BIOSYNTHESIS OF VITAMIN C DURING GERMINATION

IV. Effects of Mitotoxic Agents and Certain Enzyme Inhibitors

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The possible involvement of certain precursors in the biosynthesis of vitamin C has been indicated in earlier reports.[1,2,3] Sugars such as glucose, fructose, mannose and sucrose have been suggested as precursors by other workers as well.[4–7] Apparently unrelated with these findings was the observation of King *et al.*[8,9] that various chemicals such as terpene-like cyclic ketones, chloretone, chloral hydrate and certain barbiturates induced increased urinary excretion of ascorbic acid in experimental rats. These workers further observed a correlation between the metabolism of *d*-glucuronic and ascorbic acids, thus suggesting that stimulation in vitamin C synthesis is interlinked with detoxification mechanisms. In a later report it was shown[10] that the accelerated vitamin C synthesis did not proceed *via* utilization of the stimulating compound, methyl labelled chloretone, as precursor. It would seem therefore that the effect of the nerve-depressant may be upon the enzyme systems which control glucose oxidation. Ganapathi[11] has suggested a mechanism by which ascorbic and glucuronic acids may be formed from two 3-carbon units. Adequate thiamine nutrition has been shown to be a prerequisite to chloretone stimulation of ascorbic acid synthesis in rats.[12]

It was therefore of interest to study the effects of mitotoxic agents such as chloretone and coumarin[13,14,15] on ascorbic acid formation during germination and in presence of the precursors[1] which in themselves had stimulatory effects. That the Krebs' intermediates were involved in the transformation of the sugars into ascorbic acid[3] was further ascertained from use of certain enzyme inhibitors.

Experimental

The procedures followed for seed germination and pretreatments employed as well as for vitamin determinations were as described before.[3]

Effects of Mitotoxic Agents

Some of the results obtained using different concentrations of urethane, chloretone, barbitone and coumarin are presented in Tables I and II. In preliminary experiments it was observed that in these concentrations there was no marked effect on growth except a slight thickening of radicles with coumarin-treated seedlings. Higher concentrations resulted in stunted growth.

TABLE I

Effects of Urethane, Chloretone and Coumarin

Day of germination Treatment	Ascorbic acid content (mgm. per cent.)				
	1st	2nd	3rd	4th	5th
Nil ..	84·6	123·7	134·0	152·9	185·6
Urethane (100 p.p.m.) ..	89·2	123·7	148·2	170·5	178·6
Urethane (10 p.p.m.) ..	93·6	121·1	151·5	158·8	183·3
Chloretone (100 p.p.m.)	98·6	116·8	146·4	167·6	194·9
Chloretone (10 p.p.m.) ..	98·6	116·0	146·0	161·7	191·6
Coumarin (100 p.p.m.) ..	100·5	118·6	146·6	158·8	192·6
Coumarin (10 p.p.m.) ..	104·8	116·8	148·2	154·4	187·5

It may be seen that on the whole all the substances used had a stimulatory effect on ascorbic acid formation at some stage or other during germination. The best concentration and the stage of growth when stimulatory effect was observable varied with the mitotoxic agents. However, after five days' germination, all treated samples had higher vitamin values as compared to control except in the case of barbitone where it was equal to it. Lower concentrations of chloretone and coumarin had better effect than the respective higher concentrations.

Effects of Supplementation of Glucose and B Vitamins to Chloretone and Coumarin

Treatments were given as usual. Growth of seedlings was good in all samples. Coumarin treated seedlings had a slightly retarded growth with somewhat thickened radicles. Aliquots were taken simultaneously from the same lot for ascorbic acid and nicotinic acid determinations (Tables III and IV respectively). Vitamin estimations were carried out on the third, fourth and fifth days of germination.

TABLE II

Effects of Higher Concentrations of Urethane, Chloretone, Barbitone and Coumarin

Day of germination Treatment	Fresh basis						Dry basis					
	mgm. per 100 g.			mgm. per 100 seedlings			mgm. per 100 g.			mgm. per 100 seedlings		
	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th
Nil ..	27·16	22·1	21·07	5·28	5·4	4·8	181·1	187·2	210·0	35·2	45·7	48·0
Barbitone (1000 p.p.m.)	31·13	34·93	18·4	6·97	9·1	4·78	193·2	226·8	209·0	43·3	59·06	54·08
Urethane(1000 p.p.m.)	27·7	23·2	25·2	6·04	6·25	5·95	197·8	236·7	252·0	44·08	63·7	59·5
Chloretone (1000 p.p.m.)	27·59	25·68	24·12	6·04	5·6	5·85	171·3	237·7	241·2	37·5	51·8	58·5
Chloretone (500 p.p.m.)	30·3	26·2	22·95	5·8	6·6	5·52	202·0	272·9	283·3	38·6	68·7	68·1
Coumarin (400 p.p.m.)	29·1	24·92	27·03	5·08	5·52	6·15	158·0	235·1	262·4	27·6	52·07	59·7
Coumarin (300 p.p.m.)	28·7	23·23	28·4	5·56	5·68	7·2	147·1	187·3	278·4	28·5	48·08	70·58

TABLE III

Effects of Chloretone and Coumarin together with Glucose and B Vitamins : Ascorbic Acid

Day of germination Treatment*	Fresh basis						Dry basis					
	mgm. per 100 g.			mgm. per 100 seedlings			mgm. per 100 g.			mgm. per 100 seedlings		
	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th
Nil ..	21·99	22·49	19·34	5·0	6·14	5·72	146·6	214·1	197·3	33·3	58·4	58·3
Glucose ..	23·31	23·27	20·68	5·14	6·36	5·38	151·2	187·1	213·1	33·4	51·2	55·4
B vitamin mixture ..	24·9	22·6	21·05	5·46	6·25	5·93	168·2	185·2	191·3	36·92	51·2	53·9
Chloretone ..	23·53	23·27	21·05	5·47	6·36	5·83	149·1	217·4	212·6	34·8	59·4	58·8
Chloretone + Glucose	21·97	25·24	21·43	5·30	6·86	5·93	141·7	221·3	238·1	34·19	60·35	65·8
Chloretone + Glucose + B vitamins	26·24	24·66	24·11	5·55	6·48	6·36	169·2	186·8	229·6	35·8	49·04	60·5
Coumarin ..	23·12	22·36	20·88	5·14	5·83	6·03	144·5	164·4	200·7	32·15	42·8	57·9
Coumarin + Glucose	24·85	26·16	20·97	5·07	6·14	5·83	171·3	186·8	216·1	34·96	43·8	60·1
Coumarin + Glucose + B vitamins	23·44	26·78	23·3	4·43	6·36	6·25	141·1	201·3	230·7	25·6	47·8	61·8

* *Concentrations.*—Glucose, 500 p.p.m.; Chloretone, 500 p.p.m.; Coumarin, 300 p.p.m.; B vitamin mixture contained thiamine, riboflavin and nicotinic acid, 100 p.p.m. each.

Treatment with glucose had a favourable effect on ascorbic acid formation on the fifth day of germination and with the B vitamin mixture on the third day. Chloretone and coumarin both had maximum stimulatory effect on the fifth day, this being more marked with the former. Supplementation with glucose enhanced these effects which were better shown in presence of the B vitamins.

TABLE IV

Effect of Chloretone and Coumarin Supplemented with Glucose and B Vitamins: Nicotinic Acid

Day of germination Treatment*	Fresh basis						Dry basis					
	micrograms per 100 g.			micrograms per 100 seedlings			micrograms per 100 g.			micrograms per 100 seedlings		
	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th
Nil ..	850	925	738	200	261	208	5689	8766	7608	1339	2471	2144
Glucose ..	775	875	825	175	246	262	5021	7063	10730	1015	1985	3402
B vitamins ..	1000	1075	738	223	347	235	6736	8798	8533	1505	2842	2713
Chloretone ..	750	625	762	157	188	229	4768	5825	9232	998	1758	2770
Chloretone + Glucose	675	613	750	215	163	249	4361	5378	11080	1101	1425	3677
Chloretone + Glucose + B vitamins	738	625	712	163	188	253	4753	4740	9627	1049	1429	3423
Coumarin ..	750	712	712	162	194	221	4696	5240	8486	1014	1429	2639
Coumarin + Glucose	712	588	662	153	181	210	4900	4189	8700	1050	1290	2767
Coumarin + Glucose + B vitamins	1100	625	788	215	167	247	6363	4707	9777	1241	1259	3060

* For concentrations see foot note to Table III.

As with ascorbic acid, the influence individually of chloretone, coumarin, glucose and B vitamins was beneficial for nicotinic acid formation during germination. Glucose with chloretone as well as with coumarin induced higher stimulation after five days. Treatment with B vitamins along with chloretone and glucose had no additional effect while that with coumarin and glucose showed definite enhancement.

Since mitotoxic agents cannot obviously act as precursors of vitamin C[10] their effect could only be through stimulation at some phase of glucose metabolism which directly funnels into the biosynthetic mechanism for ascorbic acid. The following studies were undertaken to elicit further information through use of specific enzyme inhibitors whose effect if any would indicate whether a certain metabolite constitutes an essential intermediate step or not.

Effects of Certain Enzyme Inhibitors

Although tracer work[10] has shown that glucose carbon chain occurs as such in the ascorbic acid synthesized from it by the rat, the possibility is not precluded that vitamin C is actually derived from the products of metabolic breakdown of glucose[16] followed by resynthesis of simpler carbon units. Study of the effects of some of these intermediates has in fact revealed a contribution by some at least to ascorbic acid formation[3] in *müng* seedlings.

Effects of cyanide, 2:4 dinitrophenol, azide and iodoacetate

The substances selected are all inhibitors of enzymes concerned in carbohydrate breakdown. Cyanide is a general enzyme inhibitor but particularly inhibits cytochrome oxidase. Dinitrophenol and azide inhibit phosphorylation which precedes glucose breakdown as well as of several of its intermediates. Iodoacetate inhibits glycolysis. The concentrations studied (Table V) were chosen after preliminary experiments; higher concentrations, particularly of dinitrophenol, azide and iodoacetate affected growth. To accentuate the effects of inhibitors, they were studied in presence of glucose (1%), B vitamins (thiamine, riboflavin and nicotinic acid, 100 p.p.m. each) and phosphate (100 p.p.m.). Ascorbic acid determinations were carried out after four days' germination (Table V).

Except iodoacetate which could not be employed at higher concentrations on account of toxic effects, the other additions had definite inhibitory effects.

TABLE V

Effects of Cyanide, 2:4 Dinitrophenol, Azide and Iodoacetate

Treatment*	Ascorbic acid (mgm. per cent.)	Per cent. variation from control
Glucose + B vitamins + phosphate (control) ..	269·9	..
Glucose + B vitamins + phosphate + cyanide (250 p.p.m.) ..	232·1	−14
Glucose + B vitamins + phosphate + cyanide (100 p.p.m.) ..	247·1	− 8·85
Glucose + B vitamins + phosphate + dinitrophenol (45 p.p.m.) ..	231·1	−14·37
Glucose + B vitamins + phosphate + azide (50 p.p.m.) ..	228·7	−15·26
Glucose + B vitamins + phosphate + azide (25 p.p.m.) ..	260·0	− 3·67
Glucose + B vitamins + phosphate + iodoacetate (10 p.p.m.) ..	276·6	+ 2·48

* *Concentrations.*—Glucose, 1% ; B vitamins contained thiamine, riboflavin and nicotinic acid, 100 p.p.m. each ; phosphate, 100 p.p.m.

Effects of enzyme inhibitors on germinating excised embryos

The experiment was similar to the above but excised embryos were grown by the earlier technique.[3] Treatment with fluoride, another inhibitor of glycolysis, was included. Samples were taken after germination for four days. Growth of embryos was slightly retarded with fluoride, azide and iodoacetate treatments.

TABLE VI

Effects of Cyanide, 2:4 Dinitrophenol, Azide, Iodoacetate and Fluoride on Excised Embryos

Treatment*	Ascorbic acid (mgm. per cent.)	Per cent. variation from control
Glucose + phosphate (control) ..	266·3	..
Glucose + phosphate + cyanide (50 p.p.m.) ..	258·3	−3
Glucose + phosphate + dinitrophenol (10 p.p.m.) ..	252·5	−5·18
Glucose + phosphate + azide (5 p.p.m.) ..	248·8	−6·57
Glucose + phosphate + iodoacetate (1 p.p.m.) ..	262·6	−1·4
Glucose + phosphate + fluoride (10 p.p.m.) ..	252·9	−5·03

* *Concentrations.*—Glucose, 3% ; phosphate, 0·5%.

Much lower concentrations of the inhibitors had to be used for satisfactory growth than in the preceding experiments with intact seedlings (Table V) and hence, probably, the effects though definite were less pronounced.

Effects of malonate and calcium^{++}

It was reported[3] that of the Krebs' intermediates, fumarate and succinate in particular, had a pronounced stimulatory effect on ascorbic acid formation during germination. It was of interest to study the effects of malonate, which inhibits competitively the catalytic action of the enzyme succinic dehydrogenase[17] and of calcium ion which is known to activate it.[18]

Influence of malonate and calcium ion on stimulation of ascorbic acid formation due to succinate was studied by soaking the seeds as usual in different solutions of the substances singly and in combinations (Table VII). Two concentrations of malonate were tried; one equimolar to that of succinate and the other half of it (90 and 45 p.p.m. respectively of malonate against 100 p.p.m. of succinate). Samples for vitamin determination were taken on the fourth day.

TABLE VII

Effects of Malonate and of Calcium^{++} *(as Calcium Chloride) together with Succinate*

Treatment	Ascorbic acid (mgm. per cent.)	Per cent. variation from control
Nil ..	197·1	..
Succinate (100 p.p.m.) ..	205·2	+ 4·1
Malonate (45 p.p.m.) ..	190·6	− 3·3
Malonate (90 p.p.m.) ..	190·4	− 3·4
Succinate + malonate (45 p.p.m.) ..	197·2	..
Succinate + malonate (90 p.p.m.) ..	169·9	−13·8
Calcium chloride (50 p.p.m.) ..	200·5	+ 1·7
Succinate + calcium chloride (50 p.p.m.) ..	191·4	− 2·9

Treatments with malonate or calcium chloride did not result in appreciable effect on ascorbic acid formation. In view of this and the observation of Laties[19] that inhibition due to malonate is more effective at acid pH, a different method of treatment was tried.

The seeds after overnight soaking in distilled water (10 g. in 50 c.c. per sample) were allowed to germinate in separate dishes, serving water when required. After three days when rapid ascorbic acid formation commences,

succinate solution (100 p.p.m.) enough to soak the wads (45 c.c.) was served to samples 3, 6, 7 and 9 (Table VIII) and water was served to the rest. On the fourth day, malonate solutions in two concentrations adjusted at pH 4·5 were served; that of 45 p.p.m. to samples 4 and 6 and that of 90 p.p.m. to samples 5 and 7 (45 c.c. in each case). A control for pH was also kept (sample 2). Calcium chloride solution was served to samples 8 and 9. After a further day's germination, vitamin contents of all samples were determined.

TABLE VIII

Effects of Malonate (at Low pH) and of Calcium Chloride together with Succinate

Day of germination Treatment	Ascorbic acid (mgm. per cent.)		Per cent. variation from control (on 5th day)
	4th	5th	
Sample:			
1 Nil ..	167·9	170·8	..
2 pH control ..		177·0	+ 3·63
3 Succinate control (100 p.p.m.) ..	178·0	214·2	+25·4
4 Malonate (45 p.p.m.) ..		149·6	−12·4
5 Malonate (90 p.p.m.) ..		142·8	−16·4
6 Succinate + malonate (45 p.p.m.) ..		167·3	− 2·05
7 Succinate + malonate (90 p.p.m.) ..		188·4	+10·3
8 Calcium chloride (50 p.p.m.) ..		198·6	+16·28
9 Calcium chloride + succinate ..		199·0	+16·5

The effect of malonate treatment was evident at the low pH employed here. It depressed ascorbic acid formation, in succinate-treated and control samples. Calcium ion had some stimulatory effect, which was not accelerated in presence of succinate.

Effects of malonate and of calcium^{++} on excised embryos

The embryos were excised as usual and allowed to grow on the semi-solid nutrient medium containing different substances (Table IX). It was not possible to have a pH of 4·5 found necessary to observe malonate inhibition with intact seedlings on account of difficulty in obtaining a well set gel-medium. However, by use of malonic acid instead of its salt, a slight

TABLE IX

Effects of Malonate and of Calcium^{++} Together with Succinate on Embryos

Treatment	Ascorbic acid (mgm. per cent.)	Per cent. variation from control
Nil ..	142·6	..
Succinate control (0·5%) ..	168·6	+18·23
Malonate control (0·25%) ..	136·2	− 4·49
Succinate + malonate ..	145·3	+ 1·85
Calcium chloride (0·25%) ..	140·3	− 1·6
Succinate + calcium chloride ..	176·0	+23·42

lowering of the pH below neutral was achieved. Samples for vitamin estimation were taken after four days' germination.

Treatment with calcium chloride alone had no effect but it activated the stimulatory effect of succinate. Although malonate control showed only slight inhibition as compared to normal control, it reversed the enhancement due to succinate. The effect of malonate may be expected to be more pronounced at still lower pH.

Changes in Phosphatase and Pyrophosphatase Activities

On the basis of experimental results presented in the foregoing pages, it would seem that in the biogenesis of ascorbic acid from glucose, the latter may be degraded into smaller fragments which by further synthetic steps would lead to the formation of ascorbic acid. The observed changes in vitamin C as a result of cultural additions or environmental alterations and the parallel increases in nicotinic acid also suggested that the latter may be involved as its co-enzyme in some of the reactions concerned with ascorbic acid elaboration. Inhibition studies on phosphorylation also pointed to the conclusion that one of the ways in which energy was supplied to the endergonic reaction of vitamin C elaboration may be phosphorylation. Further proof of phosphorylation as an intermediate step in vitamin C biogenesis was ascertained from the following studies on phosphatase (acid) and pyrophosphatase activities; alkaline phosphatase was absent.

10–11 g. of *müng* seeds were soaked separately in 50 c.c. of (i) distilled water, (ii) fumarate solution (100 p.p.m.), (iii) solution of fumarate (100 p.p.m.) and atabrine (150 p.p.m.), (iv) 2 : 4 dinitrophenol solution (50 p.p.m.).

TABLE X

Effects of Fumarate and of Inhibitors on Nicotinic Acid, Phosphatase and Pyrophosphatase Activities

Treatment	Fresh basis						Dry basis					
	per 100 g.			per 100 seedlings			per 100 g.			per 100 seedlings		
	A	B	C	A	B	C	A	B	C	A	B	C
Nil (Dormant seeds)	1050	0·573	0·345	52·5	0·029	0·017	1188	0·65	0·39	59·4	0·033	0·02
Nil (Seedling control)	362·5	1·49	0·394	93·9	0·386	0·102	3452	14·18	3·751	894·1	3·673	0·971
Fumarate (100 p.p.m.)	375	1·51	0·58	101·6	0·409	0·157	3750	15·10	5·8	1016	4·092	1·572
Fumarate (100 p.p.m.) +atabrine (50 p.p.m.)	337·5	1·463	0·374	85	0·369	0·094	3125	13·55	3·463	787·5	3·416	0·872
Fumarate (100 p.p.m.) +atabrine(100 p.p.m.)	350	1·631	0·197	90·8	0·423	0·051	3182	14·84	1·79	825·8	3·85	0·519
Fumarate (100 p.p.m.) + 2 : 4 dinitrophenol (50 p.p.m.)	350	1·282	0·316	93·5	0·342	0·084	2781	10·42	2·57	742·5	2·781	0·726

A = Nicotinic acid (micrograms).
B = Phosphatase activity (expressed as g. of P).
C = Pyrophosphatase activity (expressed as g. of P).

Dinitrophenol and atabrine are inhibitors of phosphorylation mechanisms.[20, 21, 22] After four days' germination, 0·5 g. of seedlings were crushed with 5 c.c. of acetate buffer (pH 5·4) in a glass mortar and transferred to a 100 c.c. flat-buttom flask. Phosphatase and pyrophosphatase activities were studied in these extracts according to the methods of Sadasivan[23] using 10% solution of sodium β-glycerophosphate and 5% solution of sodium pyrophosphate respectively. Colour for phosphate was developed according to Koch[24] and using the Fiske-Subbarow reagent,[25] a Klett-Summerson photo-electric colorimeter with filter 66 being employed for colour measurements.

Additional determinations for nicotinic acid were also carried out in the same sample (Table X).

As a result of germination, phosphatase and pyrophosphatase activities increased to a great extent. There was a slight favourable effect of fumarate on phosphatase activity. Atabrine had little or no influence while dinitrophenol had appreciable depressing effect on phosphatase activity compared to control. Fumarate enhanced pyrophosphatase activity to a considerable extent. Both dinitrophenol as well as higher concentration of atabrine had a marked depressing effect on pyrophosphatase activity as compared to control.

Further data on the effects of fumarate and/or chloretone with and without the phosphorylation inhibitors, atabrine and 2:4 dinitrophenol, are given in Table XI for pyrophosphatase activity only. Results are for five days' seedlings.

Fumarate as well as chloretone stimulated pyrophosphatase activity and atabrine depressed it; the inhibition due to the latter was not observable when fumarate and chloretone were used together. However, the inhibition due to dinitrophenol was practically not influenced by fumarate or chloretone when its concentration was 50 p.p.m. At a lower concentration results similar to those with atabrine were obtained (Table XII) for five days' seedlings.

The stimulation in ascorbic acid and nicotinic acid biogeneses due to succinate and fumarate and the inhibition due to malonate reported earlier,[3] (also *cf.* Table VIII) were further studied with respect to phosphatase and pyrophosphatase activities and nicotinic acid synthesis (Table XIII). Values are for four days' seedlings.

TABLE XI

Effects of Fumarate, Chloretone and Inhibitors on Pyrophosphatase Activity

Treatment	Fresh basis		Dry basis	
	per 100 g.	per 100 seedlings	per 100 g.	per 100 seedlings
	Pyrophosphatase activity expressed as g. of P			
Nil ..	0·488	0·141	5·603	1·615
Fumarate (100 p.p.m.) ..	0·811	0·214	10·130	2·676
Chloretone (500 p.p.m.) ..	0·666	0·186	7·750	2·156
Fumarate + chloretone ..	0·832	0·321	10·140	2·819
Atabrine (150 p.p.m.) ..	0·264	0·063	2·869	0·682
Fumarate + atabrine ..	0·396	0·113	4·356	1·235
Chloretone + atabrine ..	0·354	0·082	3·978	0·928
Fumarate + chloretone + atabrine ..	0·540	0·140	6·208	1·601
Dinitrophenol (50 p.p.m.) ..	0·408	0·109	4·080	1·090
Fumarate + dinitrophenol ..	0·377	0·100	3·919	1·040
Chloretone + dinitrophenol ..	0·316	0·077	3·225	0·777
Fumarate + chloretone + dinitrophenol ..	0·445	0·117	4·542	1·200

TABLE XII

Effects of Fumarate, Chloretone and 2:4 *Dinitrophenol on Pyrophosphatase Activity*

Treatment	Fresh basis		Dry basis	
	per 100 g.	per 100 seedlings	per 100 g.	per 100 seedlings
	Pyrophosphatase activity expressed as g. of P			
Nil ..	0·616	0·166	6·046	1·631
Fumarate (100 p.p.m.) ..	1·059	0·260	10·524	2·580
Chloretone (500 p.p.m.) ..	0·609	0·134	6·143	1·351
Fumarate + chloretone ..	1·083	0·284	10·939	2·859
Dinitrophenol (20 p.p.m.) ..	0·652	0·127	5·720	1·111
Fumarate + dinitrophenol ..	0·698	0·168	6·591	1·584
Chloretone + dinitrophenol ..	0·672	0·143	6·106	1·300
Fumarate + chloretone + dinitrophenol ..	0·824	0·199	7·546	1·822

TABLE XIII

Effects of Inhibitors on Nicotinic Acid, Phosphatase and Pyrophosphatase Activities

Treatment	Fresh basis						Dry basis					
	per 100 g.			per 100 seedlings			per 100 g.			per 100 seedlings		
	(A)	(B)	(C)	(A)	(B)	(C)	(A)	(B)	(C)	(A)	(B)	(C)
Nil ..	512·5	1·98	0·394	123	0·287	0·094	6832	15·97	5·15	1640	3·834	1·26
pH (4·5) control ..	525	1·293	0·393	123	0·304	0·093	6732	16·58	5·05	1582	3·897	1·19
Fumarate (100 p.p.m.) ..	525	1·412	0·610	152	0·408	0·176	7501	20·17	8·72	2168	5·830	2·52
Succinate (100 p.p.m.) ..	537·5	1·453	0·517	138	0·374	0·133	7791	21·06	7·50	2002	5·412	1·93
Malonate (50 p.p.m.) ..	500	1·224	0·381	110	0·266	0·084	6250	15·08	4·76	1375	3·322	1·05
Fumarate + malonate ..	462·5	1·400	0·520	116	0·350	0·130	6424	19·74	7·20	1606	4·861	1·80
Fumarate + succinate ..	487·5	1·375	0·443	122	0·344	0·110	6771	19·38	6·16	1692	4·774	1·54

(A) = Nicotinic acid (micrograms).
(B) = Phosphatase activity (expressed as g. of P).
(C) = Pyrophosphatase activity (expressed as g. of P).

As may be expected fumarate or succinate stimulated biogenesis of nicotinic acid as well as phosphatase and pyrophosphatase activities. Malonate showed some inhibition both by itself and in presence of fumarate or succinate. The results were similar to those obtained with ascorbic acid (Table VIII).

DISCUSSION

That hexoses act as primary precursors in the biogenesis of ascorbic acid has been confirmed[1, 3] working with both intact seedlings and excised embryos of *miing*. It would seem that this conversion might be taking place *via* compounds having smaller carbon chains although admittedly, the fact that short chain compounds stimulate ascorbic acid formation cannot alone lead conclusively to this view.

The investigations of King and co-workers with chloretonized rats probably provide the first indications of a biosynthetic mechanism. Treatment with chloretone stimulated ascorbic acid formation *in vivo* along with glucuronic acid in rats.[8,9] From these data[8,9,16,26] Ganapathi[11] has suggested that ascorbic and glucuronic acids may be formed from two 3-carbon units by aldol condensation similar to that in the formation of fructose and sorbose from two molecules of glyceraldehyde, the two 3-carbon units in this case being glyceraldehyde and hydroxy puruvic acid or their tautomeric forms; the reactions can proceed in two directions yielding in one case ascorbic acid and in the other glucuronic acid.

In a recent communication[10] King and co-workers reported the results of experiments with labelled glucose. C^{14} glucose labelled uniformly in all the six positions was used for *in vivo* study with chloretonized rats. Partial degradation of the biosynthetic ascorbic acid and the measurement of radioactivity at all the positions indicated that conversion of glucose to ascorbic acid proceeded without appreciable differential dilution of radio activity in positions 1 and 2 as compared to the remaining carbon atoms of the molecule. Distribution of radio activity in the six carbon atoms of the biosynthetic ascorbic acid was essentially identical with that in the initial glucose molecule. It was therefore suggested that the carbon chain of glucose may not be broken before being converted to ascorbic acid; however, the possibility of recombination of the fragments without a major dilution effect could not be ruled out and would seem the probable route from King's own earlier observations on the stimulatory effects of glyceraldehyde and pyruvate in *in vitro* studies with liver slices of chloretone-treated rats[16] and from the data obtained by the present authors.

Furthermore, the depression in ascorbic acid production during germination by certain inhibitors of enzymes concerned in the Krebs' oxidative cycle also lends support to this view. The inhibitors for glycolysis, such as fluoride, depressed the formation of ascorbic acid indicating that breakdown of glucose into smaller fragments is a prerequisite to the subsequent endergonic step of ascorbic acid formation. The depressing effects on ascorbic acid synthesis of selective inhibitors of phosphorylation such as azide, 2 : 4 dinitrophenol and atabrine indicate that phosphorylation of intermediates is a necessary link in ascorbic acid elaboration. This is further supported from the concomitant changes observed in phosphatase and pyrophosphatase activities. A more specific demonstration of the involvement of at least one of the Krebs' intermediates in ascorbic acid genesis is the depressing effect of malonate.

Chloretone stimulates ascorbic acid production in animal tissues[16] and its action is indirect.[10] It is now shown that this as well as other cytotoxic agents like coumarin and urethane in appropriate concentrations stimulate ascorbic acid production by germinating seeds as well. Supplementation of glucose and B vitamins enhances this effect.

The effects of these substances may only be stimulatory, the mechanism being unknown. It has been reported that nerve depressants affect the enzyme systems concerned with oxidation of glucose in animal tissues.[27] A similar effect may be inferred in plant tissues as well. If, for instance, fumarate or pyruvate or any defined intermediate in glucose oxidation is involved in ascorbic acid elaboration, it seems possible that the mitotoxic agent inhibits oxidation in the Krebs' cycle at this point and thus helps to shunt the metabolism of this intermediate away from its normal pathway and towards steps leading to formation of ascorbic acid.

The parallelism between ascorbic acid and nicotinic acid changes reported earlier[1,3] is again observable in the foregoing studies with mitotoxic agents as well as selective inhibitors. A direct approach to the elucidation of these relationships through spectrophotometric determinations of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) is currently in progress in this laboratory.

Summary

(1) Further studies on the changes in ascorbic acid elaboration by germinating *müng* with various cultural additions have been carried out with a view to elucidate the postulated role of intermediaries of glucose metabolism.

(2) Selective inhibitors of certain enzymes concerned in glucose breakdown and in phosphorylation, such as azide, fluoride and 2 : 4 dinitrophenol adversely affect ascorbic acid formation.

(3) Malonate, a competitive inhibitor of succinic dehydrogenase, depresses ascorbic acid elaboration. Involvement of fumarate in reactions leading to synthesis of ascorbic acid is thus inferable.

(4) In proper concentrations, certain mitotoxic agents like chloretone, urethane and coumarin favour synthesis of ascorbic acid.

(5) Phosphorylation inhibitors such as atabrine and 2 : 4 dinitrophenol adversely affect phosphatase and, more particularly, pyrophosphatase activities which are stimulated by fumarate or succinate and by mitotoxic agents like chloretone in suitable concentrations. Phosphorylation of intermediates seems therefore a necessary step in ascorbic acid elaboration.

(6) In general, changes in nicotinic acid are parallel to those in ascorbic acid.

(7) The related observations have been discussed and it is concluded that metabolic breakdown of glucose is a probable prerequisite to its transformation into ascorbic acid.

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