MATERIALS

AND

METHODS

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Chemicals

The chemicals used in the experiments were obtained from the following sources :

Magnesium chloride, magnesium sulphate, ammonium sulphate, sodium dihydrogen phosphate, sodium chloride, potassium nitrate, sucrose, glucose, tris, tri-sodium phosphate, triphosphopyridine nucleotide, uracil, thymine, sodium succinate, sodium citrate and ninhydrin from British Drug House, England.

L-arginine hydrochloride, calcium chloride, calcium nitrate, diacetylmonoxime, EDTA, iodoacetic acid, DL-Isoleucine manganese sulphate, pyridoxal phosphate, sodium hydroxide, sodium nitrate, aluminium sulphate and D(+) fructose from E. Merck & Co., Germany.

AMP, orthoaminobenzaldehyde and p-chloromercury benzoate, from Fluka & Co., Switzerland.

Ferric chloride, nickel chloride, sodium azide, sodium fluoride, sodium molybdate, zinc chloride and zinc sulphate from Reidel-DeHaen; aluminium chloride, from Albright & Wilson Co., London; L-citrulline and glucose-1-phosphate from Nutritional Biochemical Corporation, U.S.A.

Adenosine, AMP, ADP, ATP, GMP, CMP, UMP, TMP, IMP, XMP, carbamyl phosphate, oxaloacetic acid, DL-ornithine, L-ornithine, triton X-100, 2-oxoglutaric acid, N-methyl-nicotinamide, diphosphopyridine nucleotide, glucose-6-phosphate, fructose-1,6-diphosphate and fructose-1-phosphate from Sigma Chemical Co., U.S.A.

Agar agar powder from Heiwa Agar Co., Japan; adenine from Paul Neuendore, Manz, Germany; malic acid from CSR. Penick & Co.; fumaric acid from Chemo Puro, N.Y., U.S.A.; Ribose from British Drug House, England and DEAE cellulose from Brown & Company, Berlin.

Source of Tissue

Normal and tumour tissues of Rumex acetosa L. were obtained from Prof. A.C. Gentile, University of Massachusetts, U.S.A.

Medium and maintenance of tissues

Normal and tumour tissues of Rumex acetosa were cultivated on solid medium as described by Gentile (1963). Different stock solutions were prepared according to the composition given in Tables 1 and 2 and stored at 5-10°C. The medium was compounded fresh from the stock solutions and diluted so as to yield the specific composition. Double glass distilled water was used for preparing the medium as

Table 1 : Composition of the culture for Rumex tumour tissue.

	Stock solutions	grams/litre	Volume taken for 1 litre of medium (ml)
1.	KNO_3	20,220	10
2.	$Ca(NO_3).4H_2O$	47,232	15
3.	$MgSO_4.7H_2O$	24.648	10
4.	KH_2PO_4	68.044	16
5.	$CaCl_2.2H_2O$	29,404	15
6.	KCl	14.912	10
7.	$MgCl_2.6H_2O$	20.332	10
8.	Thiamine hydrochloride	0.100	1
	Pyridoxine hydrochloride	0.800	
	Nicotinamide	0.800	
9.	Na_2 EDTA	0.800	3
	$Fe_2 (SO_4)_3$	0.380	
10.	H_3BO_3	0.570	1
	$MnCl_2.H_2O$	0.360	
	$ZnCl_2$	0.625	
	$Na_2MoO_4.2H_2O$	0.252	
	$CuCl_2.2H_2O$	0.028	

Table 2 : Composition of the culture medium for Rumex normal tissue.

	Stock solutions	grams/litre	Volume taken for 1 litre of medium (ml)
1.	Na_2SO_4	20.000	
	$MgSO_4.7H_2O$	73.700	
	KNO_3	8.000	
	KCl	6.500	
	$NaH_2PO_4.H_2O$	1.900	10
	$MnSO_4.H_2O$	0.500	
	$ZnSO_4.7H_2O$	0.267	
	H_3BO_3	0.150	
	KI	0.750	
2.	Ca $(NO_3)_2.4H_2O$	28.650	10
3.	Na_2 EDTA	0.800	3
	$Fe_2(SO_4)_3$	0.380	
4.	2,4-Dichlorophenoxy acetic acid	0.036	1
5.	Glycine	3.000	
	Nicotinic acid	0.500	
	Thiamine hydrochloride	0.100	1
	Pyridoxine hydrochloride	0.100	
6.	Coconut milk	20%	200

well as the stock solutions. Wherever hydrates of any salts were used, appropriate corrections were made.

Nine grams of agar was separately dissolved in 500 ml of hot water and mixed with the nutrient medium to give a final volume of 1 litre. 2% sucrose was added and the pH was adjusted to 5.2 at 40-50°C using a Systronix pH meter. 25 ml of the medium was distributed while hot to each Erlenmeyer flask of 100 ml capacity. The flasks were tightly plugged with cotton wrapped in gauge cloth and the medium was sterilized by autoclaving at 15 lbs p.s.i. for 15 minutes. After the medium was properly set, unless otherwise stated, about 30-40 days old tissues were sub-cultured into these flasks under sterile conditions and were allowed to grow at a temperature of 22-23°C in diffused light.

Preparation of calcium phosphate gel

Calcium phosphate gel was prepared according to the method described by Keilin and Hartree (1938). 150 ml calcium chloride solution (132 grams $CaCl_2.6H_2O$ per litre) was diluted to about 1600 ml with distilled water and shaken with 150 ml trisodium phosphate solution (152 grams $Na_3PO_4.12H_2O$ per litre). The mixture was brought to pH 7.4 with dilute acetic acid and the precipitate washed three or four times by decantation with large volume of water (15-20 litres). The precipitate was finally washed with distilled water and allowed

to stand for about a month. After removing the clear water layer, the gel was shaken and the dry weight per ml determined. It was found to be 22 mg per ml.

Preparation of alumina C_γ gel

Alumina C_γ gel was prepared according to the method described by Willstatter and Kraut (1923). 300 grams of ammonium sulphate was dissolved in 6.5 litres of water, heated at 60°C and 420 ml of 20% (W/W) NH_3 (i.e. 77.5 grams NH_3 (Theor. 76.6 grams) was added, the fluid remained slightly alkaline during the subsequent precipitation). Hot solution of 500 grams aluminium sulphate in 1 litre was poured with vigorous stirring. Stirring was continued for 15 minutes after addition, keeping the temperature at about 60°C. Diluted to 40 litres, precipitate allowed to settle and decanted. Precipitate was washed repeatedly by decantation. To fourth wash-water 80 ml of 20% ammonia was added. After 12 to 20 washings, the water remained opalescent, after this point is reached, precipitate was washed twice more and allowed to stand several weeks to convert the C_α form into C_γ form. The dry weight per ml was determined. It was found to be 18 mg per ml.

Preparation of hydrindantin

Hydrindantin was prepared according to the method described by Duggan (1957). 80 grams of ninhydrin was

dissolved in 2 litres of water, and ascorbic acid solution (80 grams per 100 ml water) was added to it with stirring at 90°C. The crystallized material was cooled by running tap water for 1 hour. The crystals were filtered, washed with water, dried in vacuum,protected from light and stored in dark bottle.

Estimation of ornithine

Ornithine was estimated according to the method of Ratner and Rochovansky (1956). To 1.0 ml sample was added 1.0 ml of warm ninhydrin reagent (250 milligrams of ninhydrin + 37.6 milligrams of hydrindantin dissolved in 4.0 ml of 6 M H_3PO_4 and 6.0 ml of glacial acetic acid by heating at 50-60°C) followed by 1.5 ml of glacial acetic acid. The tubes were kept in boiling water bath for 30 minutes, 2.5 ml of glacial acetic acid ~~was~~ hard added after cooling the tubes and the colour was read at 540 mμ in Klett colorimeter against a standard ornithine sample, treated in the same manner.

Estimation of citrulline

Citrulline was estimated according to the method described by Archibald (1944) as modified by Srivastava, Dholakia and Naik, 1971).

Estimation of protein

Protein was determined by the method of Lowry,Rosenbrough, Farr and Randall (1951) and in purified fractions by the method of Warburg and Christian (1941).

Enzyme studies

The enzymes studied are arginase (L-arginine ureo-hydrolase, E.C., 3.5.3.1), ornithine transcarbamylase (L-ornithine carbamoyl transferase, E.C., 2.1.3.3) and ornithine transaminase (L-ornithine : 2-oxoacid amino-transferase, E.C., 2.6.1.13). The enzyme ornithine trans-carbamylase is referred to as ornithine carbamoyl transferase hereafter.

Preparation of homogenate for enzyme assay

Tissues cultivated for 3 weeks were used for all the studies. The tissue was taken out from the culture flask, freed from adhering agar, if any, and a weighed amount ground for 7-10 minutes with 0.25% triton X-100 in a chilled mortar kept on crushed ice.

Enzyme assays

Details of the enzyme assays are given in Table 3.

Specific activity of the enzyme

Specific activity is defined as enzyme units per milligram portein.

Table 3 : Details of enzyme assays

Details of assay system and procedure	Arginase (L-arginine urec hydrolase) (E.C., 3.5.3.1)	Ornithine transaminase (L-ornithine: 2-oxoacid amino transferase) (E.C., 2.6.1.13)
1. Basis of method used	Ramaley and Bernlohr (1966)	Peraino and Pitot (1963)
2. Buffer	Carbonate-bicarbonate, pH 10.5, 50 μ moles)	Tris-HCl, pH 8.5, 50 μ moles
3. Substrate	L-Arginine hydrochloride, 20 μ moles	DL-Ornithine, 10 μ moles
4. Enzyme extract	0.2 ml (10% crude homogenate)	1.0 ml (40% crude homogenate)
5. Other components	$MnCl_2$, 10 μ moles	2-oxoglutamate, 10 μ moles; pyridoxal phosphate, 0.1 μ moles; O-aminobenzaldehyde, 0.1 ml (10 mg/ml).
6. Temperature and period of incubation	37°C, 60 minutes	30°C, 60 minutes
7. Start of reaction	Enzyme added	Enzyme added.
8. Termination of reaction	0.5 ml of 10% TCA added and centrifuged to remove the precipitated protein	0.5 ml of 10% TCA added and centrifuged to remove precipitated protein.
9. Treatment of Blank	Substrate added after terminating the reaction.	Substrate added after terminating the reaction.
10. Parameter measured	Ornithine formed was measured as described in text.	Optical density of pyrroline-5-carboxylic acid-O-aminobenzaldehyde complex formed was measured at 430 m μ
11. Enzyme unit	Amount of enzyme required to form 1 μmole of ornithine per hour under the assay conditions.	Amount of enzyme required to give a change of 0.050 in optical density per hour under the assay conditions.

Table 3 (contd.)

Details of assay system and procedure	Ornithine carbamoyl transferase (Carbamyl phosphate: L-ornithine carbamyl transferase) (E.C., 2.1.3.3)
1. Basis of the method used	Burnett and Cohen (1957)
2. Buffer	Tris-HCl, pH 9.0; 50 μ moles
3. Substrate	DL-Ornithine, 2 μ moles
4. Enzyme extract	0.2 ml (30% crude extract)
5. Other components	Carbamyl phosphate (Li Salt), 2 μ moles
6. Temperature and period of incubation	37^{o}C, 30 minutes
7. Start of reaction	Enzyme added
8. Termination of reaction	0.5 ml of 10% TCA added and centrifuged to remove the precipitated protein.
9. Treatment of Blank	Substrate added after terminating the reaction.
10. Parameter measured	Citrulline formed was measured as described in the text.
11. Enzyme unit	Amount of enzyme required to form 1 μ mole of citrulline per hour under the assay conditions.

Purification of ornithine carbamoyl transferase

Step - 1 :

A known amount of tissue was ground for 10-15 minutes with 0.25% cold triton X-100 in a chilled mortar kept on crushed ice. The homogenate was diluted with triton X-100 solution to get 30% extract (W/V) on fresh weight basis and centrifuged at 10,000 x g for 15 minutes in a Servall refrigerated centrifuge. The residue was discarded.

Step - 2 :

A known volume of alumina C_γ gel (6 mg/ml dry weight) was taken in a centrifuge tube and centrifuged at 10,000 x g for 5 minutes. To the gel residue, the enzyme supernatant from Step-1 was added in a gel to enzyme ratio of 1.5:10 and stirred in cold for 20 minutes. The suspension was centrifuged at 10,000 x g for 10 minutes. The residue containing the adsorbed enzyme was extracted for 20 minutes with 0.05 M Tris-HCl buffer pH 8.5 with stirring and then centrifuged at 10,000 x g for 10 minutes.

Step - 3 :

A known volume of calcium phosphate gel (22 mg/ml dry weight) was taken in a centrifuge tube and centrifuged at 10,000 x g for 5 minutes. To the gel residue, the enzyme fraction from Step-2 was added in a gel to enzyme ratio of

1:10 and stirred in cold for 10 minutes. The suspension was centrifuged at 10,000 x g for 10 minutes. The supernatant contains enzyme.

Step - 4 :

A DEAE-cellulose column of 40 x 1 cm. was prepared and was equilibrated with 0.05 M Tris-HCl buffer pH 8.5. Enzyme containing supernatant from Step - 3 was passed through the column. The column was washed with 5 volumes (10 ml each) of 0.05 M NaCl and the enzyme was eluted with 0.1 M NaCl. First four fractions (10 ml each) of 0.1 M NaCl were collected and used as enzyme source.