MATERIALS AND METHODS

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Chemicals :

The chemicals used in the experiments were of research grade purity and were obtained from the following sources:

DL-Alanine, L-aspartic acid, boric acid, copper sulfate, cupric chloride, L-cystine, 2:4-dichlorophenoxy acetic acid, glucose, L-glutamic acid, L-glutamine, glycine, guanine, L-hydroxy proline, indole acetic acid, magnesium chloride, magnesium sulfate, manganese sulfate, mercuric chloride, naphthalene acetic acid, potassium chloride, potassium dihydrogen phosphate, potassium hydroxide, potassium iodide, potassium nitrate, DL-phenyl alanine, L-proline, DL-serine, sodium dihydrogen phosphate, sodium sulfate, sodium chloride, sucrose, DL-threonine, thymine, L-tryptophan and urea from British Drug House, England.

Adenine, allantoin, L-arginine hydrochloride, L-ascorbic acid, L-asparagine, calcium chloride, calcium nitrate, L-cysteine hydrochloride, digitonin, disodium hydrogen phosphate, diacetyl monooxime, EDTA, iodoacetic acid, DL-isoleucine, L-leucine, L-lysine, manganese sulfate, DL-methionine, nicotinic acid,ninhydrin, pyridoxal phosphate, pyridoxine hydrochloride, pyruvic acid, sodium hydroxide, sodium nitrate, L-tyrosine, uric acid and xanthine from E.Merck & Co., Germany.

γ-Aminobutyric acid, O-amino benzaldehyde, glyoxalic acid, guanosine and DL-histidine from Fluka & Co., Switzerland; ferric chloride, nickel chloride, sodium arsenate, sodium azide, sodium fluoride, sodium molybdate, zinc chloride and zinc sulfate from Riedel-De Haen; agar-agar powder from Heiwa Agar Co., Japan; aluminium chloride and ferric sulfate from Albright and Wilson Co.,London; L-citrulline from Nutritional Biochemical Corporation, U.S.A. ; uracil and L-valine from L.Light & Co.,England; nicotinamide from Ches-Pfizzer & Co., U.S.A. and sodium deoxycholate from Difco. Penicillin, terramycin, streptomycin, aureomycin chloramphenicol, sulphaguanidine, and phenobarbitone were obtained from local market.

AMP. ADP, ATP, agmatine sulfate, canavanine sulfate, carbamyl phosphate, cytosine, guanidino acetic acid, γ-guanidino butyric acid, hapoxanthine, kinetin, 2-oxo-glutaric acid, NAD, oxaloacetic acid, DL-ornithine, orotic acid, putrescine, triton X-100, 2-thioxanthine, 6-thioxanthine, 2-amino-6-mercapto purine, 2,6-dithiopurine, 2-acetyl amino-6-hydroxy purine and 6-hydrazinopurine from Sigma Chemical Co., U.S.A.

Source of tissues :

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

Normal and crown-gall tumour tissues of Parthenocissus tricuspidata and tobacco were obtained from Prof.R.J. Gautheret, Faculty of Science, C.P.E.M.I. RE Section,Paris.

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 Tables1 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 well as the stock solutions. Wherever hydrates of any salts were used appropriate corrections in weights were made.

Seven 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 Beckman 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 p.s.i. for 15 minutes. After

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

Stock solutions	g/litre	Volume taken for 1 litre of medium (ml)
1. KNO_3	20.220	10
2. $Ca(NO_3)_2\ 4H_2O$	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	0.100	1
Pyridoxine	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	
$Na_2MoO_42H_2O$	0.252	
$CuCl_2\ 2H_2O$	0.028	

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

Stock solutions	g / litre	Volume taken for 1 litre of medium (ml)
1. Na_2SO_4	20.000	10
$MgSO_47H_2O$	73.700	
KNO_3	8.000	
KCl	6.500	
$NaH_2PO_4.H_2O$	1.900	
$MnSO_4.H_2O$	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
$Fe_2(SO_4)_3$	0.381	
4. 2,4-Dichlorophenoxy acetic acid	0.036	1
5. Glycine	3.000	1
Nicotinic acid	0.500	
Thiamine hydrochloride	0.100	
Pyridoxine "	0.100	
6. Coconut milk	20 %	200

the medium was properly set, unless otherwise stated, about 30-40 days old tissues were subcultured into these flasks under sterile conditions and were allowed to grow at a temperature of 22-23°C in diffused light.

In case, where studies were made by omitting or substituting any of the medium components, it was added or omitted during the preparation of the medium.

Normal and crown-gall tissues of Parthenocissus and tobacco were grown on the medium as suggested by Prof. Gautheret (personal communication). The composition of the medium used was as follows :

Parthenocissus tricuspidata

(a) Normal tissue

Agar 0.7 %

Macroelements of Hellers medium

Microelements of Hellers medium

Glucose 5%

Vitamin B_1, 1×10^{-6}

NAA, 5×10^{-6}

(b) Crown-gall tissue

Agar 0.7%
Macroelements of Hellers medium
Microelements of Hellers medium
Glucose 5%
Vitamin B_1, 1×10^{-6}

Tobacco

(a) Normal tissue

Agar 0.7 %
Knop 1/2
Microelements of Hellers medium
Glucose 5 %
Vitamin B_1, 1×10^{-6}
2, 4-D , 1×10^{-7}
Kinetin , 1×10^{-6}

(b) Crown-gall tissue

Agar 0.7 %
Knop 1/2
Microelements of Hellers medium
Glucose 5 %
Vitamin B_1, 1×10^{-6}

The composition of the macro and micro elements of Hellers medium and Knop 1/2 is given in Table 3.

The medium was compounded from the stock solutions in the same way as described for Rumex tissue. In cases of the media where IAA or NAA was used, they were separately sterilized by filtering through bacterial filter under sterile conditions and then added to the sterilized medium under sterile conditions. 40 ml of the media was distributed in 150 ml Erlenmeyer flasks. Unless otherwise stated about 7-8 week old tissue was used for subculturing.

Table 3. Composition of macro and micro elements of Hellers medium and Knop 1/2

Medium	Stock g/litre	Volume taken for 1 litre of medium (ml)
Hellers Macro elements		
KCl	15.00	50
$MgSO_4.7H_2O$	5.00	
$NaNO_3$	12.00	
$NaH_2\ PO_4.H_2O$	2.50	
$CaCl_2.2H_2O$	1.50	
Hellers Micro elements		
$FeCl_3.6H_2O$	1.00	1
$ZnSO_4.7H_2O$	1.00	
$MnSO_4.4H_2O$	0.10	
H_3BO_3	1.00	
$NiCl_3.6H_2O$	0.03	
$AlCl_3$	0.03	
KI	0.01	
$CuSO_4.5H_2O$	0.03	
Knop 1/2		
$Ca(NO_3)_2.4H_2O$	5.00	100
KNO_3	1.25	
$MgSO_4.7H_2O$	1.25	
KH_2PO_4	1.25	

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 g $CaCl_2 6H_2O/\ell$) was diluted to about 1600 ml with distilled water and shaken with 150 ml trisodium phosphate solution (152 g $Na_3PO_4.12H_2O/\ell$). 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 ℓ). 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/ml determined. It was found to be 22.0 mg/ml.

Preparation of Hydrindantin :

Hydrindantin was prepared according to the method described by Duggan (1957).

Eighty grams of ninhydrin was dissolved in 2 litres of water, and ascorbic acid solution (80 g/100 ml water) was added to it with stirring at 90^{o}C. The crystallised 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 mg of ninhydrin + 37.6 mg 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 acetic acid. The tubes were kept in boiling water bath for 30 min. 2.5 ml of acetic acid was added after cooling the tubes and the colour was read at 540 mµ in a Klett colorimeter against a standard ornithine sample treated in the same manner.

Estimation of Citrulline :

Citrulline was estimated according to the method of Archibald (1944). To 4.0 ml of sample 2.0 ml of a mixture of H_2SO_4:H_3PO_4 (1:3) was added followed by 0.25 ml of 3% diacetyl monooxime solution with shaking. The tubes were kept in boiling water bath for 15 min. in dark. After cooling the tubes, colour was read at 540 mµ in Klett colorimeter.

Estimation of Urea :

Urea was estimated by the method of Archibald (1944) and the colour was measured at 420 mµ. This method was used only when the sample did not contain any citrulline.

Identification and estimation of amino acids by paper chromatography :

A known amount of tissue was ground with 80% ethanol in a chilled mortar kept on crushed ice. The suspension was centrifuged and the residue was reextracted by repeating the above process two times. All the supernatants were combined and dried in vacuum. The dried sample was then extracted in a known volume of ethanol and used for chromatography.

A known volume of the sample was spotted on Whatman No.1 chromatographic paper and two dimensional descending chromatography was carried out. The solvent used for the first dimension was n-butanol-acetic acid-water (40:10:50). Three runs were given with this solvent and then phenol-acetic acid - water (74:1:19.2) was used for the 4th run in the second dimension. After drying the paper the chromatogram was developed by spraying with 0.4 % ninhydrin in acetone. The spots were allowed to develop for 30 min. at 60°. The ninhydrin positive spots were cut and the colour extracted in 5 ml of 75% ethanol saturated with $CuSO_4$. The extracted colour was read after 30 min. in a Klett colorimeter at 540 mμ, and compared with the sample of standard amino acids treated in the same manner.

In some of the enzyme assays, where amino acid was to be estimated, it was estimated by the circular paper chromatographic method of Giri, Radhakrishnan and Vaidynathan, (1953) using n-butanol - acetic acid - water (40:5:7) as solvent and treating the paper as described above for the development and estimation of amino acids.

Enzyme studies :

The enzymes studied are arginase, ornithine carbamyl transferase, ornithine-Keto acid aminotransferase, enzyme system converting glutamate to PCA, enzyme system converting proline to PCA, glutamate dehydrogenase, glutamate decarboxylase, alanine aminotransferase, aspartate aminotransferase and γ-aminobutyrate aminotransferase.

Preparation of homogenates for enzyme assay :

Unless otherwise stated, tissues cultivated for 3-4 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 min. with 0.95% potassium chloride, pH 7.4 in a chilled mortar kept on crushed ice. The homogenate was diluted with the grinding medium whenever required.

Preparation of cell fractions for localization studies :

The method of Gentile (1960) was used for the preparation of the tissue homogenate and the collection of fractions settling down at different speeds. The fractions settling down when centrifuged at 480 x g for 10 min. 5090 x g for 20 min. and 20,000 x g for 30 min. and the supernatant were used for enzyme assay.

Purification of arginase from Rumex tumour tissue :

A known amount of tissue was ground for 10 min. with 0.25% cold triton X-100 in a chilled mortar kept on crushed ice. Unless otherwise stated $MnCl_2$(0.001 M final concentration) was always included during grinding. The homogenate was diluted with triton X-100 solution to get 10% extract (W/V) on fresh weight basis and centrifuged at 10,000 x g for 15 min. in a Servall refrigerated centrifuge. The residue was reextracted with 0.25% triton once again and centrifuged. The two supernatants were combined together. A known volume of calcium phosphate gel (22 ml/ml dry weight) was taken in a centrifuge tube and centrifuged at 10,000 x g for 5 min. To the gel residue, the enzyme supernatant was added in a gel to enzyme ratio of 1:10 and stirred in cold for about 30 min. The suspension was then centrifuged at 10,000 x g for 15 min. The residue was extracted for 5 min. with 0.02 M phosphate buffer pH 7.5 with stirring and then centrifuged at 10,000 x g for 15 min. The residue was again extracted with 0.05 M phosphate buffer pH 7.5 containing $MnCl_2$(0.001 M final concentration). The suspension was stirred for 30 min. in cold and then centrifuged at 10,000 x g for 15 min. The supernatant was collected and used as enzyme source.

Enzyme assays:

Details of enzyme assays are given in Table 4.

Estimation of Protein :

Protein was determined by the method of Lowry, Rosenbrough, Farr and Randall (1951).

Specific activity of the enzyme :

It is defined as the enzyme units per mg enzyme protein.

Table 4. Details of enzyme assays

Details of assay system and procedure	Arginase (L-arginine ureohydrolase) (E.C. 3.5 3.1)	Ornithine transaminase (L-Ornithine:2Oxoacid 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 µmoles	Tris-HCl pH 8.5; 50 µmoles
3. Substrate	L-Arginine hydrochloride, 15 µmoles for purified enzyme preparation or 20 µmoles with crude homogenate	DL-Ornithine, 10 µmoles
4. Enzyme extract	0.3 ml (10% crude homogenate) or 0.2 ml (purified enzyme)	1.0 ml (40% crude homogenate)
5. Other components	$MnCl_2$, 10 µmoles	Keto acid, 10 µmoles; pyridoxal phosphate 0.1 µmole; O-aminobenzaldehyde, 0.1 ml (10 mg/ml)
6. Temperature and period of incubation	37°C, 60 minutes	37°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 the precipitated protein
9. Treatment of blank	Substrate added after terminating the reaction	Substrate added after terminating the reaction
10. Parameter measured	Ornithine or urea formed was measured as described in text	Optical density of pyrroline-5-carboxylic acid O-aminobenzaldehyde complex formed at 430 mµ
11. Enzyme unit	Amount of enzyme required to form 1 µmole of ornithine or urea 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 4 (Continued)

Details of the assay system and procedure	Ornithine carbamyl transferase (Carbamyl phosphate:L-Ornithine carbamyl transferase) (E.C. 2.1. 3.3)	Glutamate dehydrogenase (L-Glutamate:NAD oxidoreductase) (E.C. 1.4. 1.2)
1. Basis of the method used	Burnett and Cohen (1957)	Bulen (1956)
2. Buffer	Tris-HCl, pH 8.5; 50 µmoles	Tris-HCl, pH 8.5; 50 µmoles
3. Substrate	DL-Ornithine, 2 µmoles	2-Oxoglutarate, 2.5 µmoles
4. Enzyme extract	1.0 ml (20% crude homogenate)	0.2 ml (15% supernatant)
5. Other components	Carbamyl phosphate. Li Salt 2 µmoles	$(NH_4)_2.SO_4$, 300 µmoles; $NADH_2$, 0.1 µmole
6. Temperature and period of incubation	$37^{o}C$, 30 minutes	$30^{o}C$, 3 minutes
7. Start of reaction	Enzyme added	2-Oxoglutarate added
8. Termination of reaction	0.5 ml of 10% TCA added and centrifuged to remove the precipitation protein	-
9. Treatment of blank	Substrate was added after terminating the reaction	No substrate
10. Parameter measured	Citrulline formed was measured as described in text	Decrease in optical density at 340 mµ
11. Enzyme unit	Amount of enzyme required to form 1 µmole of citrulline per hour under the assay conditions	Amount of enzyme required to form 1 µmole of NAD per hour under the assay conditions

Table 4 (Continued)

Details of the assay system and procedure	Glutamate decarboxylase (L-Glutamate-1-carboxy lyase) (E.C. 4.1.1.15)	γ-Aminobutyrate transaminase (4-Aminobutyrate: 2-Oxoglutarate amino transferase) (E.C. 2.6.1.C)
1. Basis of method used	Rajalakshmi, Govindrajan and Ramakrishnan (1965)	Rajalakshmi, Govindrajan and Ramakrishnan (1965)
2. Buffer	Citrate-phosphate, pH 5.5; 50 μmoles	Tris-HCl, pH 8.5; 50 μmoles
3. Substrate	L-Glutamic acid, 10 μmoles	γ-Aminobutyric acid, 10 μmoles
4. Enzyme extract	1.0 ml (40% crude homogenate)	1.0 ml (40% crude homogenate)
5. Other components	Pyridoxal phosphate, 0.1 μmole	Keto acid, 10 μmoles; pyridoxal phosphate, 0.1 μmole
6. Temperature and period of incubation	37°C, 60 minutes	37°C, 60 minutes
7. Start of reaction	Enzyme added	Enzyme added
8. Termination of reaction	Kept in boiling water bath for 10 minutes and 1.0 ml of 95% alcohol added on cooling	Kept in boiling water bath for 10 minutes and 1.0 ml of 95% alcohol added on cooling
9. Treatment of blank	Enzyme added after incubation	Enzyme added after incubation
10. Parameter measured	γ-Aminobutyric acid formed was measured by circular paper chromatography as described in text	Amino acid formed was measured by circular paper chromatography as described in text
11. Enzyme unit	Amount of enzyme required to form 1 μmole of GABA per hour under the assay conditions	Amount of enzyme required to form 1 μmole amino acid per hour under the assay conditions

Table 4 (Continued)

Details of the assay system and procedure	Alanine aminotransferase (L-Alanine 2- oxoglutarate aminotransferase) (E.C.2.6.1.2)	Aspartate aminotransferase L-Aspartate 2-oxoglutarate aminotransferase) (E.C.2.6.1.1)
1. Basis of method used	Shah and Ramakrishnan (1963)	Shah and Ramakrishnan (1963)
2. Buffer	Tris-HCl, pH 8.5; 50 µmoles	Tris-HCl, pH 8.5; 50 µmoles
3. Substrate	Alanine, 10 µmoles	Aspartic acid, 10 µmoles
4. Enzyme extract	0.2 ml (20% crude homogenate)	0.2 ml (20% crude homogenate)
5. Other components	2-Oxoglutaric acid, 10 µmoles; pyridoxal phosphate, 0.1 µmole	2-Oxoglutaric acid, 10 µmoles; pyridoxal phosphate, 0.1 µmole
6. Temperature and period of incubation	37°C, 30 minutes	37°C, 30 minutes
7. Start of reaction	Enzyme added	Enzyme added
8. Termination of reaction	Kept in boiling water bath for 10 minutes and 1.0 ml of 95% alcohol added on cooling	Kept in boiling water bath for 10 minutes and 1.0 ml of 95% alcohol added on cooling
9. Treatment of blank	Enzyme added after incubation	Enzyme added after incubation
10. Parameter used	Glutamic acid formed was measured by paper chromatography as described in text	Glutamic acid formed was measured by circular chromatography as described in text
11. Enzyme unit	Amount of enzyme required to form 1 µmole of glutamic acid per hour under the assay conditions	Amount of enzyme required to form 1 µmole of glutamic acid per hour under the assay conditions

Table 4 (Continued)

Details of the assay system and procedure	System converting Glutamic acid to Pyrroline-5-Carboxylic acid	System converting Proline to Pyrroline-5-Carboxylic acid
1. Basis of method used	Strecker (1957)	Strecker (1957)
2. Buffer	Citrate phosphate, pH- 5.5; 50 µmoles	Citrate phosphate, pH 5.5;50 µmoles
3. Substrate	L-Glutamic acid, 50 µmoles	Proline, 50 µmoles
4. Enzyme extract	1.0 ml (40% crude homogenate)	1.0 ml (40% crude homogenate)
5. Other components	ATP, 10 µmoles; NAD, 10 µmoles; pyridoxal phosphate, 0.1 µmole; O-amino benzaldehyde, 0.1 ml (10 mg/ml)	Pyridoxal phosphate, 0.1 µmole; O-amino benzaldehyde, 0.1 ml (10 mg/ml)
6. Temperature and period of incubation	37°C, 60 minutes	37°C, 60 minutes
7. Start of reaction	Enzyme added	Enzyme added
8. Termination of reaction	0.5 ml of 10% of TCA added and centrifuged to remove the precipitated protein	0.5 ml of 10% TCA added and centrifuged to remove the precipitated protein
9. Treatment of blank	Substrate added after termination of reaction	Substrate added after termination of reaction
10. Parameter used	Optical density of the pyrroline-5 carboxylic acid O-amino benzaldehyde complex formed at 430 mµ	Optical density of the pyrroline-5 carboxylic acid O-amino benzaldehyde complex formed at 430 mµ
11. Enzyme unit	Amount of enzyme required to give a change of 0.05 in optical density per hour under the assay conditions	Amount of enzyme required to give a change of 0.05 in optical density per hour under the assay conditions