hydrolases in the particles he terms lysosomes. Besides a cathepsin, these include a ribonuclease, a deoxyribonuclease, a phosphatase, a glucuronidase and a sulphatase. As described in the first paper of this series, the action of the vitamin upon the cartilaginous rudiments grown in culture causes a complex series of changes, including loss of ribonucleic acid, amino sugars and dry weight. It seems very likely that the vitamin may release some, or all, of these bound hydrolases and that this complex of enzymes may cause many of the changes seen in the tissue treated with vitamin A.

SUMMARY

1. Particulate preparations from rat liver suspended in 0.25 M-sucrose and treated with vitamin A release a proteolytic enzyme with an acid pH optimum.

2. The action of the vitamin on the particles is dependent upon temperature and pH.

3. The enzyme, after release by hypo-osmotic treatment, is not affected by the vitamin.

4. The relationship of this effect to the action of vitamin A on intact tissue is discussed.

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REFERENCES

- Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
 Appelmans, F. & de Duve, C. (1955). *Biochem. J.* **59**, 426.
 Appelmans, F., Wattiaux, R. & de Duve, C. (1955). *Biochem. J.* **59**, 438.
 de Duve, C. (1959). *Subcellular Particles*, p. 128. New York: The Ronald Press Co.
 Dingle, J. T., Lucy, J. A. & Fell, H. B. (1961). *Biochem. J.* **79**, 497.
 Gianetto, R. & de Duve, C. (1955). *Biochem. J.* **59**, 433.
 Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961). *Biochem. J.* **79**, 500.

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A Method for the Chromatographic Detection of Penicillins and Related Compounds and of Penicillinase

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The intact penicillin molecule is not oxidized by molecular iodine, but after the β -lactam ring is opened—for example, when penicillin is inactivated by the enzyme penicillinase—the resulting penicilloic acid rapidly reacts with 8–9 equiv. of iodine (Alcino, 1946).

Iodine also reacts with many sulphur compounds related to penicillin and can be used to detect these on paper chromatograms. In combination with penicillinase it will detect those penicillins whose β -lactam ring is opened by the enzyme. Alternatively, the method can be used to detect penicillinase. The method has been developed in this Institute over the past few years.

EXPERIMENTAL

The reagents are made up as follows:

Starch-iodine solution: 0.01 M-iodine in 3 mM-potassium iodide, 10 ml.; M-phosphate buffer, pH 7.0 (54 g. of KH_2PO_4 and 84.6 g. of Na_2HPO_4 in 1 l. of water), 1 ml.; 2% (w/v) sodium starch glycollate (British Drug Houses Ltd.) in water (a crystal of thymol is added as preservative),

9 ml. The three stock solutions are stable, and the mixture is stable for several days at least.

Penicillin solution: sodium benzylpenicillin, 60 mg./ml. (100 000 i.u./ml.), freshly prepared, 1 ml.; M-phosphate buffer, pH 7.0, 1 ml.; water, 8 ml. This solution must be freshly made each day.

Penicillinase solution: Penicillinase solution of at least 1000 enzyme units/ml. A convenient method for preparing high-titre penicillinase from *Bacillus cereus* (strain 569/H or 5/B) has been given by Pollock (1957). This preparation usually contains 2000–10 000 enzyme units/ml., and is stable for some months at 4°: the enzymes from these two strains have given identical results when used in the methods described below.

Two methods are suggested for general use:

(1) Method A, for the detection of penicillin and related compounds. The paper is sprayed with starch-iodine solution and observed for 2 min. Most compounds, related to penicillin, containing potential sulphydryl groups immediately give pale spots on a deep-blue background, except for those with a closed β -lactam ring. The paper is now sprayed with a freshly made mixture of equal amounts of the starch-iodine and the penicillinase solutions. Additional pale spots will appear at the site of penicillins whose β -lactam ring is opened by the penicillinase. The

penicillinase solution will itself take up some iodine because of reducing-compounds in the medium, and should therefore be mixed as above with the starch-iodine solution before use. Generally a considerable amount of free iodine will remain in the mixture; if not, more starch-iodine solution should be added until the mixture remains dark blue for several minutes. The two penicillinases recommended above are so slowly inactivated by iodine under these conditions that the results are not affected.

(2) Method B, for the detection of penicillinase (i.e. penicillin β -lactamase). The paper is sprayed with a mixture of equal parts of the starch-iodine and penicillin solutions. The presence of penicillinase is demonstrated by the appearance of pale spots on the blue background and the rate of appearance of clear areas gives an indication of the amount of enzyme present. Any compounds which destroy iodine without enzyme treatment may be located by spraying first with starch-iodine solution, as in method A. To avoid a false negative result caused by inactivation of the enzyme by iodine the penicillin solution may be sprayed first, and followed after 10 min. by the starch-iodine solution. This immediately shows up all areas where the penicillin has been destroyed but the relative amounts of enzyme in different spots cannot now be estimated.

The blue colour on the paper will slowly fade as the iodine volatilizes, and the spots must therefore be marked. Some persons are hypersensitive to penicillin, and it is advisable to spray the penicillin solution in a suitable fume cupboard or hood.

RESULTS AND DISCUSSION

Table 1 shows the sensitivity of the method to compounds 'spotted' on Whatman no. 1 filter paper. Method A is about ten times as sensitive as the iodoplatinic acid reagents of Winegard, Toennies & Block (1948) for reduced-sulphur compounds, and has a sensitivity about equal to that of the iodine-azide spray (Chargaff, Levine & Green, 1948).

The following compounds were found to take up iodine immediately: benzylpenicilloic acid, benzylpenicilloic acid amide, D-penicillamine, thiazolidine-4-carboxylic acid, α -ethyl benzoyloxypeni-

cilloate. The following penicillins took up iodine rapidly only after spraying with *B. cereus* 569/H penicillinase: benzyl-, *p*-aminobenzyl-, penten-2-yl-, heptyl- and phenoxyethyl-penicillin and 6-(α -phenoxypropionamido)penicillanic acid; but 6-amino- and 6-(2:6-dimethoxybenzamido)-penicillanic acid did so only slowly. Cephalosporin C took up no appreciable amount of iodine even after spraying with the enzyme. *p*-Hydroxybenzylpenicillin takes up in the course of a few minutes approximately 4 equiv. of iodine (presumably due to iodination of the benzene ring) and can therefore slowly decolorize the reagent before it is split by penicillinase.

Negative results obtained with method A do not exclude the possible presence of a penicillin. Three cases may be considered where a negative result could be obtained: (i) where the penicillin is converted by the enzyme into a penicilloic acid, which does not react rapidly with iodine (however, no penicilloates derived from the thiazolidine nucleus of penicillin are yet known which do not react readily); (ii) where the penicillin has a low affinity for the enzyme and is therefore hydrolysed too slowly to give a positive response before the iodine evaporates completely from the paper; for example, 6-(2:6-dimethoxybenzamido)penicillanic acid (Celbenin) is hydrolysed only slowly by the penicillinase produced by *Staphylococcus aureus* 524 SC, for which it has a low affinity (R. Novick, personal communication); (iii) the penicillin may not be a substrate at all.

There is, however, no reason why very much larger amounts of purified penicillinase (Pollock, Torriani & Tridgell, 1956; Kogut, Pollock & Tridgell, 1956) cannot be used to increase the sensitivity for those compounds that are only slowly hydrolysed. The use of benzylpenicillin in method B seems to exclude false negative results, since all the penicillinases so far examined split benzylpenicillin very readily. The possibility of a

Table 1. *Sensitivity of the methods*

The figures in parentheses give the approximate amount of the substance per unit area when 'spotted' on Whatman no. 1 filter paper.

Substance	Amount readily detected within 2 min.	Amount just detected after 15-20 min.	Method used
Sodium benzylpenicillin	500 μ g./ml. (3 μ g./cm. ²)	100 μ g./ml. (0.6 μ g./cm. ²)	A
6-Aminopenicillanic acid	10 mg./ml. (60 μ g./cm. ²)	500 μ g./ml. (3 μ g./cm. ²)	A
<i>Bacillus cereus</i> 569/H or 5/B penicillinase (Pollock, 1957) or <i>Bacillus subtilis</i> 749 penicillinase (Kushner, 1960)	150 units/ml. (0.9 unit/cm. ²)	1.5 units/ml. (0.009 unit/cm. ²)	B
<i>Staphylococcus aureus</i> 524 SC penicillinase	200 units/ml. (1.2 units/cm. ²)	2 units/ml. (0.012 unit/cm. ²)	B

false positive result is also slight since so far no enzyme is known, other than that type of penicillinase (penicillin β -lactamase) for which the test was devised, which attacks penicillin to yield a product reacting with iodine.

SUMMARY

1. A method is described for the chromatographic detection of penicillins and related compounds and of penicillinase based on the reaction of iodine with penicilloic acids.

REFERENCES

- Alcino, J. F. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 619.
 Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
 Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 387.
 Kushner, D. J. (1960). *J. gen. Microbiol.* **23**, 381.
 Pollock, M. R. (1957). *J. Pharm., Lond.*, **9**, 609.
 Pollock, M. R., Torriani, A.-M. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
 Winegard, H. M., Toennies, G. & Block, R. J. (1948). *Science*, **108**, 506.

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Purification of Latent Phenolase from Broad-Bean (*Vicia faba*) Leaves

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The paucity of information on the nature and function of the plant phenolases is apparent from a recent review (Bonner, 1957) on this family of oxidases. An interesting contribution has been the large-scale fractionation and purification of mushroom phenolase by Kertesz & Zito (1957). More recently reports have appeared of the chromatography on cellulose ion-exchange agents of phenolase from mushroom (Frieden & Ottesen, 1959), *Polyporus versicolor* (Malmström, Fähræus & Mosbach, 1958) and from mammalian tissue (Brown & Ward, 1958). A latent phenolase is present in the leaves of the broad bean, and Kenten (1957, 1958) has studied in some detail the activation of this enzyme in a partially purified preparation. During the course of work in these laboratories on the characterization of the proteins of the broad-bean leaf, various fractions, as judged by their colour, were obviously contaminated with Kenten's latent phenolase. This brief paper describes the chromatographic method used quite early in the work for the removal of the enzyme from pigments and from the major protein components of the leaf juice.

EXPERIMENTAL

Preparation of leaf extract. The broad-bean plants (Windsor Improved) were grown to various stages of maturity in a garden or in John Innes Compost in a heated greenhouse. The leaves (50–100 g. fresh weight) were

vacuum-infiltrated with a solution containing 0.03M-potassium ascorbate, 0.2 mM-sodium ethylenediamine-tetra-acetate and 0.03M-potassium phosphate buffer, pH 7.5. Excess of solution was then squeezed from the leaves before they were ground with the aid of a little sand in a pestle and mortar at 4°. The suspension was centrifuged at 20 000g for 45 min., and the supernatant dialysed against many changes of buffer containing 5 mM-ascorbate, 4 mM-phosphate and 4 mM-citrate, adjusted to pH 6.5 with potassium hydroxide. The latent phenolase solution was freeze-dried, and the residue was taken up in 2–3 ml. of water, centrifuged if necessary to clarify, and dialysed for 1 hr. against 5 mM-potassium phosphate buffer at pH 7.

Column chromatography. Diethylaminoethylcellulose (DEAE-cellulose) was prepared from Solka Floc SW-40B (Johnsen, Jorgensen and Wettre Ltd., London, E.C. 4) according to Peterson & Sober (1956) but with only half the quantity of the reagent α -chlorotriethylamine hydrochloride. The resulting adsorbent contained 0.50 m-equiv. of basic nitrogen/g. A column of DEAE-cellulose (30 cm. $\times$ 1 cm.) was prepared from a slurry in 5 mM-potassium phosphate buffer, pH 7, and packed down under a pressure of 8 lb./in.². The phenolase solution was transferred to the column and eluted rapidly with 5 mM-potassium phosphate buffer at pH 7. After 20 ml. had passed through the column, 0.5M-potassium hydroxide was applied through a 100 ml. mixing chamber to establish a concentration gradient and a pH gradient to 8.5. Duplicate samples of 0.1 ml. were taken from 2 ml. fractions for analysis by the Folin-Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) for protein and by β -(3:4-dihydroxyphenyl)-DL-alanine-Teepol for phenolase. The latter reagent was prepared from 5 ml. of Teepol (Anionic detergent, Shell Chemical Co.), 30 mg. of β -(3:4-dihydroxyphenyl)-DL-alanine and 0.05M-potassium phosphate at pH 6 to a volume of 50 ml. This reagent (0.5 ml.) and 4 ml. of buffer, pH 6, were added to each portion, and the extinction of the

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