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4. Phocaecholic acid was degraded to norchenodeoxycholic acid (3α:7α-dihydroxynorcholanic acid), also prepared by Wieland–Barbier degradation from chenodeoxycholic acid. This work confirms the constitution suggested for phocaecholic acid by Windaus & van Schoor (1928).

5. In none of the C-23-hydroxylated bile acids described could the optical configuration at C-23 be determined by measurements of $[\alpha]_D$.

6. Although snakes of the genus *Bitis* contain C-23-hydroxylated bile acids, previously thought to be confined to the Pinnipedia, the types of bile acids present do not indicate a very close similarity between the bile salts of these snakes and those of Pinnipedia.

The author thanks Bernard Stonehouse of the Falkland Islands Dependencies Survey for the bile of the leopard seal, and also Dr W. C. Osman Hill, Mr R. N. Fiennes and the Zoological Society of London for the sealion and snake biles. He also thanks Professor S. Bergström for a specimen of his 3α:7α:12α:23-tetrahydroxycholanic acid and for friendly discussions, and Dr R. J. Bridgwater for some fractions of ethylated bile acids (sealion).

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The Breakdown of Chlorophyll by Chlorophyllase

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The first stage in the enzymic breakdown of chlorophyll *in vivo* may be the removal of the phytol side chain; chlorophyllase, the enzyme which *in vitro* catalyses its removal and attachment, has been found in most plants. The enzyme was discovered by Willstätter & Stoll (1910), and Mayer (1930) made an extensive survey of chlorophyllase activity in different species and investigated some properties of the enzyme. Krossing (1940) found that it was in the chloroplast fraction of leaves. Chlorophyllase action *in vitro* usually requires the presence of a solvent such as acetone (40–70 %, w/v) or methanol (70–80 %), but Weast & Mackinney (1940) found that in a few species at some seasons of the year there was no hydrolysis in ethanol, though an enzyme system active in water at 70° was present. Peterson & McKinney (1938) investigated the effect of mosaic viruses on the chlorophyllase activity and chlorophyll content of tobacco leaves.

Dried leaves have frequently been used as a source of enzyme for preparing chlorophyllides from chlorophyll. Fischer & Lambrecht (1938) used a leaf-meal preparation for studying the specificity of chlorophyllase action with various chlorophyll derivatives.

Mayer (1930) found that little or no chlorophyllase activity was extracted from leaf powders by water, aqueous buffer solutions or glycerol. Willstätter & Stoll (1910) obtained a trace of soluble chlorophyllase by subjecting macerated fresh leaves to 250 atmospheres pressure. Ardao & Vennesland (1960) found that a soluble chlorophyll–lipoprotein complex obtained when spinach chloroplasts are treated with digitonin had chlorophyllase activity.

The present paper is concerned with the preparation of soluble chlorophyllase, its partial purification and some of its properties.

MATERIAL AND METHODS

Plant material

Sugar beet (*Beta vulgaris* var. *saccharifera*) leaves were obtained from experimental plots on Rothamsted Farm. The leaves could be kept at 0° in polythene bags for up to a

month with little loss of chlorophyllase activity. Seedlings of pea (*Pisum sativum* var. Sutton's Improved Pilot), bean (*Phaseolus vulgaris* var. Canadian Wonder), wheat (*Triticum vulgare* var. Capelle Desprez) and barley (*Hordeum vulgare* var. Proctor) were grown in a glasshouse either in moist sand in glass tanks or in compost in pots. Leaves of other species were from plants cultivated in local gardens or growing wild.

Preparation of enzyme-containing material

(1) Leaves were dried in an oven at 40° and ground in a Casella Grain Mill with 0·5 mm. plate. Although the powder contained both enzyme and substrate, chlorophyll was not converted into chlorophyllide until it was incubated in aqueous acetone. (2) Fresh leaves were ground in a Townson and Mercer Ltd. top-drive macerator in sufficient cold acetone to give a concentration of about 80%. The suspension was filtered on a Büchner funnel; the solid portion was washed with acetone to remove as much pigment as possible, dried at room temperature and then ground finely in a Casella mill.

Method of determining chlorophyllase activity

A number of methods have been proposed for determining chlorophyllase activity. One of several described by Willstätter & Stoll (1910), which was later used by Mayer (1930) and Peterson & McKinney (1938), is fairly satisfactory as a routine method. However, it requires extraction of pigment first into ether and then into alkali and an emulsion is often formed. A chromatographic method described by Sironval (1954) and used by Gage & Aronoff (1956) is suitable where great accuracy is required but is too slow and laborious for routine use. A method used by Weast & Mackinney (1940) based on the insolubility of chlorophyllides in light petroleum was comparatively simple and formed the basis for the method used in the present investigation and also for that described recently by Ardao & Vennesland (1960).

For determining the activity of fresh leaf and of dried leaf meal the substrate was the chlorophyll already present. For acetone-dried powders and soluble preparations the substrate was an acetone extract of fresh bean leaves which in addition to the chlorophylls contained xanthophylls and carotene. It was prepared by grinding the leaves in acetone (4 ml./g. of leaf), filtering rapidly through a Büchner funnel and then storing the extract at 0° for several days before use. Carotene which precipitated out during storage was removed by filtration. The solution had a chlorophyll content of about 0·5 g./l. as determined by the method of Arnon (1949).

Powders. A weighed sample of powder (up to 500 mg.) was incubated at room temperature in the dark with 8 ml. of chlorophyll solution and 4 ml. of sodium citrate solution, the final concentration of acetone being about 50% and of citrate 0·04 M; the suspension had a pH of about 8. Powders containing both enzyme and substrate were incubated under similar conditions but without added chlorophyll. After incubation for 1, 3 or 18 hr., depending on the activity of the preparation, the powder was filtered off on a Büchner funnel and washed with acetone until all pigment was extracted. To the filtrate 10 ml. of 2% (w/v) NaCl was added and the volume made up to 50 ml. with 80% acetone. Half the solution was kept for determination of the total chlorophyll present. The other half was shaken twice with 10 ml. lots of light petroleum (b.p. 40–60°) to extract the unchanged chlorophyll. The light petroleum was discarded and the acetone layer containing the chlorophyllides was again made up to 25 ml. with 80% acetone. The absorption of the two solutions was read in an EEL photoelectric colorimeter with filter 607. Even when the conversion of chlorophyll into chlorophyllide was complete the absorption maximum at 660 mμ was decreased by less than 5%, so there was no 'bleaching' due to enzymic action or other causes under the conditions used.

The enzyme activity was expressed as a percentage of the substrate split in a given time, 1 unit being defined as the amount which will catalyse the breakdown of 10% of the substrate to chlorophyllide in 1 hr.

Soluble preparations. The final concentration of acetone was 40% and the incubation period was 1 hr. or less. It was not necessary to filter before extraction with light petroleum.

Fresh leaf. A sample of fresh leaf was ground in ten times its weight of 50% acetone and the suspension was incubated for 1, 3 or 18 hr.

Nitrogen. This was determined by a micro-Kjeldahl method with SeO_2–$CuSO_4$–K_2SO_4 (1:1:8) catalyst.

EXPERIMENTAL AND RESULTS

Distribution and formation of chlorophyllase

Although Mayer's (1930) survey of activity in different species suggested possible useful sources for obtaining the enzyme in amounts sufficient for starting purification, many plants were examined in the course of investigation. The high activity of *Heracleum*, *Lamium* and *Dahlia* and the very low activity of *Urtica*, *Cucurbita* and *Phaseolus* was confirmed. Woody nightshade (*Solanum dulcamara*) had activity comparable with that of *Heracleum*. The leaves of both spinach and sugar beet (varieties of *Beta vulgaris*) had much higher activity than all other species tested. White bryony (*Bryonia dioica*), tomato (*Lycopersicum esculentum*), tobacco (*Nicotiana tabacum*) and Brussels sprout (*Brassica oleracea gemmifera*) all had low activity. Monocotyledons mostly appear to have lower activity than dicotyledons, though the activity of wheat was comparatively high. No activity was detected in the shoots of young seedlings of barley.

Chlorophyllase distribution in pea seedlings. Although the chlorophyllase activity of pea seedlings is low, they provide convenient material for studying changes in enzyme activity during germination and growth.

Dried peas were soaked in tap water overnight and samples (10 peas) were weighed and used for chlorophyllase determinations. The soaked peas were sown in moist sand and sampled at intervals for 14 days. The temperature of the glasshouse in which the peas were grown was 16–17° and the seedlings were in daylight for about 9 hr. daily. Artificial light was provided by 'cool white'

fluorescent tubes for a further 6 hr. daily. Duplicate lots were taken at each sampling and as soon as the embryos started to develop the seedlings were divided into cotyledons (with testas discarded), roots, stems and leaves, which were weighed separately. On some occasions samples were taken for dry-matter determination. For chlorophyllase estimation each portion was ground in acetone and the acetone-dried powder was incubated with chlorophyll for 18 hr. because of the low activity. Fig. 1 shows chlorophyllase changes during the first 2 weeks of growth. The enzyme activity in the soaked cotyledons was low; the activity doubled in the next 2 days and then fell again as the embryo developed. The radicle and roots were almost devoid of activity at every sampling. At 10 days 75% of the activity was in the shoot and most of it was in the leaves. The activity of the leaves expressed on a wet-weight basis rose sharply between the fifth and tenth days and then remained nearly constant although the amount per seedling was still increasing.

Effect of light on the chlorophyllase activity of pea seedlings. Seedlings grown in the dark had much lower activity than those in the light. At 10 days, leaves from light-grown seedlings had four times as much activity as dark-grown. When dark-grown seedlings were transferred to light the enzyme activity in the leaves increased (Fig. 2). The formation of enzyme paralleled the development of chlorophyll, and after 2 days both were only slightly less than in the light-grown seedlings.

In other experiments the seedlings were grown in compost instead of sand so that they could be kept longer. The activity of dark-grown seedlings after a small initial rise did not continue to increase after the fourteenth day, but it rose rapidly when seedlings were transferred to light even after 20 days in the dark.

Soluble enzyme preparations

A variety of methods (such as the use of detergents, incubation with other enzymes etc.) which have been used for making soluble preparations of other enzymes were tried in unsuccessful attempts to prepare soluble chlorophyllase from the leaves of several species. It was only when sugar-beet leaves had been found to provide a starting material of exceptionally high activity that a method of making a soluble enzyme preparation was found. The method does not, however, appear to be of general application; soluble preparations could not be obtained from *Heracleum* leaves nor from several other species which have moderately high activity. The activity in extracts from nightshade leaves was too low to be of any use.

Some activity could be extracted from an acetone-dried powder of sugar-beet leaves by a neutral or slightly alkaline buffer solution, but clarification of the extract by centrifuging or by filtration through diatomaceous earth removed

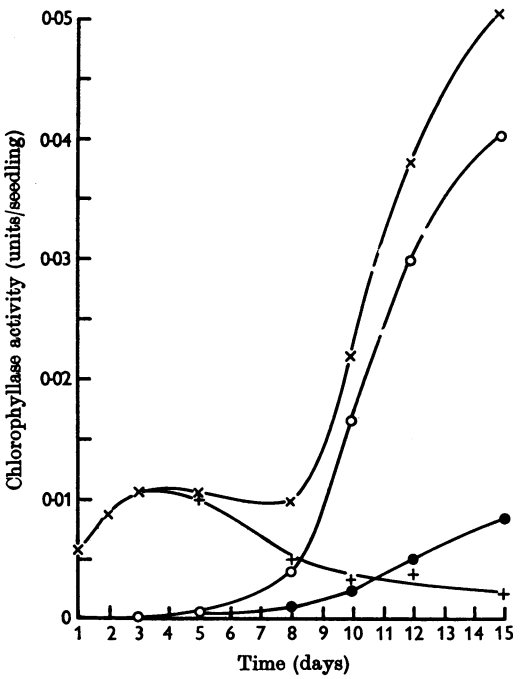

Fig. 1. Chlorophyllase activity during germination and growth of pea seedlings in the light. ×, Total; +, cotyledons; ○, leaves; ●, stem.

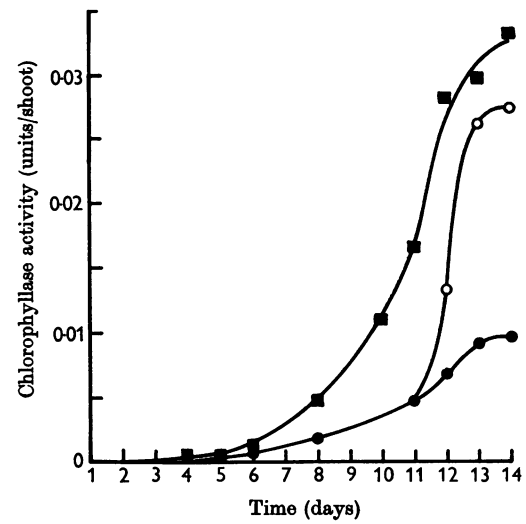

Fig. 2. Effect of light on chlorophyllase activity of pea-seedling shoots. ■, Grown in light; ●, grown in dark; ○, grown in dark and transferred to light after 11 days.

much of the activity. A soluble preparation could be made by extracting an acetone-dried powder first with 95% ethanol, followed by ethanol–ether (3:1) and finally ether, and then soaking the powder in 0·02M-sodium citrate solution (8 ml./g. of powder) at room temperature overnight. The extract was squeezed out through cotton cloth and the residue re-extracted with citrate solution (2 ml./g. of powder). The combined extracts were centrifuged at 2500 *g* for 15 min. to remove starch and other insoluble material with little loss of enzyme activity. The clear centrifuged solution had from two to six times as much enzyme activity as appeared to be present in the original powder. Even the residue after the citrate extraction had activity as high as, or even higher than, the starting material. Table 1 gives the results of a typical experiment, showing the extraction of chlorophyllase from an acetone-dried powder compared with that from a sample of the same batch of powder after extraction with ethanol and ether.

Partial purification. The enzyme could be precipitated by ammonium sulphate between 35 and 70% saturation, but the recovery was poor. Precipitation with acetone gave better recovery and a bigger increase in the specific activity because more material was rendered insoluble.

Before addition of acetone the solution was cooled to 0° and this temperature was maintained as nearly as possible during precipitation and centrifuging. Acetone (0·9 vol.) was added and, after standing for a few minutes, the precipitate was centrifuged down and taken up in water. This contained about 90% of the protein and less than 10% of the enzyme activity. To the supernatant solution from the first precipitate a further 0·9 vol. of acetone was added. The precipitate was centrifuged down and the supernatant solution discarded. The precipitate was taken up in water and centrifuged to remove a small amount of insoluble material. This fraction contained about 60% of the activity and about 1% of the nitrogen of the solvent-extracted powder. Results for one preparation are given in Table 2. Repeating the acetone precipitation increases the specific activity but so far no useful results have been obtained with adsorbents such as calcium phosphate and Celite. Partly purified preparations have low amylase activity; pectin esterase, phosphatase, amine oxidase and polyphenol oxidase are absent.

Table 1. *Extraction of chlorophyllase*

Powders (3 g.) were soaked in 40 ml. of 0·02M-sodium citrate solution at room temperature overnight. Crude extracts obtained by squeezing through cloth were centrifuged at 2500 *g* for 15 min. to give clarified extracts.

	Total units in fraction	
	Acetone-dried powder	Solvent-extracted acetone-dried powder
Starting material	61·2	60·0
Crude citrate extract	39·0	202·5
Clarified citrate extract	12·5	197·0
Residue	21·8	72·0

Properties of the enzyme

Stability. Nightshade leaves stored at −10° for 9 months retained their activity. An acetone-dried powder of sugar-beet leaves kept for 6 months at room temperature lost less than 20% of its activity. Solutions of both crude and partly purified enzyme at pH values between 6 and 8 kept at 0° lost about 2% of the activity per day; at 20° 30–50% was lost in 24 hr. The partly purified enzyme was destroyed by 10 min. at 65°; 40% of the activity was lost on heating at 60° for 10 min. It was stable for 1 hr. at 45° in citrate buffer, pH 7, but all activity was lost at this temperature in the presence of 40% acetone.

At pH 3 the enzyme was rapidly inactivated and it would stand only brief exposure to pH 4·5.

On dialysis against distilled water in the cold for 48 hr. up to 60% of the activity was lost; the original activity was not restored by adding dialysate nor by adding boiled enzyme.

Variation of activity with acetone concentration. The presence of a solvent such as acetone is essential for enzyme action. Fig. 3 compares the effect of a range of acetone concentrations on the activity of insoluble and soluble enzyme preparations. The optimum concentration was higher for both types of insoluble preparation than for the partly purified soluble preparation.

Variation of activity with pH. Fig. 4 shows the effect of pH on the activity of soluble and insoluble preparations. Michaelis veronal–acetate buffers were used for the whole range. The pH values of the

Table 2. *Partial purification of chlorophyllase*

	Total units	Activity per g. of dry matter	Activity per g. of N
Solvent-extracted acetone-dried powder (40 g.)	920	23	410
Centrifuged citrate extracts (320 ml.)	2020	—	7 200
Acetone ppt. (0–45%)	205	—	6 500
Acetone ppt. (45–65%)	1056	3600	39 000

buffers were affected both by the chlorophyll solution and by the acetone-dried powder and it was therefore necessary to determine the actual pH of a duplicate portion of each incubated system. There was no change in pH during incubation. For the soluble enzyme the optimum was between 7·5 and 8·0, but with the acetone-dried-powder preparation a higher pH of the suspension was required for maximum activity.

Variation of activity with temperature. The results in Fig. 5 show how temperature affected the enzyme activity of a partly purified preparation. The optimum temperature was between 20° and 30°.

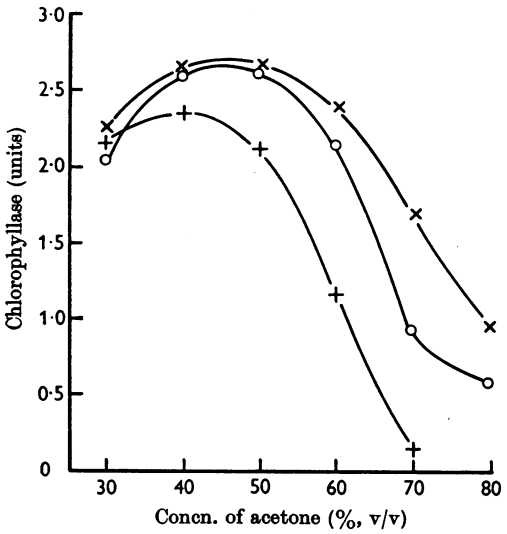

Fig. 3. Variation of chlorophyllase activity with acetone concentration. + Soluble preparation (0·10 mg. of N); ○, acetone-dried powder (200 mg. of dry matter, 13·2 mg. of N); ×, dried-leaf meal (200 mg. of dry matter, 10·6 mg. of N). Incubation, 1 hr.

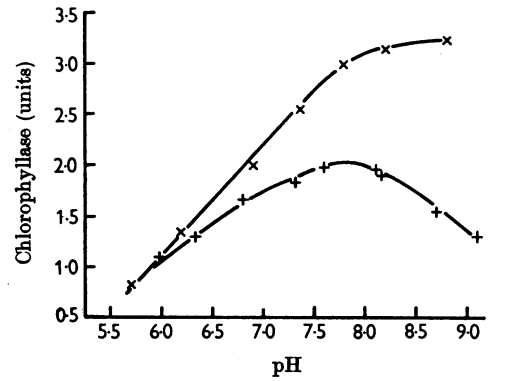

Fig. 4. Variation of chlorophyllase activity with pH. ×, Acetone-dried powder (100 mg. of dry matter, 5·8 mg. of N); +, dialysed soluble preparation (5·1 mg. of dry matter, 0·54 mg. of N). Incubation, 1 hr.

DISCUSSION

The two- to six-fold increase in enzyme activity when chlorophyllase becomes soluble is similar to that for the phenolase of sugar-beet-leaf chloroplasts found by Mayer & Friend (1960) and for aconitase and glutamic acid dehydrogenase of *Lupinus* mitochondria (Estermann, Conn & McLaren 1959). The fact that treatment with ethanol and ether makes subsequent extraction of the enzyme possible suggests that association with lipids, either physical or chemical, may be responsible for the insolubility of the chlorophyllase and also for its being unable to exert maximum activity when *in situ* in the chloroplasts. Ardao & Vennesland (1960) suggest that all the chlorophyllase of the mature spinach leaf may be present in a chlorophyll–lipoprotein complex.

Mayer (1930) found that the enzyme in leaf powders had an optimum pH near 6. This is much lower than was found here, where the activity of an acetone-dried powder increased as the pH of the suspension was raised to almost 9. The pH optimum of the soluble enzyme was between 7·5 and 8, but with a powder a higher pH of the suspension is probably necessary to achieve the optimum pH at the site of enzyme action.

The present findings confirm the need for a high concentration of organic solvent, though the optimum concentration was rather lower (40–50 %

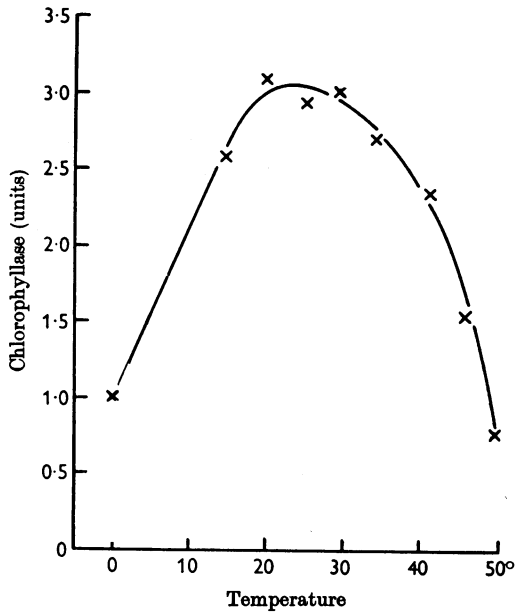

Fig. 5. Variation of chlorophyllase activity with temperature. Soluble preparation (0·10 mg. of N). Incubation, 1 hr.

acetone), than found in earlier investigations. Mayer (1930) used a concentration of 66% for his determinations, but this is definitely inhibitory, though less so for insoluble preparations than for soluble.

The enzyme is remarkably stable before it becomes soluble and the activity of soluble preparations is retained well in the cold. The optimum temperature for enzyme action is much lower than for many enzymes, presumably because the temperature at which the enzyme is inactivated is comparatively low, particularly in the presence of acetone.

The rise in chlorophyllase activity when dark-grown pea seedlings are transferred to light is similar to that found by Hageman & Arnon (1955) for triphosphopyridine nucleotide-linked glyceraldehyde phosphate dehydrogenase. Marcus (1960) induced the formation of triose phosphate dehydrogenase, without the development of chlorophyll, in kidney-bean leaves by brief exposure to red light. Booth (1960) found that a carotene-destroying enzyme system was not normally present in potatoes and carrots, but exposure to light leading to chlorophyll formation in the surface layers also caused the enzyme to appear.

SUMMARY

1. Soluble chlorophyllase preparations were made from sugar-beet leaves, which have the highest activity of all species tested.

2. For the partly purified enzyme the optimum conditions for enzyme action are pH 7·7 at 25° and an acetone concentration of 40%.

3. When dark-grown pea seedlings with very low chlorophyllase activity were transferred to daylight the activity rose in 48 hr. to a level comparable with that of light-grown seedlings.

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Pyridoxamine Phosphate-Oxidase and Pyridoxal Phosphate-Phosphatase Activities in *Escherichia coli*

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Beechey & Happold (1955) first demonstrated the conversion of pyridoxamine phosphate into the aldehyde form by cell-free extracts of *Escherichia coli*. The nature of the product was established by its ability to activate apotryptophanase, an enzyme also present in the aqueous extracts. Optimum conditions for enzyme assay were studied, and simple kinetic studies were carried out by spectrophotometric methods, by Beechey & Happold (1957), who found that *E. coli* extracts catalysed the disappearance of pyridoxal phosphate and that light-absorption at the wavelength characteristic of pyridoxamine phosphate increased during the reaction. The reaction involved thus appeared to be reversible, although only the conversion of pyridoxamine phosphate into the pyridoxal ester required Mg^{2+} ions for maximum activity.

The possibility that more than one enzyme was involved was recognized, but the apparent reversibility of the reaction, and other data, led these workers to assume that a transamination reaction was involved. Unlike Meister, Sober & Tice (1951),

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