relationship between the increased excretion and the fundamental lesion in the pathological cases, and the range of pathological cases in which this increased excretion occurs, remain to be determined.

SUMMARY

1. A metabolite excreted in small amount in some normal human urines, and in appreciably larger amounts in certain pathological urines, is shown by degradative and synthetic evidence to be 2-amino-3-hydroxyacetophenone *O*-sulphate.

2. Reasons are given for considering this substance to arise by hydrolytic scission of the side chain of hydroxykynurenine.

REFERENCES

Boyland, E. & Sims, P. (1954). *J. chem. Soc.* p. 980.
Butenandt, A. & Renner, W. (1953). *Z. Naturf.* **8b**, 454.
Dalgliesh, C. E. (1952). *Biochem. J.* **52**, 3.
Dalgliesh, C. E. (1955a). *J. clin. Path.* **8**, 73.
Dalgliesh, C. E. (1955b). *Advanc. Protein Chem.* **10**. In the Press.
Longenecker, J. B. & Snell, E. E. (1955). *J. biol. Chem.* **213**, 229.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.
Musajo, L., Spada, A. & Bulgarelli, E. (1950). *Gaz. chim. ital.* **80**, 161.
Musajo, L., Spada, A. & Casini, E. (1950). *Gaz. chim. ital.* **80**, 171.

Studies on the Acetylesterase of *Sclerotinia laxa*

By R. J. W. BYRDE and A. H. FIELDING
Long Ashton Research Station, University of Bristol

(*Received* 12 *April* 1955)

This paper deals with the hydrolysis of phenolic esters by enzyme preparations from *Sclerotinia laxa* Aderh. & Ruhl., one of the fungi causing the brown-rot diseases of fruits. The investigations were undertaken on account of the apparent role of a fungal enzyme in the mode of fungistatic action of esters of 2:3-dichloro-1:4-naphthaquinone (Byrde & Woodcock, 1952, 1953).

Macdonnell, Jang, Jansen & Lineweaver (1950), in the course of studies of pectin and acetylesterases of fruits of *Citrus* spp., showed also that a pectin esterase of fungal origin readily hydrolysed phenyl acetate and phenyl propionate at pH 6·8, and, to a lesser extent, at pH 4·5. Subsequently Jermyn (1953) demonstrated that three or four components with esterase activity could be separated by paper chromatography from cultures of *Aspergillus flavus-oryzae*. In further studies on the enzymes of this fungus, Crewther & Lennox (1953) showed that a pH of 6·0 was the optimum for esterase activity, and demonstrated that there was little or no activity in cultures under 6 days old.

Amongst the more numerous workers on esterases of non-fungal origin, Aldridge (1953), in a study of serum esterases, classified them into two types, on the basis of substrate specificity and their inhibition by diethyl *p*-nitrophenyl phosphate (paraoxon). He defined type A esterases as those hydrolysing *p*-nitrophenyl acetate more readily than the corresponding butyryl ester, while relatively resistant to inhibition by paraoxon. Type B esterases, on the other hand, hydrolysed the butyryl derivative more rapidly than the acetate, and were inhibited by concentrations of paraoxon as low as 10^{-7}–10^{-8} M.

The effect of nuclear substitution on the hydrolysis of phenyl acetate by a wheat-germ esterase was studied by Gawron, Grelecki & Duggan (1953), who suggested that electromeric factors might account for the differing rates of hydrolysis. Electron-withdrawing substituents, especially in the *meta* position, increased the hydrolysis rate, but electron-donating substituents decreased the rate.

EXPERIMENTAL

Source of enzyme

The enzyme used in most of the experiments was a crude preparation from cultures of *S. laxa* grown on autoclaved acidified potato plugs, the medium used in this laboratory for routine production of spores. The mycelium was scraped from the surface of the cultures and macerated with buffer solution or water, according to the method subsequently used for the estimation of enzyme activity. The resultant pulp was filtered under suction and the crude filtrate stored at −4°. The enzyme was roughly concentrated by successive freezing and thawing and removal of ice crystals, resulting in a preparation of moderately high activity.

Preparations of higher activity could be obtained by fractionation with ammonium sulphate in the presence of sodium oxalate, followed by dialysis, as described by Jansen, Jang & Macdonnell (1947).

Substrates

The following esters were prepared by conventional methods: phenyl acetate, b.p. 196°; *o*-chlorophenyl acetate, b.p. 103°/15 mm.; *p*-chlorophenyl acetate, b.p. 76°/0·8 mm.; 2:4:5-trichlorophenyl acetate, m.p. 66–67·5°; 2:4:6-trichlorophenyl acetate, m.p. 47–48°; pentachlorophenyl acetate, m.p. 147–149°; *p*-chlorophenyl propionate, b.p. 160–164°/8 cm.; *p*-chlorophenyl butyrate, b.p. 172–176°/8 cm.; *p*-chlorophenyl valerate, b.p. 190·8°/9 cm.; *p*-chlorophenyl hexanoate, b.p. 108–112°/0·4 mm.; *p*-chlorophenyl benzoate, m.p. 87–88°.

In order to disperse the esters in water without using a mutual solvent, each was formulated as a 20% wettable preparation on a kaolin base, by slurrying an acetone solution with kaolin and grinding the suspension in a mortar until the solvent had evaporated.

Estimation of enzyme activity

(*a*) *Colorimetric method.* This technique was similar to that described by Jansen, Nutting & Balls (1948). It was based on the colorimetric estimation of *o*-nitrophenol liberated by the enzymic hydrolysis of *o*-nitrophenyl acetate.

The test system comprised 9 ml. suitable buffer solution, 1 ml. enzyme preparation (in buffer), and 0·2 ml. 0·1 M *o*-nitrophenyl acetate (in ethanol). The development of yellow colour was estimated on a Spekker absorptiometer, a violet filter (Ilford 601, max. transmission approx. 4300 Å) being used, and with a water setting of 1·300. Necessary blanks were included. The amounts of *o*-nitrophenol liberated were estimated from a standard curve.

p-Nitrophenyl derivatives have also been used by other workers (Huggins & Lapides, 1947; Jermyn, 1953; Crewther & Lennox, 1953), but all such colorimetric methods are restricted to use with esters of coloured phenols and at pH values below 8·5, owing to excessive non-enzymic hydrolysis of the esters under alkaline conditions.

A modification of this method used for detection of esterase activity *in vivo* and in chromatographic studies depended on the hydrolysis of diacetylfluorescein to fluorescein, which, unlike its ester, fluoresces strongly in ultraviolet light.

(*b*) *Manometric method.* This technique has also found wide use in esterase studies (Gawron *et al.* 1953; Aldridge, 1953). It is based on the manometric estimation of CO_2 liberated from $NaHCO_3$ by the carboxylic acid formed on enzymic hydrolysis of the ester.

Warburg flasks (15 ml.) with single side arm were used. The main flask contained 0·75 ml. 0·03 M-$NaHCO_3$ and 1·50 ml. substrate (1% aqueous suspension of a 20% w/w preparation on kaolin). The side arm contained 0·95 ml. enzyme preparation made up in water and with pH adjusted to 7·0.

The flasks were gassed with 5% CO_2 in N_2 and equilibrated at 25° before addition of the contents of the side arm. The molarity of the $NaHCO_3$ was such as to give a pH of 7·0 in equilibrium with an atmosphere of 5% CO_2 (Umbreit, Burris & Stauffer, 1949, p. 26). Necessary blanks were included.

This method, while not restricted to esters of any specific phenol, is limited to the pH range 6·4–7·8 by virtue of the $NaHCO_3$ buffer. Gawron *et al.* (1953) also pointed out that it is necessary to use a correction factor for carbon dioxide liberated as a result of dissociation of the free phenol. This factor is expressed by $(K_a+[H^+])/(2K_a+[H^+]$, where K_a is the dissociation constant of the free phenol. At a pH of 7·0 this factor is negligible for the less acidic phenols but becomes appreciable for trichlorophenols and pentachlorophenol.

Chromatographic separations

The separation of the esterase of *S. laxa* was carried out by the method of Jermyn (1953), descending paper chromatography being used. The solvent selected was 40% acetone buffered to pH 4·2 with McIlvaine citrate–phosphate buffer (0·01–0·02 M). Whatman no. 4 paper was used to ensure rapid development. The papers were removed from the trough and dried at laboratory temperature when the solvent front had moved to a distance of 20 cm. from the initial spot. As soon as they were dry, they were sprayed with diacetylfluorescein (0·01% in 50%, v/v, aqueous acetone buffered to pH 6·5), and after 30 min. exposed to ultraviolet light to make the spots visible.

RESULTS

Substrate specificity

Effect of side chain. The relative activity of the enzyme preparation from *S. laxa* towards different side chains on the phenol nucleus was investigated by the manometric technique, with a series of *p*-chlorophenyl esters as substrates. Table 1 summarizes the results obtained in duplicate tests and shows that increasing side-chain length is associated with decreasing enzyme activity towards the ester.

Table 1. *Relative esterase activity of an extract from* Sclerotinia laxa *towards esters of* p-*chlorophenol*

Hydrolysis of the esters in the absence of the enzyme preparation was of the order of 2% of that in its presence. Mean rate of enzymic hydrolysis of acetyl ester: 11·45 μ-moles/ml. enzyme prep./hr. For conditions see text.

Ester	Relative activity
Acetyl	100
Propionyl	66
Butyryl	28
Valeryl	10
Hexanoyl	2
Benzoyl	2

Effect of nucleus. The relative activity of the esterase towards a range of chlorinated phenyl acetates of interest as fungistatic agents was investigated by the manometric technique, with phenyl acetate as a standard in each test. Table 2 summarizes the results obtained in duplicate experiments; the K_a values used for corrections for phenolic dissociation are also listed.

Table 2. *Relative esterase activity of an extract from* Sclerotinia laxa *towards acetyl esters of chlorinated phenols*

Hydrolysis of the esters in the absence of enzyme was of the order of 3% of that in its presence. Mean rate of enzymic hydrolysis of phenyl acetate: 9·57 μmoles/ml. enzyme prep./hr. For conditions see text.

Parent phenol	$10^7 K_a$*	Relative esterase activity (corr.)
Phenol	0·001	100
o-Chlorophenol	0·36	93
p-Chlorophenol	0·21	74
2:4:5-Trichlorophenol	0·47	47
2:4:6-Trichlorophenol	3·80	12
Pentachlorophenol	55·00	<1

* After Tiessens (1931).

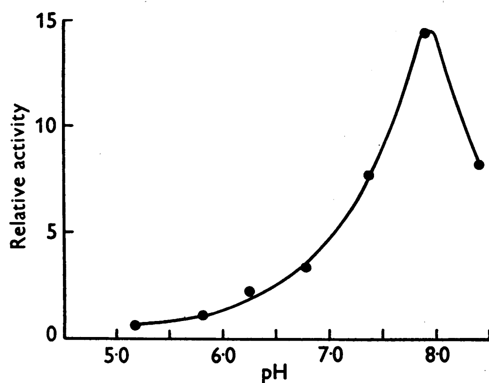

Fig. 1. Effect of pH on the activity of *S. laxa* acetylesterase. For conditions see text.

pH *relationships*

The effect of pH on enzyme activity was studied by the colorimetric method, with a McIlvaine citrate–phosphate buffer. Fig. 1 illustrates the relative activity of the enzyme over the pH range 5·2–8·4; no measurable activity was found below pH 5·0. The optimum occurs in the region of pH 7·8, a value higher than that obtained for the preparation from *A. flavus-oryzae* by Crewther & Lennox (1953).

Inhibitory effect of diethyl p-*nitrophenyl phosphate*

The effect of diethyl *p*-nitrophenyl phosphate (paraoxon) on the esterase from *S. laxa* was investigated by the colorimetric method, with a 0·1 M phosphate buffer (pH 6·65). The inhibitor (0·1 ml. of an acetone solution of appropriate concentration) was added to the enzyme (1 ml.) and buffer (9 ml.) 30 min. before the addition of substrate (0·2 ml.). The results of percentage inhibition of enzyme activity, measured over a 30 min. period, were transformed into probit units (Finney, 1952) and plotted against the logarithm of final inhibitor concentration. Fig. 2 illustrates the results obtained, together with those of similar experiments with enzymes of plant and insect origin. Clearly, the fungus enzyme, by contrast with the insect enzyme, is relatively tolerant of paraoxon.

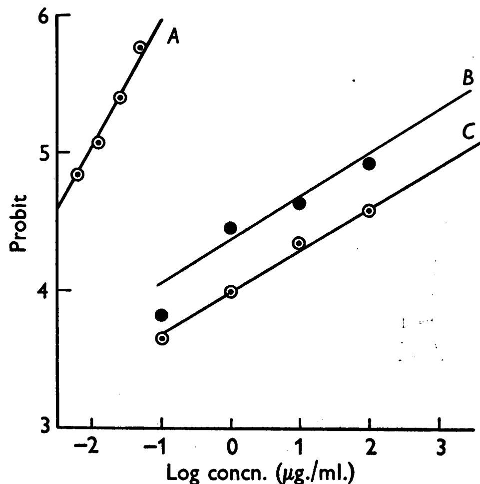

Fig. 2. Inhibtion of acetylesterases by diethyl *p*-nitrophenyl phosphate. For conditions see text. *A, Locusta migratoria migratorioides* (8×10^{-3}); *B, Vicia faba* (100). *C, Sclerotinia laxa* ($2{\cdot}2 \times 10^3$). Approximate dosages for 50% inhibition (μg./ml.) are shown in brackets.

Exocellular enzyme production by developing cultures

Cultures of *S. laxa*, from a heavy spore inoculum, were grown on 50 ml. portions of a liquid potato–dextrose medium (200 g. boiled potato, 20 g. dextrose, 1 l. water) in 250 ml. conical flasks. At intervals of a few days after inoculation the dry weight of duplicate mycelial mats was determined. At the same time, the esterase activity of the culture filtrates was estimated by the colorimetric method, with a 0·1 M phosphate buffer (pH 6·65).

Fig. 3 shows the results obtained, which indicate that exocellular esterase secretion lagged behind mycelial growth; no measurable enzyme activity developed before the sixth day. This is in accordance with the findings of Crewther & Lennox (1953), using *A. flavus-oryzae*.

In a further test, acetylesterase activity was assessed *in vivo* under the experimental conditions commonly used for toxicity trials in this laboratory. Four plates were poured from 50 ml. melted dextrose–peptone–phosphate agar (pH 6·5) to which had been added 0·5 ml. of a solution of

diacetylfluorescein (1 % in acetone). On cooling, they were inoculated at a central point with a disk (4 mm. diameter) from a culture of *S. laxa* and incubated at 25°. After 1 day a strongly fluorescent ring had developed round the disk, from which the mycelium subsequently grew out, surrounded by a fluorescent zone about 1 cm. wide. The background showed only slight fluorescence. This result suggests that, under these experimental conditions, hydrolysis of diacetylfluorescein by an exocellular esterase occurred at an early stage in the growth of the fungus, and in the proximity of the mycelium was considerably in excess of non-enzymic hydrolysis.

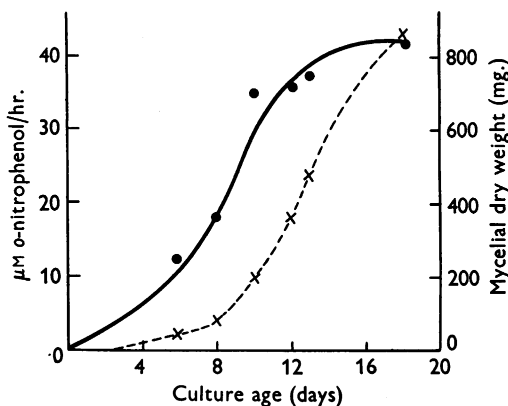

Fig. 3. Relation between acetylesterase activity and age and weight of mycelium of *S. laxa* in liquid culture. ●, Weight; ×, enzyme activity. For conditions see text.

Chromatographic separation of enzyme components

A crude *S. laxa* extract was separated into four components showing acetylesterase activity. The distances (cm.) of these spots from the starting line, after 20 cm. solvent travel, were as follows: (1) ±0·5, (2) 6·0–7·5, (3) 12·0–13·5, (4) 16·5–20·0.

Chromatography of fractions obtained by ammonium sulphate precipitation showed that, whilst the enzyme had been concentrated, the fractionation had only partially separated these four components.

An attempt was also made to examine chromatographically an esterase preparation from the gut of *Locusta*. Excessive streaking made evaluation difficult, but little evidence of difference was obtained, other than the absence of component (2).

DISCUSSION

The results suggest that the esterase of *Sclerotinia laxa* resembles the type A serum esterase described by Aldridge (1953). The fungal enzyme hydrolysed *p*-chlorophenyl acetate more readily than the butyryl derivative, and was relatively little affected by diethyl *p*-nitrophenyl phosphate.

The demonstration in cultures of *S. laxa* of an active esterase, with a range of substrates, lends support to the suggestion put forward by Byrde & Woodcock (1953, 1955) that the activity of esters of both 2:3-dichloro-1:4-naphthahydroquinone and pentachlorophenol against several fungi (including *S. laxa*) might arise from their enzymic hydrolysis to the toxic phenol. Moreover, in both series, the lower fatty acid esters showed greater fungistatic activity than the higher members of the series, whilst it has now been demonstrated manometrically, for a series of *p*-chlorophenyl esters, that increasing length of aliphatic side chain is associated with a decreasing rate of enzymic hydrolysis. The statistically significant effect of pH, over the range 5·4–7·5, on the fungistatic activity of two pentachlorophenyl esters towards *S. laxa* (Byrde & Woodcock, 1956) may similarly be ascribed to differences in the rates of enzymic hydrolysis over this range, which have been demonstrated *in vitro* by a colorimetric method.

The evidence presented also indicates that the primary site of action of the fungal enzyme in such toxicity tests is outside the cell, rather than within, and that non-enzymic hydrolysis plays a relatively small part in bringing about the fungistatic effect of these compounds. On the other hand, the possibility cannot be dismissed that hydrolysis of the phenolic esters may also occur within the fungal cell after penetration by molecules of the esters. Such a mode of action was suggested by Grove (1953) to account for the fungistatic activity of acetylgladiolic acid against conidia of *Botrytis allii* Munn.

SUMMARY

1. The presence of an acetylesterase of *Sclerotinia laxa* was demonstrated. The activity of the enzyme towards phenolic esters of fatty acids decreased with increasing molecular weight of the side chain, and was also related to the nature of the phenolic nucleus.

2. The esterase showed optimum activity at pH 7·8, and was relatively resistant to inhibition by diethyl *p*-nitrophenyl phosphate. Exocellular enzyme activity of developing cultures of *S. laxa* in a liquid medium lagged behind mycelial growth, but localized production was demonstrated at an early stage in cultures on solid medium.

3. On the basis of these results, the enzyme appears akin to type A serum esterases.

4. From the crude extract of *S. laxa* four components with esterase activity were separated by paper chromatography.

5. Exocellular fungal esterase appears to play a large part in bringing about the fungistatic activity of phenolic esters.

The authors wish to thank Dr D. Woodcock for the synthesis of phenolic esters, and Mr A. Stringer for supplying insect material and for permission to quote data on the inhibition of acetylesterase from *Locusta*.

REFERENCES

Aldridge, W. N. (1953). *Biochem. J.* **53**, 110.
Byrde, R. J. W. & Woodcock, D. (1952). *Nature, Lond.*, **169**, 503.
Byrde, R. J. W. & Woodcock, D. (1953). *Ann. appl. Biol.* **40**, 675.
Byrde, R. J. W. & Woodcock, D. (1956) *Ann. appl. Biol.* In the Press.
Crewther, W. G. & Lennox, F. G. (1953). *Aust. J. Biol. Sci.* **6**, 410.
Finney, D. J. (1952). *Probit Analysis*, 2nd ed. Cambridge University Press.
Gawron, O., Grelecki, C. J. & Duggan, M. (1953). *Arch. Biochem. Biophys.* **44**, 455.
Grove, J. F. (1953). *Biochem. J.* **54**, 664.
Huggins, C. & Lapides, J. (1947). *J. biol. Chem.* **170**, 467.
Jansen, E. F., Jang, R. & Macdonnell, L. R. (1947). *Arch. Biochem.* **15**, 415.
Jansen, E. F., Nutting, M. D. F. & Balls, A. K. (1948). *J. biol. Chem.* **175**, 975.
Jermyn, M. A. (1953). *Aust. J. Biol. Sci.* **6**, 77.
Macdonnell, L. R., Jang, R., Jansen, E. F. & Lineweaver, H. (1950). *Arch. Biochem.* **28**, 260.
Tiessens, G. J. (1931). *Rec. Trav. chim. Pays-Bas*, **50**, 112.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis: Burgess Publishing Co.

Changes in Lens During the Formation of X-Ray Cataract in Rabbits

3. PHOSPHATES*

BY ANTOINETTE PIRIE, RUTH VAN HEYNINGEN AND P. H. FLANDERS
Nuffield Laboratory of Ophthalmology, University of Oxford, and Medical Research Council Experimental Radiopathology Research Unit, Hammersmith Hospital, London

(*Received* 20 *April* 1955)

The first biochemical change caused by irradiation of tissues is unknown. There is some evidence, reviewed by Ord & Stocken (1953), that changes take place in the high-energy phosphate ($\sim$P) compounds but assessment of the results is complicated by the fact that in most situations it is difficult to separate primary from secondary effects. Gross histological change may occur in an organ within a few hours of whole-body irradiation, with lysis of some cells and influx of others from the blood stream. No valid biochemical comparison is possible between the tissue in this state and in the normal state before irradiation. Where cell population has changed one can expect large changes in chemical and enzymic make-up.

From this point of view a study of the effect of irradiation on the lens may be peculiarly valuable. It is an isolated non-vascular organ, derived from a single type of embryonic ectodermal cell, and any observed early effect of radiation is more likely to be a primary effect than it is in other tissues where the cell population is complex to start with and may change after irradiation. We have already shown that certain changes in the composition and enzymes of lens occur during the early stages of the formation of X-ray cataract (Pirie, van Heyningen & Boag, 1953; van Heyningen, Pirie & Boag, 1954). The changes may not be primary effects, however, since their onset occurred some weeks after irradiation. Since changes in $\sim$P may be an early result of irradiation, we have examined the effect of X-rays on the phosphate compounds of the lens.

We have used rabbits of 14–28 days in this study for two reasons. First, it is known that the concentration of high-energy phosphate compounds is greater in the lens of the young animal than it is in the old (Nordmann & Mandel, 1952; Müller & Kleifeld, 1953) and, secondly, the development of cataract for a given dose of X-rays is far quicker in the young (Cogan & Donaldson, 1951). Both these characteristics are probably related to the higher proportion of dividing cells and nucleated fibres in the young lens. The irradiated lens was compared directly with its normal fellow, as it has been shown that the composition and weight of the two lenses of a single rabbit are very similar, although values may differ widely from animal to animal.

A known early change in the rabbit lens after X-ray treatment is a fall in glutathione (Pirie *et al.* 1953; van Heyningen *et al.* 1954). We have therefore estimated glutathione in some of the lenses, considering that any change in it was a yard-stick by which to assess other changes.

* Part 2: van Heyningen, Pirie & Boag (1954).