce conformational changes of benzylmalformin were not successful.

Malformin A

Conformational changes of malformin A were also studied. Although the biological activity of malformin A was decreased after heating in alkaline solutions, differences were

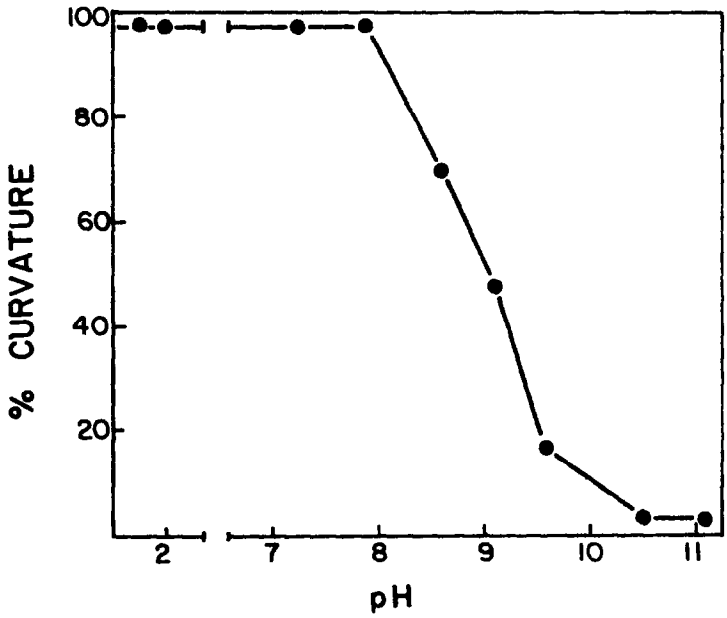
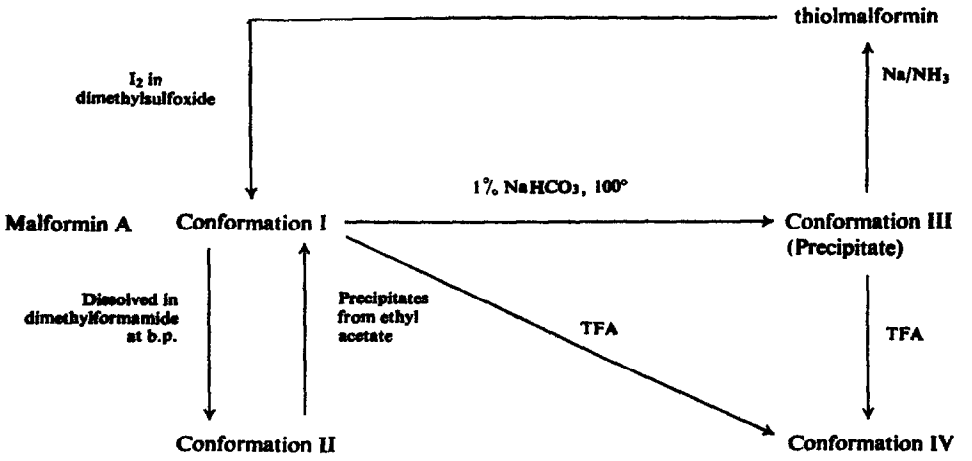


FIG. 3. Effect of heating at 100° for 1 hr in buffers at various values of pH on biological activity of malformin A (0·1 ppm).

noted when compared with the behavior of thiolmalformin. (1) More alkaline solutions were required for steric conversion of malformin A (Fig. 3). (2) Biological activity was not completely lost. After heating in alkaline solution the optimum concentration for corn-root curvature shifted from 0·1 to about 5·0 ppm. (3) Biological activity diminished by heating in alkali was not recovered under conditions successfully applied to regenerate inactive thiolmalformin.

A *less* active malformin A (conformation III, scheme II) was isolated after malformin A (conformation I, scheme II) was heated at 100° for 3 hr in 1 % $NaHCO_3$ or left in 1 % Na_2CO_3 at room temperature for 24 hr. Although the i.r. spectrum of the less active isomer was identical with that of malformin A, the optical rotation differed markedly (conformation I, $[\alpha]_D^{23} = -18°$, $c = 1$, trifluoroacetic acid; conformation III, $[\alpha]_D^{23} = +150°$, $c = 1$, trifluoroacetic acid). The optimum concentration for induction of corn-root curvatures by less active malformin A (conformation III) ranged between 2 to 10 ppm, whereas malformin A (conformation I) was most effective at about 0·1 ppm.

Two experiments indicated that the less active form of malformin A (conformation III, scheme II) was actually another form of malformin A (conformation I, scheme II). (1) The less active form was converted to the more active form of malformin A (conformation I) via thiolmalformin (scheme II). (2) The active form of malformin A (conformation I), the less active form (conformation III), isomalformin A,[6] and thiolmalformin (conformation I,



SCHEME II

scheme I) were disssolved in trifluoroacetic acid and their $[\alpha]_D$ values observed at intervals for 100 days. Whereas the $[\alpha]_D$ values of isomalformin A and thiolmalformin A were essentially constant throughout the experiment, the $[\alpha]_D$ values of malformin A and its less active form changed markedly and reached the same value after 6 days ($[\alpha]_D^{23} = +63°$, $c = 1$) (Fig. 4). The optimum concentration for induction of root curvatures by both samples after 100 days was essentially the same, i.e., about 0·5 ppm. Apparently, both malformin A and its less active form assumed a different conformation in trifluoroacetic acid. The biological activity, specific rotation, and solubility of isomalformin A and various conformations of malformin A are summarized in Table 1. Although the i.r. spectra of the different conformations were almost identical, slight differences were noted in the 3300 cm^{-1} region. These differences are probably related to hydrogen bonding of amido groups.

We concluded that both malformin A and thiolmalformin A are capable of assuming various conformations with varying degrees of stability. Conformational changes of straight-chain peptides can occur rapidly, even at room temperature. Our results suggest that the

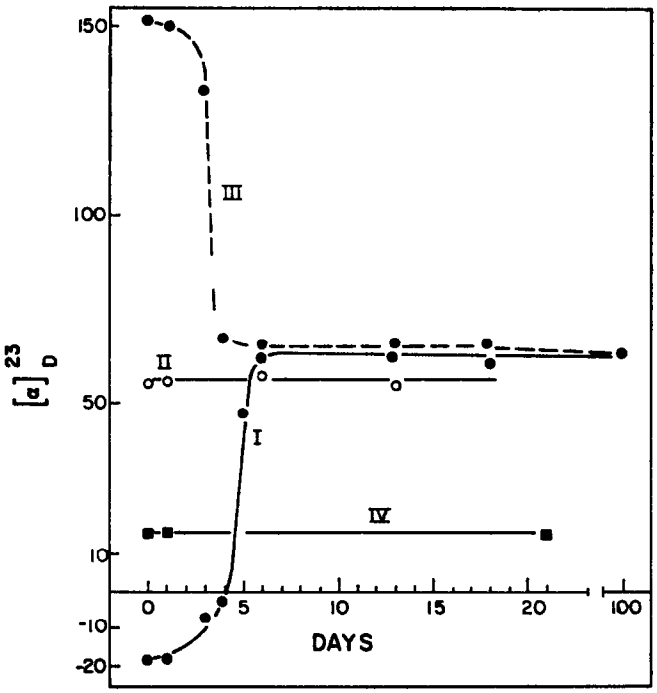


Fig. 4. Specific rotation of malformin A isomers and thiolmalformin A.
$c = 1$, trifluoroacetic acid.
I. Malformin A—conformation I.
II. Thiolmalformin A.
III. Malformin A—conformation III.
IV. Isomalformin A.

energy barrier for conformational changes of cyclo-peptides is greater than that required for oligomeric and polymeric straight-chain peptides, possibly because of the greater rigidity of the ring structure. The presence of three ring systems in malformin A, including an S—S bond, may explain the more stringent conditions necessary to induce conformational changes when compared with thiolmalformin A.

TABLE 1. BIOLOGICAL ACTIVITY, OPTICAL ROTATION, AND SOLUBILITY OF ISOMERS OF MALFORMIN A

	Optimum conc. for corn-root curvatures (ppm)	$[\alpha]_D^{23}$ ($c = 1$, trifluoroacetic acid)	Solubility in dimethylformamide and dimethylsulfoxide
Malformin A			
Conformation I	0·1	−18°	Soluble
Conformation II	0·1	−18°	Soluble
Conformation III	2–10	+150° ($c = 0·5$)	Scarcely soluble
Conformation IV	0·5–1·0	+62°	Scarcely soluble
Isomalformin A	2–10	+15°	Scarcely soluble

Although our studies on synthetic thiolmalformin were limited by scarcity of sample, we attempted unsuccessfully to activate this compound by methods successfully employed to activate previously-inactivated natural thiolmalformin. The reason for the differences in biological activity and $[\alpha]_D$ values of natural and synthetic thiolmalformin were not clarified but we believe that conformational differences are responsible. Dimethylformamide is known as an α-helix solvent for straight-chain peptides,[12] and in the synthesis of thiolmalformin, *p*-nitrophenyl-*S*-benzyl-D-cysteinyl-L-valyl-*S*-benzyl-D-cysteinyl-D-leucyl-L-isoleucinate was cyclicized in a mixture of dimethylformamide and pyridine. If the straight-chain pentapeptide derivative assumed a more or less α-helical conformation, the cyclic product might take a "basket" conformation containing four seven-membered hydrogen-bonded rings. However, using molecular models, we were unable to construct four hydrogen-bonded rings in malformin A because of the S—S bond. Furthermore, our studies indicated that the conformation of malformin A is not changed by reduction in liquid ammonia to thiolmalformin. If the conformation of the synthetic benzylated cyclic peptide was also unchanged by reduction in liquid ammonia, then synthetic thiolmalformin should have a stable conformation different than that of the natural product.

EXPERIMENTAL

General Methods

The biological activity of malformin A and thiolmalformin A was assayed by their ability to induce corn-root curvatures using the filter paper method.[13] Because the solubility of thiolmalformin in water is very low (40 ppm or less) the use of a surface active agent, Tween 80, was necessary when conformational changes were attempted at higher concentrations. Despite separation of Tween 80 from the aqueous phase at 100°, it prevented precipitation. Experiments were usually performed at two concentrations of thiolmalformin, 400 and 10 to 40 ppm. Tween 80 was usually excluded at the lower concentrations. Biological activity of thiolmalformin was determined at 1, 2, 5, and 10 ppm; malformin A was also assayed at 0·1 ppm.

Effect of Urea and Arginine on Biological Activity of Thiolmalformin

Thiolmalformin (4 mg/ml in dimethylsulfoxide), 0·1 ml, was diluted with aqueous solutions of urea or arginine·HCl (0·01 to 5 N), 0·9 ml, containing Tween 80, 1 drop/ml. The solutions were heated at 100° for 2 hr in sealed tubes or left at 38° for 5 days. Biological activity gradually diminished as the concentration of urea increased from 0·1 to 0·5 N and was virtually eliminated in 1 N urea. Similar results were obtained when the concentration of thiolmalformin during treatment with urea was decreased to 40 ppm and Tween 80 was excluded. Arginine was ineffective.

Air Oxidation of Cysteine in Acidic and Basic Solution

Cysteine, 20 to 30 mg, was heated at 100° for 1 hr in 5 ml of various acidic or basic solutions. The solutions were neutralized, filtered, and the amount of cysteine remaining in the solution determined by iodometry (Table 2). When oxidation of cysteine was marked, cystine was precipitated by neutralization, filtered, washed with water, dried, and weighed to confirm the results obtained by iodometry. In these experiments larger quantities (100 mg) of cysteine were employed.

TABLE 2. EFFECT OF pH ON OXIDATION OF CYSTEINE

Solution	Per cent cysteine after heating at 100°, 1 hr
0·2 N Pyrophosphate buffer, pH 8·3	93
0·2 N K_2HPO_4–Na_3PO_4, pH 9·5	91
0·01 N NaOH	90
0·1 N NaOH	80
1·0 N NaOH	77
Acetic acid: conc. HCl (1:1)	62
Acetic acid: conc. HCl:H_2O (30:4:1)	95

Effect of pH on Biological Activity of Thiolmalformin A

Thiolmalformin, 400 ppm, was heated at 100° for 2 hr in the following solutions: 0·05 N HCl (pH 1·8), 0·01 N HCl (pH 2·1), 0·01 N citric acid (pH 3), 0·2 N citrate buffer (pH 4·25), 0·2 N phosphate buffer (pH 4·55, 5·25, 5·9, 6·55, 7·2), 0·1 N K_2HPO_4 (pH 9·1), 1% $NaHCO_3$ (pH 9·6), 10^{-3} N NaOH (pH 10·6), 1% Na_2CO_3 (pH 11·1). All solutions contained Tween 80, 1 drop/ml. After heating the solutions were neutralized, diluted and assayed for biological activity. When heated at pH 6·55 or greater, biological activity was completely lost (Fig. 2). Although similar results were obtained when the concentration of thiolmalformin was decreased during heating to 20 ppm, the higher buffer concentrations of the assay solutions inhibited root growth. No loss of activity occurred when thiolmalformin was heated in water.

Recovery of Biological Activity of Thiolmalformin

Thiolmalformin in dimethylsulfoxide, 4 mg/ml, was diluted to 40 ppm with 0·1 N phosphate buffer, pH 7·2, containing Tween 80, 1 drop/ml, and heated at 100° for 2 hr. As described previously, loss of biological activity was complete. The solution was adjusted to pH 4·5 to 5·0 with 0·1 N citric acid and again heated at 100° for 2 hr. Recovery of biological activity was essentially complete.

When thiolmalformin was inactivated by heating in 0·1 N K_2HPO_4 (pH 9·1) or in 10^{-3} N NaOH (pH 10·6), no recovery was observed when subsequently heated at pH 4·5 to 5·0.

In a second experiment, thiolmalformin was inactivated by heating at 100° for 2 hr in 4 N urea, 0·1 N phosphate buffer (pH 7·2), or 0·1 N K_2HPO_4 (pH 9·1). The concentration of thiolmalformin in these solutions was 20 ppm. One drop of Tween 80 was added to each 20 ml of solution. The inactive solutions were extracted once with equal volumes of butanol. The butanol layer was removed and evaporated *in vacuo* with occasional addition of water until butanol-free aqueous solutions were obtained. In some experiments the butanol was removed under nitrogen. The optimum concentration for induction of corn-root curvatures was approximately 1 to 2 ppm, indicating almost complete recovery of activity after extraction with butanol.

Less Active Thiolmalformin (Conformation II)

Malformin A was boiled in dimethylformamide, 5 mg/ml, for 10 min and dried *in vacuo*. The amorphous residue was reduced with sodium in liquid ammonia to obtain less active thiolmalformin. The specific rotation ($[\alpha]_D^{23} = +50{\cdot}4°$, $c = 1$, trifluoroacetic acid) differed slightly from that of thiolmalformin (conformation I, $[\alpha]_D^{23} = +57{\cdot}4°$, $c = 1$, trifluoroacetic acid) obtained by direct reduction of malformin A, and the biological activity varied from 20

to 50% of that of the more active form. After the less active form was heated in dimethylsulfoxide at 100° for 1 hr no difference in biological activity was observed.

Conformations I and II differed markedly when oxidized by iodine.[6] When conformation II, 350 mg, was oxidized with iodine in dimethylsulfoxide the yields of malformin A and *iso*-malformin A were 20 mg, and 250 mg, respectively. From conformation I, 300 mg, we obtained 140 mg and 50 mg, respectively.

Inactive Thiolmalformin (Conformation IV)

Thiolmalformin, 50 mg, in dimethylformamide, 30 ml, was treated with 10^{-3} N NaOH, 1 l, with vigorous stirring to decrease precipitation. The solution (pH 10·9) was filtered to remove a small amount of precipitate and heated at 100° for 2 hr. The biologically-inactive solution was neutralized with HCl, dried *in vacuo*, the residue washed repeatedly with water, and dried *in vacuo*. Yield 40 mg, $[\alpha]_D^{23} = +110°$ ($c = 1$, trifluoroacetic acid).

When conformation 1 (active) was suspended in conc. HCl and shaken for 2 days at room temperature, thiazoline-containing malformin was observed after 2 days.[1] Conformation IV (inactive) was not dissolved in conc. HCl and formation of the thiazoline chromophore was not detectable. The solubility of conformation IV in dimethylsulfoxide was considerably less than that of conformation I. None the less, biological activity of conformation IV was completely recovered after boiling for 5 min in dimethylsulfoxide.

In the second experiment, thiolmalformin, 200 mg, in dimethylsulfoxide, 50 ml, was treated with hot 1% Na_2CO_3, 1 l, with stirring, heated for 10 min at 100°, and allowed to stand at room temperature overnight. The precipitate was filtered and washed with water and alcohol. Yield 100 mg; $[\alpha]_D^{23} = +130·2°$ ($c = 1$, trifluoroacetic acid). This sample was scarcely soluble in dimethylsulfoxide but recovered about 20 per cent biological activity after boiling in dimethylsulfoxide for 5 min.

Thiolmalformin heated in pyridine, dimethylformamide, ethanol, or butanol did not decrease in activity. In each experiment, piperidine, 1 drop/ml, was present. No decline in activity was observed after heating in dimethylformamide: pyridine (1:1) or dimethylsulfoxide:ethanol:H_2O (1:4:5). When 0·2 N phosphate buffer (pH 7·2) replaced water in the latter solvent biological activity was lost.

Effect of pH on Biological Activity of Malformin A

Malformin A, 1000 ppm, in dimethylsulfoxide was diluted to 10 ppm with each of the following solutions and heated at 100° for 1 hr: 0·1 N HCl, 0·05 N HCl, 0·01 N HCl, 0·1 N citric acid (pH 3), 0·2 N citrate buffer (pH 4·2, 5·5), 0·2 N phosphate buffer (pH 7·2, 7·9), 0·2 N pyrophosphate buffer (pH 8·6), 0·1 N K_2HPO_4 (pH 9·1), 1% $NaHCO_3$ (pH 9·6), 0·1 N K_2HPO_4–Na_3PO_4 (pH 10·5), 1% Na_2CO_3 (pH 11·1). Biological activity decreased gradually as the pH increased from 8 to 10·5 (Fig. 3). At the higher pH activity at 0·1 ppm was eliminated but could be demonstrated at 2 to 10 ppm. The following conditions were necessary for transformation of malformin (conformation I) to the less active form (conformation III): 0·1 N K_2HPO_4 (pH 9·1) at 100° for 5 hr; 1% $NaHCO_3$ at 100° for 2 hr; 0·1 N K_2HPO_4–Na_3PO_4 (pH 10·5) at 100° for 1 hr; 1% Na_2CO_3 at 100° for 5 min; 1% Na_2CO_3 at room temperature for 24 hr.

Less Active Malformin A (Conformation III)

Malformin A, 200 mg, in dimethylformamide, 50 ml, was treated with 1% $NaHCO_3$, 1 l, and heated at 100° for 4 hr. The isomer, which precipitated, was filtered and washed repeatedly

with water and alcohol. Yield 190 mg; m.p. over 300°; $[\alpha]_D^{23} = +151°$ ($c = 1$, trifluoroacetic acid); optimum concentration for corn-root curvatures, 2 to 10 ppm. The same compound was obtained when malformin A in 1% Na_2CO_3 stood at room temperature for 24 hr. As compared with conformation I, conformation III was scarcely soluble in dimethylsulfoxide and dimethylformamide. Trifluoroacetic acid was the only solvent capable of dissolving conformation III. The i.r. spectra of both compounds were similar, but conformation I had adsorption at 3280 and 3380 cm^{-1} whereas conformation III had absorption only at 3280 cm^{-1} in this region.