Carbon Assimilation by *Pseudomonas oxalaticus* (OX1)

6. REACTIONS OF OXALYL-COENZYME A*

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(Received 16 November 1962)

Work by Quayle, Keech & Taylor (1961) suggested that oxalyl-CoA can be formed by oxalate-grown *Pseudomonas oxalaticus* and reduced to glyoxylate. An enzyme, 'glyoxylic dehydrogenase', was obtained by Quayle & Taylor (1961) from oxalate-grown *P. oxalaticus* which catalyses reaction (1). To explain the catalytic requirement

for CoA it was assumed that the reaction proceeded via oxalyl-CoA as intermediate, this compound then being hydrolysed either chemically or enzymically to give oxalate and CoA. The demonstration that 'glyoxylic dehydrogenase' actually catalyses reaction (2) followed the chemical

synthesis of oxalyl-CoA (Koch & Jaenicke, 1962; Quayle, 1962). By analogy with the systematic name aldehyde-nicotinamide-adenine dinucleotide oxidoreductase (acylating coenzyme A) (EC 1.2.1.10), adopted for the enzyme that reduces acetyl-CoA to acetaldehyde (Burton & Stadtman, 1953), it is now suggested that 'glyoxylic dehydrogenase' be named glyoxylate-nicotinamide-adenine dinucleotide phosphate oxidoreductase (acylating coenzyme A). Because of the metabolic role proposed, the trivial name oxalyl-coenzyme A reductase is suggested.

Some further studies of this reaction and of properties of oxalyl-CoA are described below.

MATERIALS AND METHODS

Special chemicals. CoA (75% pure), lactate dehydrogenase, NAD⁺, NADH, NADP⁺ and NADPH were obtained from C. F. Boehringer und Soehne, Mannheim, Germany, and sodium glyoxylate from Sigma Chemical Co., St Louis, Mo., U.S.A. The last-named was found to be 96% pure by enzymic assay involving the reduction of glyoxylate by NADH in the presence of purified lactate dehydrogenase.

Growth of the organisms. *P. oxalaticus* was grown in liquid culture (10 l. batches) in a growth medium consisting

of sodium oxalate and mineral salts, harvested by centrifuging and stored at -15°. The experimental details have been reported by Quayle & Keech (1960).

Oxalyl-coenzyme A reductase. The enzyme used in these studies was that purified by Quayle & Taylor (1961) and since stored at -15°.

Preparation of oxalyl-coenzyme A. This followed the procedure outlined by Quayle (1962), a typical preparation being carried out as follows: 24.5 μmoles of thiocresyl hydrogen oxalate was dissolved in 4 ml. of ether, shaken for 15 min. with 14 μmoles of CoA dissolved in 4 ml. of water and adjusted to pH 7.5 with NaHCO₃. The ethereal layer was drawn off, the pH of the aqueous layer adjusted to 4.0 with N-HCl and then washed twice with 4 ml. portions of ether. The final volume of the oxalyl-CoA solution was 3.3 ml.; assay (see below) showed it to be 4.4 mM, which represents a 100% yield based on the CoA used. The solution was stored at -15°.

Chromatographic analysis. The papers (Whatman no. 4; 14 in. × 6 in.) were developed in an ascending direction. Acyl thiol compounds were chromatographed in ethanol-0.1 M-sodium acetate, pH 4.5 (1:1, v/v), and in propan-2-ol-0.1 M-sodium acetate, pH 5.0 (1:1, v/v) (Koch & Jaenicke, 1962). Hydroxamates were chromatographed in butan-2-ol-formic acid-water (75:13:12, by vol.) (Vagelos, Earl & Stadtman, 1959) and in propan-2-ol-pyridine-water (1:1:1, by vol.) (Vagelos, 1960), and detected by spraying with 5% (w/v) FeCl₃·6H₂O in ethanol (0.1 N with respect to HCl) (Stadtman & Barker, 1950).

Decarboxylation of oxalate with oxalate decarboxylase. Oxalate decarboxylase was obtained from *Collybia velutipes* by the method of Shimazono & Hayaishi (1957). For the determination of oxalate, 0.5 ml. of the purified enzyme solution was placed in the main compartment of a Warburg flask together with 400 μmoles of potassium citrate-citric acid buffer, pH 3.0, in a total volume of 1 ml. Up to 1 ml. of the oxalate solution was placed in the side arm of the flask, and this was tipped after 5 min. of equilibration at 30°. Carbon dioxide evolution was then followed during incubation for 1 hr. at 30°.

Assay of oxalyl-coenzyme A. Oxalyl-CoA may be assayed by three different methods: (a) Alkaline hydrolysis to oxalate and CoA, followed by manometric assay of oxalate by using oxalate decarboxylase (see above). (b) Colorimetric determination as hydroxamate by the method of Lipmann & Tuttle (1945). In this method to 0.5-3 μmoles of oxalyl-CoA in 2 ml. of water was added 1 ml. of 2 M-hydroxylamine (prepared by neutralizing 4 M-hydroxylamine hydrochloride to pH 7.0 with 4 N-KOH) and the solution incubated for 10 min. at room temperature. Ferric chloride reagent (3.0 ml.) [5% (w/v) FeCl₃·6H₂O in 0.1 N-HCl-12% (w/v) trichloroacetic acid-3 N-HCl; 1:1:1, by

* Part 5: Quayle & Taylor, 1961.

vol.] was then added. The extinction was read at 540 μ m and compared with that produced from a sample of a standard solution prepared by dissolving 1 g. of succinic anhydride in 40 ml. of the neutralized hydroxylamine reagent. (c) Spectrophotometric assay by using oxalyl-CoA reductase. This method is described in the Results section.

Preparation of buffer solutions. Phosphate buffer solutions were prepared by mixing equimolar solutions of NaH_2PO_4 or KH_2PO_4 with Na_2HPO_4 or K_2HPO_4 to give the required pH. Citrate buffers were prepared by mixing equimolar solutions of trisodium citrate and citric acid to give the required pH. Sodium pyrophosphate solution was adjusted to the required pH by the addition of hydrochloric acid. Tris buffer was made by mixing solutions of tris and hydrochloric acid.

RESULTS

Preparations and characterization of oxalyl-coenzyme A

Oxalyl-CoA, prepared by ester interchange as detailed in the Materials and Methods section, was characterized on the basis of its hydrolysis to oxalate and CoA, and its formation of oxalyl monohydroxamate on treatment with hydroxylamine.

Hydrolysis of oxalyl-coenzyme A. To 1 ml. of the solution of oxalyl-CoA, whose preparation is detailed in the Materials and Methods section, was added 0.1 ml. of N-sodium hydroxide and the solution was incubated at 30° for 5 min. The pH of the solution was then adjusted to 4.5 with hydrochloric acid. The free oxalic acid was determined by manometric measurement of the carbon dioxide evolved on the addition of oxalate decarboxylase (see the Materials and Methods section). It was found that 4.8 μ moles of carbon dioxide were evolved from the alkali-treated sample as compared with 0.4 μ mole from an untreated sample. Chromatography of the untreated sample in the propan-2-ol-sodium acetate system (see the Materials and Methods section), followed by examination under ultraviolet light, revealed a single absorbing spot at R_F 0.45; after alkali treatment a single spot was visible at R_F 0.6. The latter was coincident with authentic CoA. Thiocresyl hydrogen oxalate had R_F 0.95 in this system. The R_F values for oxalyl-CoA, CoA and thiocresyl hydrogen oxalate in the ethanol-sodium acetate system (see Materials and Methods section) were 0.60, 0.65 and 0.92 respectively.

These results show that the product of the ester interchange reaction is split by alkali to give oxalic acid and CoA.

Formation of oxalyl hydroxamate. To 0.3 ml. of 5 mM-oxalyl-CoA solution was added 0.02 ml. of 0.2M-hydroxylamine, pH 7.0. Samples of this solution were chromatographed alongside markers of formyl hydroxamate and oxalyl monohydroxamate prepared chemically from the monoethyl

esters of the parent acids by the method of Hestrin (1949). In the chromatographic system butan-2-ol-formic acid-water, the observed R_F values were: formyl hydroxamate, 0.60; oxalyl monohydroxamate, 0.15; hydroxamate from oxalyl-CoA, 0.15. In the system propan-2-ol-pyridine-water, the observed R_F values were: formyl hydroxamate, 0.84; oxalyl monohydroxamate, 0.51; hydroxamate from oxalyl-CoA, 0.51.

Spectral characteristics of oxalyl-coenzyme A. Koch & Jaenicke (1962) studied the ultraviolet-absorption spectra of several oxalyl thiol compounds and noted that the absorption maximum at 234 μ m characteristic of the group $\text{R}\cdot\text{S}\cdot\text{CHO}$ was displaced to 260 μ m for the group $\text{R}\cdot\text{S}\cdot\text{CO}\cdot\text{CO}_2^-$. This effect is also seen in the absorption spectrum of thiocresyl hydrogen oxalate, which exhibits a weak maximum at 265 μ m (Fig. 1). With oxalyl-CoA, a maximum in this region of the spectrum is masked by the relatively high absorption maximum of CoA itself at 260 μ m. Koch & Jaenicke (1962) showed from difference spectra that the acyl thiol grouping of oxalyl-CoA shows an absorption maximum at 260 μ m with extinction coefficient of 2400. This observation has been confirmed in the present work (Fig. 1), where an extinction coefficient of 2300 at the absorption maximum has been found. However, under the experimental conditions used the peak was at 269–271 μ m. Since the CoA molecule absorbs nine times as much at this wavelength as the acyl thiol group, the quantitative determination of the difference spectrum may not be very accurate.

Stability of oxalyl-coenzyme A. Solutions of oxalyl-CoA at pH 3–6.5 may be stored for weeks at –15° with little decomposition. It is hydrolysed in alkaline solution, completely in a few minutes in 0.1N-sodium hydroxide, and by about 5% in 1 hr. at 25° in 20 mM-pyrophosphate buffer, pH 8.6.

Enzymic reduction of oxalyl-coenzyme A to glyoxylate

When oxalyl-CoA was incubated with NADPH at pH 6.5 in the presence of purified oxalyl-coenzyme A reductase, a stoichiometric conversion into glyoxylate according to reaction (2) was observed (Koch & Jaenicke, 1962; Quayle, 1962). For the reverse of the reaction, previous work (Quayle & Taylor, 1961) had failed to demonstrate the formation of oxalyl-CoA as a product; instead, oxalate was the product and CoA was needed in catalytic quantities only. It was assumed that the explanation for this would lie in the chemical or enzymic hydrolysis of oxalyl-CoA under the conditions of reaction. Examination of the properties of oxalyl-CoA has now shown neither of these explanations could be correct, since oxalyl-CoA is not hydrolysed rapidly enough at pH 8.6 nor could any

deacylase activity be detected in the oxalyl-coenzyme A reductase. An explanation for this has been found in terms of a non-enzymic ester interchange between oxalyl-CoA and thiol compounds.

In the presence of a limiting amount of CoA, a stoichiometric reduction of NADP^+ was catalysed in the presence of glyoxylate by oxalyl-coenzyme A reductase at pH 8.6 (Fig. 2). When 2 μmoles of cysteine were then added, reduction of NADP^+ recommenced. This effect diminished with decreasing pH, being barely detectable at pH 7.0, and also diminished with decreasing concentration of cysteine, concentrations of less than 0.2 mM showing little effect (Fig. 3). Glutathione behaved in exactly the same way. These experiments indicated that an ester interchange was occurring between oxalyl-CoA and the cysteine, forming, presumably, oxalyl-cysteine and free CoA. That this interchange was non-enzymic was shown by placing 0.14 μmole of oxalyl-CoA in each of two tubes together with 6 μmoles of sodium pyrophosphate buffer, pH 8.6, and water to 1 ml. To one

Fig. 1. Absorption spectra of thiocresyl hydrogen oxalate and oxalyl-CoA. *A*, Spectrum of thiocresyl hydrogen oxalate dissolved in 50 mM-potassium phosphate buffer, pH 6.5. *B*, Difference spectrum between oxalyl-CoA and CoA. To 0.5 ml. of 10 mM-oxalyl-CoA was added 0.05 ml. of 1N-NaOH, the solution maintained at 30° for 10 min. and then made up to 50 ml. with 50 mM-potassium phosphate buffer, pH 6.5. This solution was placed in the blank cell (1 cm. light-path) of a Zeiss spectrophotometer (PMQ II) and a solution of 0.1 mM-oxalyl-CoA in the same buffer placed in the test cell. The difference spectrum of the two solutions was plotted.

Fig. 2. Effect of cysteine on the reduction of oxalyl-CoA at: *A*, pH 8.6; *B*, pH 7.5; *C*, pH 7.0. The reaction was followed by change of extinction at 340 m μ in a Cary recording spectrophotometer. The reaction system, contained in 1 ml. quartz cells (1 cm. light-path) consisted of: 25 μmoles of sodium pyrophosphate buffer (pH as indicated), 0.02 μmole of CoA, 0.88 μmole of NADP^+ , 0.92 μmole of sodium glyoxylate, 0.1 ml. of oxalyl-coenzyme A reductase, and water to give a final volume of 1 ml. The reaction was started by the addition of the enzyme. CoA was omitted from the blank cell. A portion (0.02 ml.) of 0.1 M-cysteine was added at the times indicated by the arrows.

Fig. 3. Effect of various concentrations of cysteine on the reduction of oxalyl-CoA. The reaction was followed by the change of extinction at 340 m μ in a Cary recording spectrophotometer. The reaction system, contained in 1 ml. quartz cells (1 cm. light-path) consisted of: 25 μmoles of sodium pyrophosphate buffer, pH 8.6, 0.02 μmole of CoA, 0.88 μmole of NADP^+ , 0.92 μmole of sodium glyoxylate, 0.1 ml. of oxalyl coenzyme A reductase, and water to give a final volume of 1 ml. The reaction was started by the addition of the enzyme. CoA was omitted from the blank cell. To test and blank cells were added: at *A*, 0.02 ml. of 1 mM-cysteine; at *B*, 0.02 ml. of 10 mM-cysteine; at *C*, 0.02 ml. of 100 mM-cysteine.

of them 15 μ moles of cysteine were added. After incubation at 25° for 30 min., no oxalyl-CoA could be detected by enzymic assay (see below) in the tube containing cysteine, whereas the concentration of oxalyl-CoA in the control tube remained almost unchanged. Similar behaviour has been observed in the effect of thiol-containing compounds on the stoichiometry of the phosphotransacetylase (Stadtman, 1952) and aldehyde-dehydrogenase reactions (Burton & Stadtman, 1953).

In previous experiments with glyoxylate oxidation cysteine was always added to the reaction mixture to preserve the CoA against autoxidation; hence the reason for the catalytic requirement for CoA is now apparent. In the absence of cysteine, oxalyl monohydroxamate was obtained from the reaction of glyoxylate with NADP⁺ and CoA in the presence of oxalyl-coenzyme A reductase. In this experiment, the complete reaction system contained: 50 μ moles of sodium pyrophosphate buffer, pH 8.6, 1 μ mole of sodium glyoxylate, 2 μ moles of NADP⁺, 2 μ moles of CoA, 0.1 ml. of oxalyl-coenzyme A reductase, and water to give a final volume of 1.1 ml. After incubation at 25° for 30 min., 0.5 ml. of 2M-hydroxylamine, pH 7.0, was added and the mixtures were left at room temperature for 10 min. Acetone (25 ml.) was then added and the resulting precipitate centrifuged and discarded. The supernatant solution was evaporated to dryness, the residue extracted with 2 ml. of acetone and the extract evaporated to dryness. This procedure was repeated. Samples of the final extracts were chromatographed in propan-2-ol-pyridine-water and hydroxamates located with ferric chloride spray (see Materials and Methods section). One hydroxamate spot, R_F 0.71, was on the chromatogram from the complete system. Authentic oxalyl monohydroxamate run as marker on the same paper had the same R_F . No hydroxamate could be detected on the chromatograms from control incubation mixtures from which CoA had been omitted, or from complete incubation mixtures in which boiled enzyme was used in place of the active enzyme.

Optimum pH of oxalyl-coenzyme A reductase. The optimum pH for the reduction of oxalyl-CoA to glyoxylate by oxalyl-coenzyme A reductase was 6.75 (Fig. 4); this compares with 8.6 for the reverse reaction (Quayle & Taylor, 1961).

Inhibition of oxalyl-coenzyme A by tris buffer. Quayle & Taylor (1961) found that preincubation of oxalyl-coenzyme A reductase with tris buffer caused an inhibition of activity towards the catalysis of glyoxylate oxidation. This also occurred with the enzyme catalysing the reverse reaction. Preincubation of oxalyl-coenzyme A reductase in 0.1M-tris-hydrochloric acid buffer, pH 7.0, for 30 min. caused an 87% inhibition in the rate of

reduction of oxalyl-CoA by NADPH compared with the rate observed with the enzyme at the start of the preincubation period. This result, together with the discovery by Koch & Jaenicke (1962) that the speed of hydrolysis of oxalyl thiol compounds is considerably higher in amino buffers such as tris-hydrochloric acid or triethanolamine than in inorganic buffers such as phosphate, makes tris buffer unsuitable for use with oxalyl-coenzyme A reductase.

Equilibrium constant of oxalyl-coenzyme A-reductase reaction. The equilibrium constant for reaction (2) at 25° in sodium hydrogen phosphate buffer has been measured (Table 1). Six separate determinations were made and from these the mean value for K was $1.38 \times 10^5 \pm 0.05 \times 10^5$ (s.d.).

Specificity of oxalyl-coenzyme A reductase. The substrate specificity of the enzyme has been examined by Quayle & Taylor (1961) and it was stated that the CoA requirement for the reversal of reaction (2) could not be met with other thiol-containing compounds including pantetheine. During the present study of the effect of thiol compounds on oxalyl-CoA reduction, it was observed that pantetheine could indeed be substituted for the CoA requirement in glyoxylate reduction. The failure to observe this previously was probably due to prior autoxidation to pantetheine of the sample of pantetheine used. *N*-Acetyl-cysteamine reacts very slowly in place of CoA in the reaction. Cysteamine itself does not react at all.

Fig. 4. Effect of pH on the reduction of oxalyl-CoA to glyoxylate. The reaction was followed by change of extinction at 340 $m\mu$ in a Cary recording spectrophotometer. The reaction system, contained in 1 ml. quartz cells (1 cm. light-path) consisted of: 50 μ moles of sodium hydrogen phosphate buffer, 0.4 μ mole of NADPH, 0.16 μ mole of oxalyl-CoA, 0.01 ml. of oxalyl-coenzyme A reductase, and water to give a final volume of 1 ml. The reaction was started by the addition of the enzyme.

Table 1. *Determination of the equilibrium constant of the oxalyl-coenzyme A-reductase reaction*

To a spectrophotometer cuvette were added: 50 μ moles of sodium hydrogen phosphate buffer, a known amount of sodium glyoxylate (0.7–1.8 μ moles), a known amount of CoA (0.3–1.4 μ moles), a known amount of NADP⁺ (0.3–1.6 μ moles), and water to give a final volume of 0.95 ml. The extinction at 340 m μ was read and 0.05 ml. of enzyme solution (240 μ g. of protein) was added. The increase in extinction was followed in a Cary recording spectrophotometer until equilibrium was reached (1–1.5 hr.). The temperature was 25°. Controls lacking glyoxylate or CoA showed negligible extinction changes. The final concentrations of reactants were calculated from the extinction changes.

$$K = \frac{[\text{Glyoxylate}][\text{NADP}^+][\text{CoA}]}{[\text{Oxalyl-CoA}][\text{NADPH}][\text{H}^+]}$$

Concentration at equilibrium

pH	[H ⁺] (μ mm)	[Glyoxylate] (mm)	[CoA] (mm)	[NADP ⁺] (mm)	[Oxalyl-CoA] (mm)	[NADPH] (mm)	10 ⁻⁵ K
6.70	199	0.827	1.38	0.327	0.115	0.115	1.42
6.85	141	1.75	0.367	0.532	0.133	0.133	1.37
6.70	199	1.23	0.568	1.14	0.182	0.182	1.21
6.80	158	0.793	0.851	0.736	0.149	0.149	1.41
6.60	251	0.748	1.31	1.58	0.194	0.194	1.62
6.98	105	0.791	1.35	0.291	0.151	0.151	1.30
Mean ... 1.38 $\pm$ 0.05 (s.d.)							

Use of oxalyl-coenzyme A reductase in the assay of oxalyl-coenzyme A, glyoxylate and coenzyme A. From the value of the equilibrium constant for reaction (2) conditions may be established for pushing the reaction to completion in either direction in the presence of limiting concentrations of any of the reactants. Thus, in the presence of a tenfold excess of CoA and NADP⁺ over glyoxylate at pH 8.6, the enzyme may be used for the determination of glyoxylate. This method of assay is not as convenient as that based on the reduction of glyoxylate by NADH in the presence of glycolate-nicotinamide-adenine dinucleotide oxidoreductase (EC 1.1.1.26). For the latter assay (Klotzsch & Bergmeyer, 1962) the enzyme is readily available and the relatively expensive co-factors NADP⁺ and CoA are not involved. However, oxalyl-coenzyme A reductase might be useful in those cases where hydroxypyruvate is present. Glycollate-nicotinamide-adenine dinucleotide oxidoreductase is not completely specific and reacts with this compound, in contrast with oxalyl-coenzyme A reductase, which, as far as is known, is specific for glyoxylate.

Similarly, CoA in limiting concentrations can be determined under these conditions. The limitations of this assay are that no other thiol compounds must be present, owing to thiol ester interchange reactions; also certain CoA analogues such as pantetheine will react.

At pH 6.5 the enzyme may be used to determine oxalyl-CoA if a fivefold excess of NADPH is present. Under these conditions, the reaction proceeds to more than 99 % completion. The limitations of the assay are that some oxalyl-CoA analogues, e.g. oxalyl-pantetheine, will also react. Little ester interchange takes place at this pH in the presence of other thiol compounds.

DISCUSSION

The present work shows that oxalyl-CoA may be readily synthesized and describes some of its chemical properties. Its reversible enzymic reduction to glyoxylate and CoA has been demonstrated. This reaction has been suggested as a key reaction in the microbial synthesis of cell constituents from oxalate (Quayle *et al.* 1961). Use of the measured equilibrium constant gives a value of +2.54 kcal. for $\Delta G'$ at pH 7.0 of reaction (2), proceeding from left to right. This compares with the value -0.9 kcal. calculated from thermodynamic data (Quayle & Taylor, 1961). However, some of the values used in the latter calculation, e.g. the heat of combustion of oxalic acid and the free energy of hydrolysis of oxalyl-CoA, are of doubtful accuracy. The value of +2.54 kcal. for the free energy of reduction of oxalyl-CoA may be compared with the value of +4.2 kcal. for the analogous reaction (3) catalysed by the aldehyde dehydrogenase from *Clostridium kluyveri* (Burton & Stadtman, 1953).

SUMMARY

1. Oxalyl-CoA has been prepared by ester interchange between thiocresyl hydrogen oxalate and CoA.

2. Oxalyl-CoA has been shown to be the product of the CoA- and NADP-linked oxidation of glyoxylate in the presence of oxalyl-coenzyme A reductase. The reverse of this reaction had been demonstrated previously.

3. The enzymic reduction of oxalyl-CoA to glyoxylate has a pH optimum at 6.75. The enzyme is inhibited in the presence of tris buffer.

4. The equilibrium constant for the reduction of oxalyl-CoA to glyoxylate by oxalyl-coenzyme A reductase is 1.38×10^5 at 25° . From this value, $\Delta G'$ at pH 7.0 for the reaction is calculated to be $+2.54$ kcal.

5. A non-enzymic ester interchange between oxalyl-CoA and thiol compounds such as cysteine and glutathione has been found. This accounts for the previous inability to isolate oxalyl-CoA as the reaction product from glyoxylate oxidation.

6. The use of oxalyl-coenzyme A reductase for the direct spectrophotometric determination of glyoxylate, oxalyl-CoA and CoA is discussed.

I thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement and Miss A. West for technical assistance. This work was supported in part by the Rockefeller Foundation, the United States Public Health Service, and by the Office of Scientific Research, OAR, through the European Office, Aerospace Research, United States Air Force, under contract no. AF 61 (052)-180.

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Biochem. J. (1963) **87**, 373

The Inhibition of Ribonuclease by Extract of Privet Leaves and by α -Angelica Lactone

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(Received 14 September 1962)

A low-molecular-weight thermostable inhibitor of pancreatic ribonuclease was extracted from the leaves of lilac (*Syringa vulgaris*) and privet (*Ligustrum vulgare*) by Bernheimer & Steele (1955). These workers showed that its effect increased on preincubation with ribonuclease, which suggested to us that it might be an alkylating agent analogous to bromoacetate (Barnard & Stein, 1959), or possibly an acylating agent. For a review of the action of alkylating and other agents on ribonuclease see Stein (1960). The amino acids usually attacked are lysine, histidine or methionine, according to pH. This paper describes some of the properties of the privet inhibitor, and its reaction with ribonuclease and histidine. Both wild privet (*Ligustrum vulgare*) and the species used for hedges (*L. ovalifolium*) were used and gave apparently indistinguishable extracts. *L. ovalifolium* was particularly convenient since it keeps its leaves throughout the winter. The extracts throughout the year had approximately constant activity. Ash (*Fraxinus excelsior*), also a member of the family Oleaceae, was found to contain an inhibitor, but to have no special advantage.

A guess that the inhibitor might be a lactone led to the discovery that α -angelica lactone also inhibits, but by a different mechanism.

EXPERIMENTAL

Materials. Ribonuclease, deoxyribonuclease and α -angelica lactone were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. The lactone was redistilled three or four times before use. RNA was from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany, and DNA was isolated from calf thymus by Mrs D. M. Temple, of this Unit. *N*-Ethylmaleimide was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Specpure (spectrographically pure) KH_2PO_4 was from Johnson Matthey and Co. Ltd. 'Copper-free' potassium phosphate buffer was made from KH_2PO_4 (British Drug Houses Ltd.) recrystallized from EDTA, and conc. volumetric KOH (British Drug Houses Ltd.).

Centrifuging. Centrifugal separations are specified (cf. de Duve & Berthet, 1953) by S_{min} , the uncorrected sedimentation coefficient in Svedberg units (10^{-13} sec.) of particles that would just be completely sedimented, ignoring convection. The corresponding speeds etc. are given in Table 1. With angle heads R_{min} is measured to