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.

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REFERENCES

- Burton, R. M. & Stadtman, E. R. (1953). *J. biol. Chem.* **202**, 873.
 Hestrin, S. (1949). *J. biol. Chem.* **180**, 249.
 Klotzsch, H. & Bergmeyer, H. U. (1962). In *Methoden der Enzymatischen Analyse*, p. 300. Ed. by Bergmeyer, H. U. Weinheim: Verlag Chemie.
 Koch, J. & Jaenicke, L. (1962). *Liebigs Ann.* **652**, 129.
 Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
 Quayle, J. R. (1962). *Biochim. biophys. Acta*, **57**, 398.
 Quayle, J. R. & Keech, D. B. (1960). *Biochem. J.* **75**, 515.
 Quayle, J. R., Keech, D. B. & Taylor, G. A. (1961). *Biochem. J.* **78**, 225.
 Quayle, J. R. & Taylor, G. A. (1961). *Biochem. J.* **78**, 611.
 Shimazono, H. & Hayaishi, O. (1957). *J. biol. Chem.* **227**, 151.
 Stadtman, E. R. (1952). *J. biol. Chem.* **196**, 535.
 Stadtman, E. R. & Barker, H. A. (1950). *J. biol. Chem.* **184**, 769.
 Vagelos, P. R. (1960). *J. biol. Chem.* **235**, 346.
 Vagelos, P. R., Earl, J. M. & Stadtman, E. R. (1959). *J. biol. Chem.* **234**, 765.

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

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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

Table 1. *Centrifuging data*

All centrifuge types were MSE refrigerated.

Separation	$S_{\min.}$	Centrifuge	Head	$R_{\max.}$ (cm.)	$R_{\min.}$ (cm.)	$10^{-3} \times \text{Speed}$ (rev./min.)	Time (min.)
Privet extract from chloroplasts etc.	3850	Superspeed 25		7.37	4	22	5
Cells and nuclei from liver homogenate	172000	Major	no. 6875	20.5	11.8	3.5	4
Acid-precipitated DNA	5880	Major	no. 6885	15.65	13.58	6	10
Calcium ribonucleate from ribonuclease digestion mixture	3940	Major	no. 6885	15.65	13.58	6	15
Mitochondria (including lysosomes) from liver homogenate	1890	Major (superspeed attachment 69401)	no. 69402	5.52	3.04	20	12
Microsomes from liver homogenate	156	Major (superspeed attachment 69401)	no. 69402	5.52	3.04	20	145

the meniscus and $R_{\max.}$ is taken as $R_{\min.}$ plus the maximum horizontal distance from the meniscus to the inside of the outer wall of the tube.

Preparation of inhibitor. The leaves (usually privet) were pulled off their twigs and macerated with 2–5 vol. of water in a Waring Blendor. The resulting sludge was filtered through muslin and then centrifuged (Table 1). Inactive acid-precipitable material was removed from the supernatant by addition of HCl or H_2SO_4 until no further precipitate was formed. Usually 16–18 m-equiv. of acid/100 g. was needed, which brought the pH to about 2.0. The precipitate was centrifuged down and the supernatant adjusted to pH 6.5–7.0 by addition of NaOH. It was then either stored at -15° and used in this form, or dried. In the latter case it was evaporated with a stream of air on a water bath; its temperature was not allowed to exceed 40 – 50° . When it had reached a toffee-like consistency it was freeze-dried. The yield from 100 g. of leaves was about 7.5 g. of brown powder, of which about 1 g. was salt introduced by the acid precipitation followed by neutralization. The acid precipitation removed about the same weight of material. In early preparations cations were removed, with no loss of activity, on a Dowex 50 column, after the acid precipitate had been removed and before neutralization. The standard concentration of extract used in inhibitor tests was 20 mg. of dried powder/ml. The concentrated extract smelled like blackcurrant jam. For the measurement of u.v. absorption the leaf extract at the standard concentration was diluted 1:100 in 0.1N-NaOH. The extinction at $280\text{ m}\mu$ was measured in a 1 cm. cell in a Uvispek spectrophotometer, for comparison with other values obtained during fractionation.

Preparation of rat-liver fractions. Perfused rat liver was homogenized in a non-rotating cylinder with a piston that forced the undiluted liver through filters and later forced the 1:3 diluted mush through a 0.001 in. gap. The apparatus was made of aluminium alloy that had been pickled in dilute HNO_3 in an attempt to avoid possible contamination of the homogenate by copper. The piston was sealed with a Perbunan O-ring. The filters were disks of pickled aluminium alloy with progressively finer holes, from 0.0413 to 0.0138 in. diameter. The 0.001 in. gap was at the bottom, between optically flat faces held apart by a screw thread. The homogenate was finally diluted 1:10 or 1:20 in cold 0.3M-sucrose and centrifuged as shown in Table 1 to give

a mitochondrial (+lysosomal) fraction (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) and a supernatant fraction. The mitochondrial fraction was washed twice with 0.3M-sucrose.

Measurement of ribonuclease inhibition. (a) Pancreatic ribonuclease. A modification of the method of Bernheimer & Steele (1955) was used. Up to 0.2 ml. of leaf extract (i.e. 4 mg. of powder), 0.2 ml. of M-potassium phosphate buffer, pH 7.8, and 0.2 ml. of a solution of 0.5 $\mu\text{g.}$ or 1 $\mu\text{g.}$ of pancreatic ribonuclease/ml., in a total volume of 0.6 ml., were preincubated for 20 min. at 37° . Then 5 ml. of 0.1% RNA was added and the mixture was incubated for 10 min., after which samples (2 ml.) were mixed with portions (2 ml.) of N-HCl at room temperature and the turbidity was measured exactly 10 min. afterwards at $420\text{ m}\mu$ in a Unicam SP. 600 spectrophotometer with a 1 cm. cell. A tube containing neither leaf extract nor ribonuclease was run simultaneously. This gave a maximum value for RNA turbidity from which the other turbidity readings were subtracted. The differences were plotted against the volumes of leaf extract taken to give a calibration curve for determining inhibitor activity, defined as the quantity of a standard preparation needed to produce the observed effect. With partially inactivated inhibitor the term 'percentage of inhibitor activity' is defined as $100[(\text{activity of treated preparation})/(\text{activity of untreated preparation})]$. If it was necessary, as in kinetic experiments, to determine the ribonuclease activity, a calibration curve with up to 0.2 ml. of ribonuclease (1 $\mu\text{g./ml.}$) was prepared under similar conditions.

(b) Alkaline ribonucleases of rat liver. A turbidity method could not be used for measuring inhibition of the alkaline ribonucleases of rat liver because of the larger amounts of protein in the liver fractions. A u.v. method was used, which was less convenient because of the high u.v. absorption of the leaf extract. A further complication was that the alkaline ribonuclease of the supernatant fraction is in an inactive form, combined with the Roth inhibitor, from which it may be released by treatment with sulphhydryl reagents (Roth, 1956). We used N-ethyl maleimide in the following method. A mixture of 0.1 or 0.2 ml. of 1:10 or 1:20 rat-liver fraction, 0.1 ml. of 0.01M-N-ethylmaleimide or 0.1 ml. of water and 0.1 ml. of M-potassium phosphate buffer, pH 7.8, was preincubated for $\frac{1}{2}$ hr. at 37° for maximum activation of ribonuclease

when necessary. Then 0.1 ml. of leaf extract or 0.1 ml. of water was added and the preincubation was continued for $\frac{1}{2}$ hr. or more. Ribonuclease (5 ml. of 0.01% soln.) was then added and the mixture incubated for $\frac{1}{2}$ or 1 hr. To stop the reaction 0.2 ml. of $m\text{-CaCl}_2$ was added. This precipitated undegraded RNA and protein, but not nucleotides. Blank (non-incubated) tubes were also prepared, in which CaCl_2 was added before the RNA. The tubes were cooled in ice for 20 min. and then centrifuged as shown in Table 1. The extinction of the supernatants was measured at 260 $m\mu$ after dilution (1:2) if necessary.

(c) Acid ribonuclease of rat liver. A portion (0.2 ml.) of a twice-washed mitochondrial fraction diluted 1:10 and 0.2 ml. of $m\text{-potassium phosphate buffer}$, pH 5.8, with or without 0.1 ml. of leaf extract, in a total volume of 0.5 ml., were preincubated at 37° for times up to 120 min. Then 5 ml. of 0.01% RNA was added and the mixture was incubated for 30 min. A portion (0.1 ml.) of $n\text{-NaOH}$ and 0.2 ml. of $m\text{-CaCl}_2$ were then added (the NaOH was added to bring the mixture to the pH for precipitation of RNA and liver fractions by CaCl_2). The rest of the procedure was as in (b) above.

Measurement of deoxyribonuclease inhibition. (a) Pancreatic deoxyribonuclease. A portion (0.2 ml.) of pancreatic deoxyribonuclease solution (75 $\mu\text{g./ml.}$), 0.1 ml. of 0.2 $m\text{-potassium phosphate buffer}$, pH 7.0, 0.1 ml. of 0.3 $m\text{-MgCl}_2$, with or without 0.2 ml. of leaf extract, in a total volume of 0.6 ml. were preincubated at 37° for times up to 60 min. Then 0.4 ml. of calf-thymus DNA (0.5 mg./ml.) and 1 ml. of water were added and the mixture was incubated for 90 min. Ice-cold 10% HClO_4 (2 ml.) was added and the tubes were cooled in ice for 10 min. and centrifuged as shown in Table 1. The extinction of the supernatants was measured at 260 $m\mu$, after dilution (1:5).

(b) Acid deoxyribonuclease of rat liver. A twice-washed mitochondrial fraction was used as the source of acid deoxyribonuclease. A portion (0.2 ml.) of this fraction, 0.4 ml. of 0.5 $m\text{-sodium acetate buffer}$, pH 5.2, with or without 0.02 ml. of leaf extract, in a total volume of 0.62 ml. was preincubated at 37° for times up to 60 min. The rest of the procedure was as described above for alkaline deoxyribonuclease.

Inactivation of leaf extracts by acid or alkali. The acid or alkaline inhibitor solutions were neutralized, after cooling in ice if the experiment had involved heating, and NaCl was added to the control inhibitor solutions to bring them to the same ionic strength as the acid- or alkali-treated solutions. They were then evaporated to dryness by use of an air-blower at 37°, and made up to their original volumes for testing.

Inactivation of leaf extracts by acidic alcohol. Up to 1 ml. of ethanol containing various concentrations (usually 0.04 n) of H_2SO_4 or HClO_4 was added to weighed samples of about 4 mg. of the dried extract, which were then heated in stoppered or sealed tubes for times up to 3 hr. The tubes were cooled in ice, and the sealed tubes centrifuged to remove liquid from the top before they were opened. The contents were carefully neutralized with $n\text{-}$ or 2 $n\text{-Na}_2\text{CO}_3$, a magnetic stirrer being used, and evaporated to dryness as described above. Water (0.2 ml.) was added to give approximately the same concentration as in the 20 mg./ml. standard, and the inhibitor activity was measured.

Reactivation of leaf extract. (a) In aqueous acid. Aqueous HCl at concentrations stated in the text was added to the

dried inactivated material, which was then reheated at 60° for various times. The solutions were cooled and neutralized with $n\text{-Na}_2\text{CO}_3$ and the rest of the procedure was as described above.

(b) In acidic alcohol. After the extracts had been inactivated and cooled, various volumes of aqueous acid (usually HClO_4) were added to the solutions to give whatever final concentrations of ethanol and acid were wanted, and the tubes were then reheated as described above. Control tubes were prepared containing the same amounts of acid and alcohol. They were not heated but immediately neutralized and evaporated to dryness, and 0.2 ml. of water was added as described above. These procedures caused little or no inactivation, and the activities in the experimental tubes were expressed as percentages of those in the control tubes.

Estimation of histidine. The method of Newman & Turnbull (1960) was used with the concentration of the KI_2 reagent doubled (0.15 g. of I_2 in 0.8 g. of KI in 10 ml. of water; AnalaR solids). Sodium thiosulphate (AnalaR) was used instead of sodium dithionite to check further colour formation. To adjust the mixture to pH 11.0–12.0, 0.05 ml. of 0.2 $n\text{-NaOH}$ was needed when the sample contained histidine alone, and 0.04 ml. of $n\text{-NaOH}$ when the sample (0.2 ml.) also contained 0.33 $m\text{-phosphate buffer}$, pH 7.8. Under these conditions the calibration curve was linear over the range used, 0.2 μmole of histidine giving an extinction coefficient of 0.396 at 560 $m\mu$. In experiments with privet a calibration curve was prepared in which the appropriate amount of privet extract was added to histidine immediately before sampling. Privet extract (1 mg.) itself gave an extinction coefficient of 0.037. After this value was subtracted, the extract was seen to reduce the extinction with 0.15 and 0.3 μmole of histidine by 30 and 33% respectively.

RESULTS

Storage of inhibitor. The inhibitor remained active indefinitely in frozen neutral solution at -15° , and in the dry powdered form in a desiccator at room temperature. After storage for 4 weeks at pH 2.1 and -15° some solid material failed to redissolve and the inhibitory activity of the solution was reduced to 43% of the original. In neutral solution at room temperature the activity was reduced to 65% of the original after 5 weeks.

Stability to heat, acid and alkali. Fig. 1 shows that the inhibitor from privet is not inactivated by heating in 0.1 $n\text{-HCl}$ for 15 min. at 100°. It is rather less stable in neutral solution, and rapidly inactivated in 0.1 $n\text{-NaOH}$. These results are in contrast with the finding of Bernheimer & Steele (1955) that the inhibitor from lilac was stable on heating at 100° for 15 min. in either 0.1 $n\text{-HCl}$ or 0.1 $n\text{-NaOH}$. Their material had been partially purified, so that impurities in our material could have made it less stable. Possibly also the inhibitors in the two plants are different substances. Inactivation by alkali was investigated further. In the first experiment, in which several concentrations of NaOH were used, the solutions were stirred

to avoid high local concentrations of alkali during the addition of NaOH, and neutralization with HCl was completed about 15 sec. later. Nevertheless the activity was reduced to 50 % of the original by 0.16N-NaOH at room temperature and the relation between residual activity and concentration of NaOH was linear. Fig. 2 illustrates the time-course of inactivation by 33 mN-NaOH at 38°.

Fig. 1. Stability of inhibition from privet at 100°. Results are expressed as percentage of activity of untreated inhibitor. (A) 0.1N-HCl; (B) water; (C) 0.1N-NaOH; (D) ethanolic 0.04N-HClO₄.

Fig. 2. Inactivation of inhibitor from privet by 33 mN-NaOH at 38°.

Reversible inactivation by alcohols. One preparation of privet extract was dried on a rotary evaporator at about 40° while still acid (0.04N-HCl, pH 2.1) and the drying was completed by several additions of small volumes of ethanol. This preparation was stored as a dried powder, and when tested was completely inactive. The inhibitor was inactivated by heating in alcohols; 10 min. at 100° in ethanol, methanol or propanol reduced the activity to about 50 % of its original value. Acidic alcohol was even more destructive of inhibitor activity (Fig. 1D). On addition of more HCl so that the final concentration of additional HCl was about 0.04N, and heating for 2 min. in a boiling-water bath, the activity was restored to 30–60 % of the original. Heating for longer, or in more concentrated acid, caused no further reactivation. Heating with no additional acid, or in alkali, or addition of acid without heating, caused no reactivation.

Fig. 3 shows the time-course of inactivation and reactivation under more carefully controlled conditions (see Experimental section). After 3 hr. at 60° in 1 ml. of ethanolic 42 mN-H₂SO₄ the activity was reduced to about 5 % of the original. The solution was then neutralized, evaporated to dryness and 0.24 ml. of aqueous 0.17N-HCl was added. After a

Fig. 3. Inactivation of acidified inhibitor from privet in ethanol and reactivation in water. Inactivation (left-hand curve) was in ethanolic 42 mN-H₂SO₄ and reactivation (right-hand curve) in aqueous 0.17N-HCl.

further 3 hr. at 60° the activity had returned to 72 % of the original.

If ethanol was present in the reactivation mixture the amount of reactivation was reduced, and the ratio of active to inactive material in the equilibrium mixture was determined by the concentration of ethanol present. Fig. 4 illustrates an experiment in which this equilibrium was approached from both sides, for two concentrations of ethanol. The equilibrium constant

$$[K = (\text{active/inactive}) \times (\text{ethanol/water})]$$

was 2.9 for 60 % ethanol and 3.1 for 83 % ethanol. In both cases the concentration of perchloric acid was 0.04N.

Solubility. Water was the best solvent found for both the dried solid and the inhibitor it contained. About 8 g. of solid dissolved in 100 ml. of water at room temperature. Comparatively small percentages of the activity went into other solvents, and these had about the same ratio of activity to u.v. absorption, so that there was no evidence of purification.

The activity could not be extracted by dry butanol, propanol or ethanol, but it could by butanol or propanol containing 15 % of water. For example, 3 ml. of butanol containing 15.8 % (v/v) of water extracted 30 % of the activity from 200 mg. of dry powder, and doubled the ratio of activity to extinction at 280 m μ in 0.1N-NaOH.

In butanol-water partition experiments, when the initial volume ratio of butanol to water was 3.33 the activity was equally distributed between

butanol and water at pH 7.0. As the pH was lowered more activity went into the butanol layer, 70–90 % going into butanol at pH 3–4.

Precipitation. Ethanol and acetone precipitation were also tried as methods of freeing the inhibitor from impurities. The same volume (3 ml.) of both ethanol and acetone precipitated 34 % of the total activity from 0.5 ml. of leaf extract containing 80 mg. of solid/ml., but in the redissolved precipitates the ratio of activity to u.v.-absorbing material was not improved.

According to Bernheimer & Steele (1955) impurities were preferentially precipitated from lilac extract by lead acetate. We found with privet extract that the inhibitor was precipitated together with the impurities. At pH 4.3 50 % of the inhibitor was precipitated from 2 ml. of a solution containing 20 mg. of solid/ml. by 0.008 ml. of m-lead acetate, and at pH 2.1 by 0.08 ml. The ratio of inhibitor activity to extinction at 280 m μ was not increased more than threefold by any of the purification methods tried. In the precipitation experiments the dry weights of the various fractions ran parallel with their u.v. absorption, so that the ratio of activity to neither quantity was improved. The specific extinction coefficient ($E_{1\text{ cm.}}^{1\%}$) is 43.7 in 0.1N-NaOH at λ_{max} , 285 m μ , and 35.0 in 0.1N-HCl at λ_{max} , 280 m μ .

Inhibition of pancreatic ribonuclease

Effect of pH. Fig. 5 shows curves for pancreatic ribonuclease activity in the absence and presence of privet inhibitor. The non-inhibited activity shows the maximum at about pH 7.8 expected at I 0.1 (Kalmitsky, Hummel & Dierks, 1959). The percentage inhibition by privet appears to be maximal at a slightly more alkaline pH.

Kinetics of reaction between enzyme and inhibitor. Bernheimer & Steele (1955) found that their material inhibited ribonuclease only if it was allowed to react with the enzyme for some time before addition of RNA. We confirmed this with privet, and found that the reaction between ribonuclease and inhibitor appears to obey first-order kinetics. Presumably the inhibitor concentration is large compared with the enzyme concentration, and may be considered constant. The bimolecular rate constant k for the reaction from the first-order equation $\ln(x_0/x) = kct$, where x_0 is initial activity of ribonuclease, x is activity of ribonuclease at time t (sec.) after the start of the preincubation and c is concentration of unpurified inhibitor (mg./ml. of preincubation mixture), was determined from three graphs which were all satisfactorily linear, at least in the earlier stages. The plot of $\log(x_0/x)$ against c for constant t gave k 8.8×10^{-4} l.g.⁻¹ sec.⁻¹, that of $\log(x_0/x)$ against t for constant c gave k 9.1×10^{-4} l.g.⁻¹ sec.⁻¹, and that of c plotted against $1/t$

Fig. 4. Approach to equilibrium from both sides. Initial inactivation (two lowest points) was by heating for 1 hr. at 60° in 0.04N-HClO₄ in absolute ethanol. To reactivate, 0.04N-HClO₄ in water was added to give the final ethanol concentration stated, and the tubes were reheated: ○, inactivation and reactivation in 0.04N-HClO₄ in 60 % (v/v) ethanol; ●, inactivation and reactivation in 0.04N-HClO₄ in 83 % (v/v) ethanol.

for constant $\log (x_0/x)$ (1.0) gave $k\ 8.3 \times 10^{-4}\ \text{l.g.}^{-1}\ \text{sec.}^{-1}$.

Reaction of privet extract with histidine. Histidine protects ribonuclease against the inhibitor to some extent (Fig. 6, curve A) when added with the enzyme, and better (Fig. 6, curve B) when it is allowed to react with privet before the addition of ribonuclease. The reaction between privet extract and histidine was studied directly by histidine estimations. Two facts emerged: the reaction is proportional to privet concentration but independent of histidine concentration if the latter is not too low, as in the ' $S_N I$ ' mechanism of Hughes & Ingold (1935), and secondly the reactive component in privet is transformed in phosphate buffer, pH 7.8, into a less reactive substance, which can still react with the same amount of histidine but much more slowly. The independence of histidine concentration is shown in Fig. 7 by the fact that the curves relating concentration of histidine after various times to initial concentration are at 45° to the axes in their upper regions. The transformation to a less reactive substance is shown in Fig. 8, relating histidine remaining to time of

Fig. 5. Effect of pH on ribonuclease activity and its inhibition by privet extract. O, Ribonuclease activity curve (values are percentages of value at pH 7.85); x, activity of ribonuclease preincubated for 20 min. with privet inhibitor (values are percentages of non-inhibited value at pH 7.85); ●, percentage inhibition by privet. Preincubation mixture contained 0.1 $\mu\text{g.}$ of ribonuclease $\pm$ 1.2 mg. of privet extract and sufficient M-phosphate buffer to give I 0.1 in final incubation mixture. Total vol. 0.725 ml.

Fig. 6. Protection by histidine of ribonuclease from inhibition by privet extract. (A) Preincubation mixture contained 0.1 $\mu\text{g.}$ of ribonuclease, 1.0 mg. of privet extract, 0.2 m-mole of phosphate buffer, pH 7.8, and various amounts of histidine; total vol. 0.6 ml., preincubation time 3 hr. (B) Preincubation mixture contained privet extract, phosphate and histidine as above; total vol. 0.45 ml.; after 3 hr. 0.2 $\mu\text{g.}$ of ribonuclease was added and preincubation continued for a further 3 hr.; final vol. 0.65 ml.

Fig. 7. Effect of incubation with privet extract at 37° for various times on histidine present initially: (A) no incubation; (B) $\frac{1}{2}$ hr.; (C) 1 hr.; (D) 2 hr.; (E) $77\frac{1}{2}$ hr. Incubation mixture contained 1.0 mg. of privet extract, 0.2 m-mole of phosphate buffer, pH 7.8; total vol. 0.6 ml.

reaction with privet extract at 100° before and after the privet had been heated at 100° for 30 min. in phosphate buffer, pH 7.8. At 37°, with the concentrations of reactants shown in Fig. 8, half the histidine is destroyed by untreated privet in about 18 min. These later experiments taken in conjunction with the earlier stability tests in neutral solution suggest that the rate of transformation into the less reactive substance is highly pH-dependent between pH 6 and 8.

The histidine equivalent of our solid privet extract is about 2×10^{-4} mole/g., as can be determined by extrapolating Fig. 7 to infinite time and zero final histidine concentration. This makes the bimolecular constant for inhibition of ribonuclease about $4.5 \text{ l.mole}^{-1} \text{ sec.}^{-1}$, corresponding to $9 \times 10^{-4} \text{ l.g.}^{-1} \text{ sec.}^{-1}$ as quoted above. The bimolecular constant for the less reactive transformation product is about one-twentieth of this, i.e. about $0.2 \text{ l.mole}^{-1} \text{ sec.}^{-1}$.

Inhibition of rat-liver ribonucleases

Mitochondrial ribonucleases. The mitochondrial fraction contains two ribonucleases with overlapping pH-activity curves (Roth, 1957). Figs. 9 and 10 show inhibition by privet extract at their pH optima, 5.8 and 7.8. At both pH values the degree of inhibition depends on both preincubation time and inhibitor concentration. The apparent inhibition at zero preincubation time in Fig. 9 is due to the action of the inhibitor on the enzyme during the 30 min. incubation time.

Fig. 8. Destruction of histidine at 100° by: ●, untreated privet extract; ○, privet extract previously treated for 30 min. at 100° in phosphate buffer, pH 7.8. Reaction mixture contained histidine 60×10^{-8} m-mole, privet 3.0 mg., phosphate buffer, pH 7.8, 0.2 m-mole; total vol. 0.6 ml. (Privet 'blanks', due to increase in $E_{560 \text{ m}\mu}$ with time caused by heating privet alone, were subtracted.)

Supernatant ribonuclease. The alkaline ribonuclease of the supernatant fraction released from the Roth inhibitor by incubation with *N*-ethylmaleimide is also inhibited by privet extract, as shown in Fig. 11. Table 2 gives the results of experiments in which histidine was added to the preincubation mixture in concentration sufficient to inactivate the privet extract (0.1 ml. of 0.08 M-histidine added to 0.1 ml. of privet extract). In Expt. 1 all the components were preincubated

Fig. 9. Inhibition of rat-liver mitochondrial ribonuclease by privet inhibitor at pH 5.8. ○, No inhibitor; ●, 2 mg. of inhibitor/0.5 ml. of preincubation mixture.

Fig. 10. Inhibition of rat-liver mitochondrial ribonuclease at pH 7.8. Preincubation time 135 min.

together for 2.5 hr. In Expts. 2 and 3 the components were added consecutively to the preincubation mixture in the order in the Table, with delays after each addition as follows: supernatant + *N*-ethylmaleimide 30 min., privet extract 2 hr., histidine 30 min., *N*-ethylmaleimide 30 min. Line (b) of Table 2 shows inhibition by privet extract of the enzyme liberated by *N*-ethylmaleimide, and (d) shows that this inhibition is prevented by simultaneous preincubation with histidine (Expt. 1) but is not reversed when histidine is added after the privet extract is allowed to act on the enzyme (Expts. 2 and 3). Expt. 3(e) shows that privet extract can even attack 'inactive ribonuclease' (Roth, 1958), i.e. ribonuclease that

has not been released from its inhibitor by *N*-ethylmaleimide. The privet extract was added before *N*-ethylmaleimide, and excess of privet was then inactivated by incubation with histidine. Subsequent addition of *N*-ethylmaleimide gave reduced ribonuclease activity.

Inhibition of deoxyribonucleases

Privet extract did not inhibit either pancreatic deoxyribonuclease or the acid deoxyribonuclease of rat liver, even when preincubated with the enzyme before addition of the substrate.

Other inhibitors of pancreatic ribonuclease

The finding that the privet inhibitor could be reversibly inactivated by heating in acidic alcohol suggested that it might be a lactone. The following lactones were therefore tested (usually at a concentration of 20 mg./ml.) for their ability to inhibit ribonuclease: glucuronolactone, γ -butyrolactone, γ -valerolactone, β -propiolactone, D-galactono- γ -lactone, α -D-gluconoheptono- γ -lactone, 1,4-gluconolactone, D-arabonic acid- γ -lactone, D-ribonic acid lactone, DL-homocysteine thiolactone, DL-pantoyl-lactone, α - and β -angelica lactones. Of these only α -angelica lactone was found to inhibit.

As with the privet inhibitor, a preliminary incubation period of the lactone with ribonuclease was necessary. The lactone did not inhibit when low concentrations of EDTA were present in the preincubation mixture, or when the phosphate buffer was made from KH_2PO_4 that had been recrystallized from EDTA. Its inhibitory activity was increased by the addition of very low concentrations (1–10 μM) of CuSO_4 to the preincubation mixture. Addition of histidine to the preincubation mixture abolished this copper-catalysed inhibition by α -angelica lactone.

Since both Zittle (1946) and Davis & Allen (1955) found that zinc was almost as inhibitory as copper to ribonuclease, zinc was tested both as an inhibitor and for its effect on inhibition by privet and α -angelica lactone. In our assay system concentrations of ZnSO_4 or ZnCl_2 up to 3.5 mM did not inhibit and did not augment the inhibition by privet or α -angelica lactone.

Fig. 11. Inhibition of ribonuclease from rat-liver supernatant at pH 7.8. (A) Supernatant fraction; (B) supernatant + *N*-ethylmaleimide; (C) supernatant + privet inhibitor; (D) supernatant + *N*-ethylmaleimide + inhibitor. Preincubation time 1 hr. Inhibitor and *N*-ethylmaleimide blanks were subtracted.

Table 2. Effect of privet extract, with or without histidine, on ribonucleases of rat-liver supernatant fraction

Components of preincubation mixture	Ribonuclease activity (arbitrary units)		
	Expt. 1 (all components preincubated together)	Expt. 2 (components added consecutively)	Expt. 3 (components added consecutively)
(a) Supernatant + <i>N</i> -ethylmaleimide	469	231	425
(b) Supernatant + <i>N</i> -ethylmaleimide + privet extract	66	60	0
(c) Supernatant + <i>N</i> -ethylmaleimide + histidine	—	279	403
(d) Supernatant + <i>N</i> -ethylmaleimide + privet extract + histidine	570	20	70
(e) Supernatant + privet extract + histidine + <i>N</i> -ethylmaleimide	—	—	130

*Comparison of privet extract
and α -angelica lactone*

As is shown in Figs. 12 and 13, the inhibitory activity of α -angelica lactone is catalysed by copper salts and abolished by EDTA, but this is not the case with privet extract. Without copper, angelica lactone gave no inhibition, and privet gave 54% inhibition. Fig. 12 shows that as the copper concentration was increased, its catalytic effect on the inhibition by angelica lactone started abruptly at concentrations at which copper gave little inhibition by itself. On the other hand, copper added to privet gave the degree of inhibition that might have been expected from their combined inhibitory effects. Fig. 13 shows the effect of swamping the added CuSO_4 ($10^{-4.3}$ m-mole) by adding increasing concentrations of EDTA to the preincubation mixture. When enough EDTA had been added to combine with all the copper, the inhibitory activity of the angelica lactone was reduced to zero, but privet had about the same inhibitory activity as it had with no added copper. Both the lactone and the phosphate buffer were

used in unnecessarily high concentration in these two experiments, as the optimum phosphate concentration for inhibition by lactone had not then been determined.

Another difference between privet extract and α -angelica lactone was the fact that although both were inactivated by heating in acidic alcohol, α -angelica lactone being presumably converted into laevulinic ester, privet extract could be re-activated by heating in aqueous acid, whereas α -angelica lactone could not. That is, although the opening of the α -angelica lactone ring is irreversible in aqueous solution, the privet substance, if a lactone, can apparently undergo reversible ring opening.

A further difference was that oxygen was found to be necessary for the inhibition of ribonuclease by α -angelica lactone, but not by privet extract.

Other compounds tested for inhibition of ribonuclease

It was thought that the inhibitory substance in privet might be a coumarin, so the following

Fig. 12. Inhibition of ribonuclease by Cu^{2+} ions alone ($\circ$) and in combination with $0.56 \mu\text{mole}$ of α -angelica lactone ($\times$), or 0.4 mg. of privet extract ($\bullet$). Preincubation mixture also contained 0.2 m-mole of copper-free potassium phosphate buffer, pH 8.0; total vol. 0.65 ml. Preincubation time 30 min.

Fig. 13. Effect of EDTA on inhibition of ribonuclease by $10^{-4.3} \text{ m-mole}$ of CuSO_4 alone ($\circ$), and in combination with $1.12 \mu\text{moles}$ of α -angelica lactone ($\times$), or 0.4 mg. of privet extract ($\bullet$). Preincubation mixture also contained 0.2 m-mole of Speccure potassium phosphate buffer, pH 8.0; total vol. 0.7 ml. Preincubation time 30 min.

coumarins or coumarin glycosides were tested for inhibition of ribonuclease at a concentration of 20 mg./ml., or less, depending on solubility: 4-methylaesculetin, aesculin, umbelliferone, coumarin, 4-hydroxycoumarin, xanthotoxin and the related substance rotenone. None had any ribonuclease inhibitory activity. Other substances tested and found to be without inhibitory activity were: catechol, the flavonol aglycones quercetin and morin and glycosides quercetrin and rutin, 1,4-naphthaquinone, tetrahydroxyquinone, ascorbic acid, thioglycollic acid, cysteine, kojic acid, colchicine, penicillin, dehydroacetic acid, usnic acid, ethyl orthoformate, phenolphthalein, strophanthins G and K, digitalin, vinyl acetate monomer, diethyl carbonate, citraconic anhydride, cisaconitic anhydride, glycine anhydride, creatinine, meconic acid, piperonaldehyde, conidendrin, benzimidazole, 2-aminobenzimidazole, chelidonic acid and spermine phosphate. Three substances that did inhibit, probably by acting as acylating agents, were ethyl chloroformate, benzyl chloroformate and isocyanatobenzene. They reacted rapidly with ribonuclease and no appreciable preincubation period was necessary.

DISCUSSION

We think that the active substance in privet is of unusual chemical interest and should be isolated. For the isolation we recommend butanol-water partition chromatography and/or temporary conversion into the alcohol-inactivated product. The reaction with histidine suggests that the substance is an alkylating or acylating agent. The two properties suggesting that it is a very unusual one are its reversible inactivation by acidified alcohols and its transformation at pH 7.8 into another less active alkylating or acylating agent. Preliminary tests with mice suggest that its toxicity is very low; but if the active group were identified and incorporated into a bifunctional compound results might be different. If such a compound were made it would obviously be worth testing as an antileukaemic agent. As an inhibitor of ribonuclease, e.g. for cell homogenates, the privet substance is likely to be of limited usefulness because its action on histidine groups is unlikely to be confined to ribonuclease.

There is also some chemical and possibly biological interest in the discovery that α -angelica lactone inhibits ribonuclease when incubated with it in the presence of air and a catalytic copper salt.

SUMMARY

1. An extract of privet leaves that inhibits ribonucleases from rat liver and pancreatic ribonuclease does not inhibit deoxyribonuclease.
2. The inhibition is abolished by histidine, which is itself destroyed.
3. Hot acidified alcohols abolish the inhibitory activity, and hot acidified water causes it to reappear. This suggests reversible opening of a lactone or lactam ring.
4. α -Angelica lactone, alone among several lactones tried, inhibits ribonuclease, but its inactivation by alcohols was not reversed by water.
5. The inhibitory power of α -angelica lactone, but not of privet extract, is suppressed by ethylenediaminetetra-acetate and enhanced by traces of copper salts.
6. The presence of oxygen is necessary for the inhibitory activity of α -angelica lactone but not of privet extract.

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REFERENCES

- Barnard, E. A. & Stein, W. D. (1959). *J. molec. Biol.* **1**, 339, 350.
- Bernheimer, A. W. & Steele, J. M. (1955). *Proc. Soc. exp. Biol., N.Y.*, **89**, 123.
- Davis, F. F. & Allen, F. W. (1955). *J. biol. Chem.* **217**, 13.
- de Duve, C. & Berthet, J. (1953). *Nature, Lond.*, **172**, 1142.
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955). *Biochem. J.* **60**, 604.
- Hughes, E. D. & Ingold, C. K. (1935). *J. chem. Soc.* p. 244.
- Kalnitsky, G., Hummel, J. P. & Dierks, C. (1959). *J. biol. Chem.* **234**, 1512.
- Newman, P. M. & Turnbull, J. H. (1960). *Biochem. J.* **74**, 379.
- Roth, J. S. (1956). *Biochim. biophys. Acta*, **21**, 34.
- Roth, J. S. (1957). *J. biol. Chem.* **227**, 591.
- Roth, J. S. (1958). *J. biol. Chem.* **231**, 1097.
- Stein, W. H. (1960). *Brookhaven Symp. Biol.* **13**, 104.
- Zittle, C. A. (1946). *J. biol. Chem.* **163**, 111.