The Reduction of Quinic Acid to Dihydroshikimic Acid by Certain Lactic Acid Bacteria

By J. G. CARR, A. POLLARD, G. C. WHITING and A. H. WILLIAMS
Research Station, Long Ashton, Bristol

(*Received* 13 *July* 1956)

Phillips, Pollard & Whiting (1956) showed that the amount of quinic acid present in juices of English cider apples is second only to that of malic acid. In addition they showed that in juices fermented by the naturally occurring yeasts and bacteria, quinic acid was metabolized. Earlier, Carr, Phillips, Pollard, Whiting & Williams (1954) isolated from cider a heterofermentative *Lactobacillus* which, unlike the type species of *L. brevis*, *L. buchneri*, *L. pastorianus*, *L. plantarum* and *L. arabinosus* 17–5, was able to convert quinic acid into a substance tentatively identified as dihydroshikimic acid. The isolate from cider was able to carry out this conversion either in apple juice or in a semi-synthetic medium containing quinic acid. The present investigation describes the conversion by this organism of both quinic and shikimic acids into dihydroshikimic acid and the characterization of this compound.

METHODS

The organism used was the strain previously isolated from cider in the preliminary experiments (Carr *et al.* 1954). It has been identified as a variety of *Lactobacillus pastorianus* and given the epithet *quinicus*. A full description of its biological characteristics will be published elsewhere.

Two methods were used for studying the metabolism of quinic and shikimic acids: (1) inoculation of growing cells into a semi-synthetic medium containing quinic acid or shikimic acid and (2) addition of washed cells to solutions of these substances. Cells for method (1) were initially grown in 5 ml. portions of apple juice–yeast-extract medium (Carr, 1952) at 25° for 72 hr., and centrifuged and suspended in an equivalent volume of saline (0·85%). This suspension (0·5 ml.) containing 4×10^{10} organisms/ml. (total count by haemocytometer) was then inoculated into 100 ml. of medium of the following composition (%, w/v) and incubated in the presence of air without agitation or aeration for 12 days at 25°: quinic or shikimic acid 0·25 g. Allen and Hanbury's vitamin-free casein hydrolysate 0·5 g.; fructose, 0·25 g.; Difco yeast extract, 0·4 g.; KH_2PO_4, 0·055 g.; KCl, 0·0425 g.; $CaCl_2$, 0·0125 g.; $MgSO_4$, 0·0125 g.; $FeCl_3$, 0·25 mg.; $MnSO_4$, 0·25 mg. Before inoculation the pH was adjusted to 4·2 with dilute HCl and the medium sterilized at 15 lb./in.^{2} for 15 min. An uninoculated control was treated similarly.

For method (2) cells from 450 ml. of the apple-juice medium mentioned above were harvested after growth for 48 hr. at 25°, and washed twice by dispersion in sterile saline followed by centrifuging. The resultant bacterial paste was suspended in 4 ml. of sterile 0·02 M-aminotrishydroxymethyl methane (tris) buffer, adjusted to pH 7·5 with dilute HCl. Equal volumes of this suspension containing $2·5 \times 10^{10}$ viable cells/ml. and a 0·004 M solution of sterile quinic or shikimic acid were mixed in an evacuated Thunberg tube and incubated at 25° for 24 hr. A control tube, also containing equal volumes of suspension (previously heated at 100° for 20 min.) and the test substrate, was treated similarly.

The acids present before and after incubation were investigated by paper chromatography with the descending technique, on sheets of Whatman no. 1 paper. All chromatograms were run at room temperature without temperature control. The two solvents used were: (1) benzyl alcohol–*iso*propyl alcohol–*tert.*-butyl alcohol–water (3:1:1:1, by vol.) containing 2% (v/v) of formic acid (Stark, Goodban & Owens, 1951), run for 24 hr.; (2) phenol–water (3:1, w/v) containing 1% (v/w) of formic acid (Stark *et al.* 1951), run for 30 hr. and allowed to run off the end of the paper.

After incubation, liquors from both types of experiment were centrifuged, and those for paper-chromatographic examination with solvent (2) were treated with Amberlite IR-120 resin in the hydrogen form; a small volume was stirred with 25% (w/v) of the resin for 5 min. The chromatograms before spraying were examined under ultraviolet light for the presence of 5-dehydroshikimic acid, which appears as a dark spot (Weiss, Davis & Mingioli, 1953). The following spray reagents were used: (1) bromocresol green, 0·08% in ethanol; (2) sodium metaperiodate, followed by sodium nitroprusside and piperazine (Cartwright & Roberts, 1955). Reagent (2) reacts not only with quinic acid but also with shikimic and dihydroshikimic acids (Phillips *et al.* 1956). Shikimic acid appeared more rapidly in the cold than the other two acids so, as an additional aid to identification, the chromatograms were left at room temperature for 30 min. and then heated at 100° for a few minutes until maximum intensity was attained. In order to estimate the amounts of each acid present a series of standard spots were run on the same paper (Phillips *et al.* 1956).

Materials

Quinic acid was obtained from L. Light and Co. of Poyle, Colnbrook, Bucks. Shikimic acid was extracted from the carpels of *Illicium verum* (B.P.Codex *Anisum stellatum*) (B.D.H. Ltd.).

RESULTS

Growing cells

Cells grown in the presence of quinic acid completely metabolized this acid, and dihydroshikimic acid appeared in the medium; the latter acid was isolated by the following method. The culture fluid after

12 days' incubation, from which the quinic acid had completely disappeared as shown by paper chromatography, was first centrifuged and 200 ml. was then passed through a column 50 cm. long, 1·3 cm. internal diameter, containing 35 g. of Amberlite IR-120 resin in the hydrogen form, at the rate of 1 ml./min. The percolate and water washings (about 300 ml., continued until neutral to Universal Indicator paper) were passed through an identical column of Amberlite IRA-400 resin in the acetate form at the same rate. After thorough washing with CO_2-free water, dihydroshikimic acid was obtained by fractional elution with 0·1N-acetic acid. Fractions (25 ml.) were collected and examined by paper chromatography. Dihydroshikimic acid was the first acid to appear and was present, chromatographically pure, in fractions 6–18 inclusive, with a broad peak at fractions 9–12. The fractions were bulked, concentrated *in vacuo* (below 40°) and finally in a vacuum desiccator over NaOH and P_2O_5. The yield from 0·5 g. of quinic acid was 0·443 g. of dihydroshikimic acid (96 % theoretical), m.p. 132·5–134°. It was characterized as follows: crystallized as long prisms from acetone–chloroform (1:1); m.p. 135–136° (Found: C, 47·47 %; H, 6·73. $C_7H_{12}O_5$ requires C, 47·70 %; H, 6·82 %). Oxidation of the acid, in which 20 mg. was heated with 1 ml. of H_2O_2 (100 vol.) for 3 hr. at 115°, yielded tricarballylic acid as main product (Hulme, 1951). Optical rotation $[\alpha]_D^{25} - 63°$; *c* 10 in water. The melting point and the optical rotation of the acid indicated that it was not the epidihydroshikimic acid of Grewe & Lorenzen (1953), which had m.p. 178–179° and $[\alpha]_D^{26} - 13·45°$. These authors obtained dihydroshikimic acid as a syrup but described the *iso*propylidene derivative of the lactone, m.p. 98–99°. The bacterial product gave an *iso*propylidene derivative of the lactone of identical m.p. (Found: C, 60·87 %; H, 7·29. $C_{10}H_{14}O_4$ requires C, 60·59 %; H, 7·12 %). A mixed melting point of the *iso*propylidene derivative of the lactone with an authentic specimen showed no depression, confirming that the bacterial product was the dihydroshikimic acid with the same structural configuration as (−)-quinic acid. The acid formed a lactone on heating above the melting point. Sublimation occurred after heating at 175–180° at 0·1 mm. pressure. The sublimate on cooling formed needle-like crystals, m.p. 129·5–130·5°. The lactone, on standing overnight with cold dilute NaOH solution, was hydrolysed to the acid.

Cells grown in the presence of shikimic acid metabolized this acid completely and gave dihydroshikimic acid (yield 60 % of theoretical) isolated by the method described above. Its identity with the acid formed from quinic acid was shown by the melting point of the acid as compared with that of a specimen of the compound formed from quinic acid: a mixed melting point showed no depression. The *iso*propylidene derivative of the lactone also showed no depression of the melting point when mixed with an authentic specimen.

Resting cells

These investigations were carried out with the aid of paper chromatography, and solvent (2) in conjunction with Cartwright & Roberts's (1955) reagent proved of great value. The R_F values and R_Q values (distance moved relative to quinic acid) were as in Table 1. Cells incubated for 24 hr. with quinic acid showed a conversion of half the quinic acid into shikimic acid and dihydroshikimic acid, in the proportion of 1:6 as estimated by spot size and intensity. Cells incubated with shikimic acid yielded a trace of quinic acid and a large amount of dihydroshikimic acid, in the proportion 1:10; all the shikimic acid was metabolized. Control tubes containing quinic or shikimic acid together with cells which had been heated for 20 min. at 100° showed no change. In these tests approximately 40–50 % of the quinic and shikimic acids originally added gave rise to products as yet unidentified. No 5-dehydroshikimic acid was detected at pH 7·5 or at pH 6·2, where this acid is more stable.

DISCUSSION

The results show that certain lactic acid bacteria present in ciders possess an enzyme system capable of reducing quinic acid to dihydroshikimic acid. Both growing cells and resting-cell suspensions converted shikimic acid and quinic acid into the same end product, namely, dihydroshikimic acid: with resting cells there was also some conversion of shikimic acid into quinic acid. These results therefore go some way to support the hypothesis of Hulme (1951), who suggested a quinic acid–shikimic acid–dihydroshikimic acid enzyme system analogous to the malic acid–fumaric acid–succinic acid system. It is not possible from the present results to state whether shikimic acid is an intermediate in the conversion of quinic acid into dihydroshikimic acid, whether shikimic acid is first converted into quinic acid and thence to dihydro-

Table 1. *Chromatographic separation of quinic acid and related acids*

	R_F values		R_Q values (distance moved relative to quinic acid), solvent (2)
	Solvent (1)	Solvent (2)	
Quinic acid	0·22	0·50	1·00
Shikimic acid	0·42	0·58	1·16
Dihydroshikimic acid	0·42	0·66	1·32
5-Dehydroshikimic acid	0·51	0·73	1·46

shikimic acid or whether the reduction of the two compounds follows separate routes. The results show, however, that quinic acid and shikimic acid are interconvertible. In the scheme studied by Davis (1955) with *Escherichia coli* mutants it appears that quinic acid is not in the direct sequence leading from carbohydrate through shikimic acid to the aromatic metabolites. In this scheme the precursors of shikimic acid are 5-dehydroquinic acid and 5-dehydroshikimic acid. In the present tests it has not been possible to demonstrate the formation of either of the latter two compounds. The possible participation of quinic acid in a sequence leading to aromatization is, however, not excluded since in the wild-type *Aerobacter aerogenes* quinic acid is a precursor of 5-dehydroquinic acid. In the lactic acid bacterium investigated there is as yet no evidence to suggest the role of shikimic acid as a precursor of aromatic amino acids.

SUMMARY

1. Growing and resting cells of an organism occurring in cider, *Lactobacillus pastorianus* var. *quinicus*, are capable of converting quinic and shikimic acids into dihydroshikimic acid.

2. The quinic acid to shikimic acid step is reversible.

The authors are indebted to Professor Wilson Baker, F.R.S., and Dr W. D. Ollis for helpful discussions on the configuration of dihydroshikimic acid. We also wish to thank Dr B. D. Davis and Professor Grewe for gifts of 5-dehydroshikimic acid and the *iso*propylidene derivative of the lactone of dihydroshikimic acid respectively.

REFERENCES

Carr, J. G. (1952). *Rep. agric. hort. Res. Sta., Bristol*, p. 144.

Carr, J. G., Phillips, J. D. (the late), Pollard, A., Whiting, G. C. & Williams, A. H. (1954). *Chem. & Ind.* p. 1515.

Cartwright, R. A. & Roberts, E. A. H. (1955). *Chem. & Ind.* p. 230.

Davis, B. D. (1955). *A Symposium on Amino Acid Metabolism*, p. 779. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.

Grewe, R. & Lorenzen, W. (1953). *Chem. Ber.* **86**, 928.

Hulme, A. C. (1951). *J. exp. Bot.* **2**, 298.

Phillips, J. D. (the late), Pollard, A. & Whiting, G. C. (1956). *J. Sci. Fd Agric.* **7**, 31.

Stark, J. B., Goodban, A. E. & Owens, H. S. (1951). *Analyt. Chem.* **23**, 413.

Weiss, U., Davis, B. D. & Mingioli, E. S. (1953). *J. Amer. chem. Soc.* **75**, 5572.

Studies on the Carbohydrate Metabolism of Skin

By C. N. D. CRUICKSHANK, M. D. TROTTER and J. R. COOPER
Medical Research Council Unit for Research on the Experimental Pathology of the Skin, University of Birmingham

(*Received* 10 *September* 1956)

One of the classical methods of determining the metabolic pathways in a tissue is the investigation of the effects of various possible substrates upon the oxygen uptake *in vitro*. This method is open to the criticisms that some substances may not adequately penetrate the cell membrane (particularly if tissue slices are used) and that unknown factors may be present *in vivo* but absent from the *in vitro* preparation. Nevertheless, positive findings may be regarded as reliable provided that catalytic or hormonal effects are excluded. This investigation is concerned with the metabolism of glucose and fructose and with the effects of various possible intermediate metabolites upon the respiration of skin slices *in vitro*.

MATERIALS AND METHODS

Oxygen uptake was measured by means of the differential capillary respirometer designed by Cruickshank (1954) and used for a previous investigation of the glucose and lactic acid metabolism of skin (Cruickshank & Trotter, 1956). The skin slices were cut free-hand from the dorsum of the guinea-pig ear, and, to minimize animal variation, each experiment was designed to include its own control and to permit of statistical evaluation. The control medium consisted of Krebs–Ringer phosphate (Field, 1948) and for experiments lasting 24 hr. streptomycin sulphate was added to a final concentration of 50 μg./ml. Substrates (either sodium salts or free acids) were prepared in solutions ten times more concentrated than required, adjusted to pH 7·4 with NaOH where necessary and sterilized by boiling or Seitz filtration. The potassium salt of glucose 1-phosphate was used. The substrates were added to the basic medium as 10% of the final volume to give the required concentration. All experiments were carried out in an atmosphere of air and at 37°. Respiration and hexose utilization rates were expressed in relation to wet weight of tissue.

Glucose analyses were carried out by the method of Somogyi (1952) and Nelson (1944), and fructose estimations by the method of Roe (1934). Lactate was estimated by the method of Barker & Summerson (1941).