ON THE EXTRACELLULAR ACCUMULATION AND ISOLATION OF D-RIBULOSE IN CULTURES OF AGROBACTERIUM TUMEFACIENS[1]

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During growth of Agrobacterium tumefaciens on D-glucose as the only source of carbon it was observed that free D-ribulose accumulated in the medium with time. The purpose of this communication is to record these findings since they represent the first extracellular accumulation of ribulose by a bacterium grown on glucose and to draw attention to a further observation that there is a highly significant quantitative difference between certain tumorogenic and non-tumorogenic strains of this organism with respect to the formation of free ribulose.

Materials and Methods

Agrobacterium tumefaciens strains B_6 (tumorogenic), $IIBV_7(k)$ (tumorogenic) and $IIBNV_6$ (non-tumorogenic) were isolates originally obtained from the laboratory of Dr. A.C. Braun, Rockefeller Institute. D-xylulose was prepared by hydrolysis of monoacetone-D-xylulose (Levene and Tipson, 1936). D-ribulose-o-nitrophenylhydrazone was purchased from California Biochemical Corporation and free D-ribulose was obtained from the latter by hydrolysis (Schmidt and Treiber, 1933).

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The synthetic growth medium of McIntire, Peterson and Riker (1942) was used throughout with the omission of manganese, ferric and phosphate ions. Growth from an initial 24 hour inoculum (1 ml added to a total volume of 30 ml of fresh medium) was carried out in shake culture at 26°C and was determined turbidimetrically.

For descending paper chromatography, water-saturated phenol (Chang and Knight, 1961) and n-propanol-ethyl acetate-water (Fukui, Hochster Durbin, Grebner and Feingold, 1963) were used. Aldoses were detected on chromatograms with aniline phthalate (Partridge, 1949) while ketopentoses were located and distinguished by means of the trichloroacetic acid-orcinol and aniline phthalate combination sprays (Hochster, 1955). The Urea phosphate spray (Wise, Dimler, Davis and Rist, 1955) was used to distinguish ribulose from 3-ketoglucose since it reacts only with the latter.

Pentose was estimated quantitatively according to Mejbaum (1939) using a 40 minute heating period (Albaum and Umbreit, 1947) and ribulose by the method of Dische and Borenfreund (1951). Glucose was estimated by the "glucostat" technique (Saifer and Gerstenfeld, 1958).

Results

Isolation of D-ribulose (as o-nitrophenylhydrazone) from the culture medium

In preliminary experiments D-ribulose was first identified on large filter paper sheets after polysaccharide had been removed from the culture fluid by precipitation with ethanol. The supernatent solution was then concentrated to a syrup *in vacuo*. A typical result of such chromatograms showing the relative positions of related sugars as controls is given in Table I. Authentic D-ribulose and the material isolated both gave identical colors when sprayed with the combination spray for ketopentoses (Hochster, 1955).

TABLE I

Chromatography of Sugars

Sugars	Rf-Values Solvent 1*	Solvent 2
D-Glucose	0.40	0.19
3-Keto-D-Glucose	-	0.24
D-Xylose	0.47	0.27
D-Ribose	0.64	0.33
D-Xylulose	0.60	0.42
D-Ribulose	0.68	0.38
Material isolated	0.68	0.37

*Solvent 1 - Water-saturated phenol; Solvent 2 - n-propanol: Ethyl acetate:water, 7:1:2.

Subsequently, 300 ml of culture medium removed after growth of the non-tumorogenic organism IIBNV$_6$, was concentrated *in vacuo* (at 35°C) to 50 ml and 250 ml of 95% ethanol was added. Following removal of the precipitated polysaccharide by centrifugation the supernatant solution was concentrated to 5 ml and a further quantity of ethanol (50 ml) added in the same manner to remove small amounts of polysaccharide remaining. The final supernatant solution was concentrated *in vacuo* to a thick syrup. The latter was dissolved in 10 ml of 0.05 M sodium tetraborate and this solution was passed through a 2 x 23 cm Dowex (1 x 2) column in the borate form at a flow rate of 25 ml/10 min. The column was washed with 100 ml 0.05 M sodium tetraborate following which elution with 0.02 M tetraborate was begun. Cysteine-carbazole-positive material emerged after about 2-4 litres of effluent had come through the column and was collected over an elution span of 1 liter. Glucose was not eluted under these conditions, but remained on the column until much

later. The combined systeine-carbazole-positive fractions were treated with Dowex 50 (H^+) to remove sodium and then concentrated to dryness at 35°C. Borate was then removed with methanol (Zill, Khym and Chenial, 1953) and the residue reduced to a small volume. Following decolorization with charcoal (Darco G-60 previously washed with concentrated HCl) and concentration to a syrup the crystalline o-nitrophenylhydrazone was prepared as previously described (Hochster, 1955).

After several recrystallizations, first from absolute alcohol and then from ethyl acetate, the following constants were obtained: melting points of authentic ribulose-o-nitrophenylhydrazone, 165-166°C, isolated material, 166-167°C, mixed melting point, 165-166°C. The $[\alpha]_D^{20}$ of the above derivative was -47° (authentic sample: -45.5°) which is in agreement with the original work of Glatthaar and Reichstein (1935). Free ribulose was then prepared from the o-nitrophenylhydrazone as described by Schmidt and Treiber (1933). Spectrophotometrically, the ratio of optical densities at 540/670 mμ of authentic free ribulose was 0.90 after orcinol treatment (Mejbaum, 1939) while the ratio for the isolated, free ribulose was 0.89 (Seegmiller and Horecker, 1952). The optical rotation of the free ribulose isolated as above was -17.2° compared with the literature value of -16.3° (Glatthaar and Reichstein, 1935). On the basis of the above physical constants the product obtained from the culture medium was D-ribulose.

In the microbiological conversion to D-ribulose by this organism it was observed that only D-glucose served as a precursor, while D-fructose, 2-keto-D-gluconate, D-xylose, D-ribose, D-arabinose and D-arabitol gave completely negative results. The rate of conversion of D-glucose to D-ribulose is given in Table II. It should be pointed out that highly significant conversion appears to be a characteristic of the non-tumorogenic strain used while the tumorogenic strains produced relatively little D-ribulose under these experimental conditions. This

TABLE II

Formation of Ribulose by Intact Cells of
Agrobacterium tumefaciens

Biological Activity	Strains		Time (Hours) 0	10	24	48	60
Tumorogenic	A. tumefaciens (B_6)	Turbidity*	30	84	128	195	210
		Glucose; x 10^{-3}M**	68.0	58.5	56.7	49.0	38.7
		Ribulose; x 10^{-3}M***	0.33	0.55	0.60	0.45	0.38
	A. tumefaciens ($IIBV_7K$)	Turbidity	30	56	90	160	190
		Glucose; x 10^{-3}M	68.0	57.5	55.2	49.0	36.5
		Ribulose; x 10^{-3}M	0.51	1.20	1.72	1.38	0.83
Non-tumorogenic	A. tumefaciens ($IIBNV_6$)	Turbidity	20	60	107	170	190
		Glucose; x 10^{-3}M	68.0	54.0	40.2	29.3	21.5
		Ribulose; x 10^{-3}M	0.70	5.40	8.25	3.85	3.02

* Klett Units
** Glucose concentration of medium (glucostat technique)
*** Ribulose concentration of medium (cysteine-carbazole method)

interesting difference as well as the mechanism of conversion of D-glucose to D-ribulose is now under investigation. Preliminary data suggest the existence of a new non-phosphorylative pathway from D-glucose to D-ribulose in which 3-keto-D-glucose (Fukui and Hochster, 1963; Fukui, 1965) is the intermediate.

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