BIOSYNTHESIS OF VITAMIN C DURING GERMINATION

Part III. Effects of Sugars, Krebs' Intermediates, Amino Acids and B Vitamins and Correlation with Biogenesis of Nicotinic Acid

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Received April 5, 1952

It has been reported from this laboratory that germination of legume seeds in the dark results in a stimulation of ascorbic acid formation along with increase in reducing sugars through enhanced amylolysis.[1] These observations concur with the view that hexoses are precursors in the biosynthesis of ascorbic acid.[2, 3] However, it has not been ascertained whether the conversion of sugars, specifically glucose, to ascorbic acid, takes place by a direct oxidative pathway or through mediation of the glycolytic route. In this investigation evidence is presented to show that the oxidative breakdown steps are catalysed by certain of the B vitamins and that the acids involved in the intermediary metabolism of glucose, particularly fumaric and succinic acids, exert greater stimulation of vitamin C formation than comparable quantities of glucose itself. It is also shown that there is a remarkably close parallelism between the elaboration of ascorbic acid and of nicotinic acid under a variety of experimental conditions. These conclusions have been pursued further in the following communication which confirms, through use of cytotoxic agents, selective enzyme inhibitors and phosphorylation studies, that the metabolic breakdown of glucose into smaller fragments is an essential prerequisite to the subsequent endergonic step of ascorbic acid biogenesis.

Experimental

All the studies reported here have been carried out with *müng* (*Phaseolus radiatus*) seeds. The procedures followed for seed germination, sampling and ascorbic acid determinations, etc., were all as described before.[1] Treatment with a substance was given by overnight soaking of 10 gm. of the seeds in 50 c.c. of its solution in concentrations as shown. Soaked seeds were then germinated as usual, glass-distilled water being served periodically when needed.

EFFECT OF PRETREATMENT WITH GLUCOSE AND B VITAMINS

Seeds pretreated by soaking with glucose alone in 3% concentration gave 13·1% increase in ascorbic acid elaboration during four days' germination over the corresponding value for untreated seeds of 217·1 mgm. per 100 gm. At ½ and 1% concentrations, glucose had only small stimulatory effect (1·4 and 1·9% respectively) while beyond the 3% level it caused stunted growth of seedlings with bunched short roots. In contrast to glucose, hexose diphosphate (Schwarz Laboratories, New York) had a pronounced enhancing effect on vitamin synthesis even in small concentrations. Thus with 100 p.p.m., the per cent. difference over untreated four days old seedlings was 21·3. This effect was not attributable to the phosphate component alone since sodium β-glycerophosphate even at 3% concentration gave only 4·7% stimulation.

The effect of supplementation of glucose (1%) with thiamine, riboflavin and nicotinic acid (100 p.p.m. each) which are associated with carbohydrate metabolism as components of enzyme systems is shown in Table I for four days' seedlings. Phosphate (100 p.p.m.) as the trisodium salt was also employed in these studies, phosphorylations being essential steps in glucose breakdown. The data include values for dehydroascorbic acid and ascorbic acid oxidase, determined by earlier procedures.[4, 5]

TABLE I

Effects of Glucose Together with B Vitamins and Phosphate

Treatment	Ascorbic acid	Dehydro ascorbic acid	Total ascorbic acid	Ascorbic acid oxidase activity
	mg. per 100 g.			
Nil	188·9	35·67	224·5	7·12
Glucose (1%)	192·4	37·5	229·9	6·73
Glucose (1%)+B vitamin mixture*	201·2	59·4	260·6	5·58
Phosphate (100 p.p.m.)	196·8	41·2	238·0	6·86
Glucose (1%)+Phosphate (100 p.p.m.)	193·2	39·9	233·1	5·15
Glucose (1%)+B vitamin mixture*+ Phosphate (100 p.p.m.)	195·7	60·1	255·8	5·47

* Consisting of 100 p.p.m. each of thiamine, riboflavin and nicotinic acid.

† Units expressed as in (5).

Glucose at the concentration employed had only a slight effect but with added B vitamins marked stimulation was observed. Phosphate, either alone or in combination with glucose, had an influence only in presence of the B vitamins. Studies using the latter only as cultural additions are reported later (Table XIV). The values for dehydroascorbic acid were also higher in the presence of the B vitamins with or without phosphate. Oxidase activity was lower in all cases as compared to the untreated seedlings; the effects of cultural supplements on total ascorbic acid are not explicable solely on this basis.

Effects of Sugars on Germinating Embryos

Seeds were disinfected with 0·5% formalin solution, washed thoroughly with sterile glass-distilled water and after overnight soaking allowed to germinate under aseptic conditions for 24 hours. The embryos were next separated from the cotyledons and transplanted on to a nutrient agar medium containing Knopp's solution with 3% concentration of the appropriate sugar. The best concentration of agar that gave a gel soft enough for the radicles to pierce through was 0·8%. The semi-solid gel was set in a wide-mouthed, flat-bottomed flask plugged with cotton-wool.

General growth of seedlings was better in media containing added sugars as compared to the untreated lot which on the fourth day after transplantation analysed to 110·2 mgm. per cent. of vitamin C. The percentage increases in vitamin C brought about by glucose, mannose, fructose and sodium β-glycerophosphate were 59·2, 56·9, 55·1 and 27·2 respectively. The better effect due to the sugars here is due to the exclusion of the stored reserves when intact seeds are employed (of 1, 6). The comparatively lower stimulatory effect of glycerophosphate may be due to its not being an intermediate in hexose metabolism.

Effect of Glucose on Germination in the Dark

Procedures for treatment with glucose and germination in the dark were as described before. Necessary controls were kept. Results obtained on the fourth day of germination are given in Table II.

Germination in absence of light resulted, as reported earlier,[1] in increased ascorbic acid synthesis. However glucose treatment had a considerably less beneficial effect under these conditions as compared to germination in diffused light. This was to be expected in view of the fact that during sprouting in darkness there is enhanced sugar formation through amylolysis.[1]

TABLE II

Effect of Glucose and Germination in Darkness

Treatment	Germinated in diffused light		Germinated in the dark		
				Per cent. difference	
	Ascorbic acid (mg. %)	Per cent. difference over control	Ascorbic acid (mg. %)	over seedlings germinated in the dark	over seedlings germinated in diffused light
Nil ..	217·0	..	232·9	..	+ 6·8
Glucose (1%) ..	221·3	+ 1·9	242·7	+4·0	+11·8
Glucose (3%) ..	245·6	+13·1	244·4	+4·7	+12·8

EFFECT OF COLD TREATMENT DURING STEEPING AND OF GERMINATION IN THE DARK ON NICOTINIC ACID BIOGENESIS

The formation of nicotinic acid during germination has been reported by several workers[7, 8, 9] and has evoked interest with respect to its possible precursors.[10] In other studies[11] the changes in nicotinic acid and its related metabolites have been followed during germination. Unpublished data have shown a close parallelism between the elaboration of vitamin C and of nicotinic acid. In particular, cold treatment during steeping prior to germination as well as germination in absence of light which have marked stimulatory effect on vitamin C formation[1] also influence nicotinic acid synthesis. The data for four and six days' old seedlings are presented in Table III.

TABLE III

Effects of Cold Treatment and Germination in the Dark on Nicotinic Acid Formation

Day of germination	Fresh basis				Dry basis			
	micrograms per 100 g.		micrograms per 100 seedlings		micrograms per 100 g.		micrograms per 100 seedings	
	4th	6th	4th	6th	4th	6th	4th	6th
Untreated (normal steeping and germination	726	957	242	319	6914	8545	2305	2848
Cold treated ..	1056	1089	302	311	10866	8643	3104	2470
Germinated in the dark ..	990	1023	396	409	11000	12949	4400	5180

TABLE IV

Effects of Krebs' Intermediates : Ascorbic Acid

Day of germination Treatment*	Fresh basis						Dry basis					
	mg. per 100 g.			mg. per 100 seedlings			mg. per 100 g.			mg. per 100 seedlings		
	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th
Nil	28·81	26·99	21·61	5·17	6·25	5·55	154·8	182·9	200·1	27·79	42·5	51·3
Citrate	31·73	27·37	23·99	6·25	6·67	6·81	146·2	180·1	226·3	28·8	43·8	64·2
Succinate	33·31	26·71	22·74	6·12	6·67	5·55	174·3	211·9	214·5	32·04	52·71	52·3
Fumarate	31·04	29·85	26·08	5·26	6·66	6·0	164·2	304·5	246·0	27·8	68·01	56·6
Malate	32·4	27·81	23·95	6·52	6·97	6·0	163·6	186·6	202·9	32·9	46·8	50·84
Apartate	30·75	28·53	22·79	5·88	6·52	6·12	146·2	212·9	219·1	28·9	48·6	58·8

* Concentrations : 100 p.p.m. each.

TABLE V

Effects of Krebs' Intermediates : 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 ..	888	688	637	151	200	197	4818	4686	5892	836	1366	1821
Citrate ..	925	788	625	185	234	169	4268	5198	8896	853	1544	2410
Succinate ..	738	675	612	125	184	184	3865	5357	5758	656	1456	1728
Fumarate ..	950	850	600	178	194	140	5047	8692	5682	946	1979	1323
Malate ..	688	637	637	128	210	194	3473	4269	5379	646	1117	1635
Aspatate ..	888	688	838	185	208	275	4366	5153	8089	910	1556	2654

* Concentrations : 100 p.p.m. each.

Pre-treatment during steeping was given for 24 hours at 0–4° C. Nicotinic acid determinations were carried out by a cyanogen bromide procedure.[11, 12]

When compared on dry basis, both treatments augment nicotinic acid synthesis as in the case of ascorbic acid. Nicotinic acid precursor, determined by an extraction procedure at pH 10 for 6 hours at 37° C.[11] also showed increases at almost parallel rates.

Effects of Intermediates in the Krebs' Cycle

The established involvement of hexoses as precursors of ascorbic acid suggested a study of the influence of the tricarboxylic acids resulting from the oxidative breakdown after glycolysis by the Embden-Parnas-Meyerhof route. The acids (Table IV and Table V) were used as sodium salts in a concentration of 100 p.p.m. Aspartate has been included in place of its deamination product oxalacetic acid which is highly unstable. Values for ascorbic acid and nicotinic acid (total) are given separately (Table IV and Table V respectively).

It may be seen that all the intermediates in general have varying degrees of stimulatory effect on formation of both the vitamins; fumarate influence is especially noteworthy. The metabolic importance of nicotinamide is as co-enzymes (Diphosphopyridine nucleotide or DPN and Triphosphopyridine nucleotide or TPN) in various dehydrogenation systems; it is co-dehydrogenase in the oxidation of iso-citric acid (TPN) and of malic acid (DPN). Evidently, therefore, conditions influencing oxidation of the Krebs' acids would also involve increased formation of nicotinamide. The distribution of the nicotinamide synthesized during germination in terms of its various forms requires to be studied.

Higher concentrations (250 p.p.m.) of the foregoing metabolite intermediates had somewhat inhibitory effect on growth and hence on biosynthesis of the vitamins (Tables VI and VII).

It should be stated here that under normal conditions of germination and assuming the formation of these intermediates as a prerequisite to vitamin elaboration, their concentrations will at no time reach high values on account of rapid and continuous mobilisation.

Effects of Krebs' Intermediates on Excised Empbryos

The foregoing studies were extended by growing excised *miing* embryos on semi-solid nutrient media containing the sodium salts of the different metabolites. Glucose was used at 3% concentration and other salts

TABLE VI

Effects of Higher Concentrations of Krebs' Intermediates : Ascorbic Acid

Day of germination Treatment*	Fresh basis				Dry basis			
	mg. per 100 g.		mg. per 100 seedlings		mg. per 100 g.		mg. per 100 seedlings	
	3rd	5th	3rd	5th	3rd	5th	3rd	5th
Nil ..	22·86	18·48	3·97	4·69	125·9	193·6	21·95	49·4
Citrate ..	26·73	18·62	5·08	4·92	139·5	184·3	26·45	48·7
Succinate ..	27·17	20·01	4·76	5·25	138·6	174·0	23·4	45·65
Fumarate ..	26·96	19·42	4·42	4·76	135·5	188·5	22·2	46·3
Malate ..	25·69	20·22	4·92	4·76	138·8	190·5	26·6	44·99
Aspartate ..	23·33	20·40	4·24	5·00	130·3	191·0	23·72	46·7

* Concentrations : 250 p.p.m. each.

TABLE VII

Effects of higher concentrations of Krebs' Intermediates : 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	5th	3rd	5th	3rd	5th	3rd	5th
Nil ..	500	475	90	140	2759	5010	496	1471
Citrate ..	450	375	95	120	2347	3702	495	1189
Succinate ..	425	325	82	89	2097	2829	402	776
Fumarate ..	475	338	96	101	2392	3273	483	982
Malate ..	375	275	78	83	2091	2596	421	779
Aspartate ..	450	413	90	121	2511	3875	500	1131

* Concentrations : 250 p.p.m. each.

in equimolar proportions of their respective acids to this glucose concentration. Thus calculated, the percentage concentrations of the acids

were citric, 3·2; succinic, 1·96; fumaric, 1·93; malic, 2·23; pyruvic, 1·5; and aspartic, 2·21. Seedlings were sampled for vitamin C determination on fourth day (Table VIII).

TABLE VIII

Effects of Krebs' Intermediates on Biosynthesis of Ascorbic Acid by Embryos

Treatment	Ascorbic acid mg. per 100 g.	Per cent. difference over untreated
Nil ..	126·5	..
Glucose (3%) ..	192·6	+52·3
Citrate (3·2%) ..	134·2	+ 6·1
Succinate (1·96%) ..	143·8	+13·7
Fumarate (1·93%) ..	155·6	+23·0
Malate (2·23%) ..	140·9	+11·4
Pyruvate (1·5%) ..	243·8	+92·7
Aspartate (2·21%) ..	160·0	+26·5

With most salts, there was retardation in growth of embryos to some extent; this was especially so with citrate, malate and aspartate. Nevertheless, a favourable influence on ascorbic acid formation was noticeable in all cases, being pronounced with pyruvate which had also less deleterious effect on growth.

EFFECTS OF KREBS' INTERMEDIATES SUPPLEMENTED WITH B VITAMINS

Seeds were soaked in solutions containing appropriate salts (100 p.p.m. each) and/or a mixture of vitamins (100 p.p.m. each) as detailed in Table IX prior to germination as usual. Dehydroascorbic acid, total ascorbic acid and ascorbic acid oxidase activities were also followed. Determinations were carried out on fourth day.

The B vitamins in themselves had a small stimulatory effect but did not appreciably add to the influence of the salts of the organic acids employed. Oxidase activities were somewhat higher in presence of the salts and were not again influenced by the vitamin addition.

EFFECTS OF CERTAIN AMINO ACIDS

A number of amino acids could serve as sources of glycogen or in gluconeogenesis in the animal organism. They also form keto acids by deamination

TABLE IX

Effects of Citrate, Succinate and Fumarate together with B Vitamins

Treatment	Ascorbic acid	Dehydro-ascorbic acid	Total ascorbic acid	Ascorbic acid oxidase activity†
	(mg. per 100 g.)			
Nil ..	172·1	22·2	194·3	3·86
B vitamin mixture* ..	198·0	20·1	218·1	3·74
Citrate (100 p.p.m.) ..	188·7	21·2	209·9	4·26
Citrate (100 p.p.m.) + B vitamin mixture*	195·4	12·7	208·1	3·66
Succinate (100 p.p.m.)	187·5	19·8	207·3	4·12
Succinate (100 p.p.m.) + B vitamin mixture	215·1	7·3	222·4	3·62
Fumarate (100 p.p.m.)	222·9	36·7	259·6	4·45
Fumarate (100 p.p.m.) + B vitamin mixture*	202·1	60·7	262·8	4·73

* B vitamin mixture contained thiamine, riboflavin and nicotinic acid (100 p.p.m. each).

† Units expressed as in (5).

in plant metabolism. Several amino acids such as glycine, serine, glutamic acid and aspartic acid are known to be good glucose formers; glutamic and aspartic acids readily funnel into the Krebs' cycle by deamination. Hence, certain amino acids were studied for their influence on biosynthesis of ascorbic acid by seedlings. Effect of aspartate has been referred to earlier (Table IV) and was also studied by Mapson and Cruickshank[13] on cress seedlings. Tryptophane was tried because of its established relationship to nicotinic acid.[14, 15] Glycine participates as precursor for formate[16, 17] and in porphyrin[18] and nucleic acid synthesis.[19] Serine on deamination could form hydroxy pyruvic acid and on decarboxylation will yield ethanolamine. Tyrosine was included as a non-specific general type amino acid. The values for ascorbic acid (Table X) and nicotinic acid (Table XI to XIII) are presented separately.

Considering the vitamin values on dry basis, the amino acids employed had a general accelerating effect on vitamin C formation, especially on the fourth day of germination. On the other hand, the responses to nicotinic acid synthesis were rather varied and were positive only in a few cases, namely, glutamic acid (500 p.p.m.) on the fourth day and serine (500 p.p.m.)

TABLE X

Effects of Certain Amino Acids : Ascorbic Acid

Day of germination Treatment*	Fresh basis						Dry basis					
	mg. per 100 g.			mg. per 100 seedlings			mg. per 100 g.			mg. per 100 seedlings		
	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th
Nil ..	41·92	29·95	23·17	8·72	6·14	5·73	279·4	167·3	260·3	58·1	34·3	64·4
Glycine ..	38·44	35·83	24·47	9·32	9·21	5·73	247·7	243·7	239·9	60·1	62·6	65·9
Serine ..	39·85	30·4	26·36	8·72	7·6	5·74	313·7	266·6	289·6	68·6	66·6	63·0
Tryptophane ..	38·93	31·38	26·48	9·03	7·14	6·25	252·7	293·2	296·6	58·6	66·7	70·2
Tyrosine ..	40·00	29·96	25·32	8·48	7·75	5·8	322·5	356·6	287·7	68·3	92·2	65·9

* Concentrations of amino acids : 1000 p.p.m.

TABLE XI

Effects of Certain Amino Acids : Nicotinic Acid

Day of germination	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 ..	500	512	488	170	163	127	5862	6640	6902	2000	2112	1801
Tryptophane (1000 p.p.m.) ..	400	375	450	135	116	142	4421	4475	5555	1494	1378	1750
Glutamic acid (1000 p.p.m.) ..	438	388	500	150	102	138	4655	4374	6075	1592	1150	1670
Ornithine (1000 p.p.m.) ..	388	500	612	116	153	171	4953	5611	8160	1486	1723	2285
Serine (1000 p.p.m.) ..	362	400	475	111	110	126	4340	4706	6955	1328	1294	1850
Tryptophane (500 p.p.m.) ..	462	562	562	160	183	166	4575	5676	6730	1582	1850	1992
Glutamic acid (500 p.p.m.) ..	425	588	538	139	192	165	4942	7444	6482	1615	2434	1990
Ornithine (500 p.p.m.) ..	400	488	588	125	152	141	4132	5723	6570	1285	1848	1577
Serine (500 p.p.m.) ..	362	388	538	123	116	156	3815	4475	7079	1301	1343	2046

TABLE XII

Effects of Different Concentrations of Tryptophane together with Fumarate : 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	6th	3rd	4th	5th	6th	3rd	4th	5th	6th	3rd	4th	5th	6th
Nil ..	525	537·5	487·5	109·7	109·7	125·3	104·8	70·8	3500	3982	4391	4447	731·5	928	944	685
Fumarate (100 p.p.m.) ..	500	525	587·5	487·5	105	137·6	144	105·8	4238	5198	6317	5943	889·9	1362	1543	1290
Tryptophane (50 p.p.m.)	600	412·5	587·5	525	143·5	108·5	120·5	97·7	4616	3538	5063	5469	1099	943·4	1039	1017·5
Tryptophane (50 p.p.m.) +Fumarate (100 p.p.m.)	500	412·5	525	412·5	121·5	93·6	141·8	83·3	4545	4005	5000	5030	1105	909	1350	1019
Tryptophane (100 p.p.m.)	437·5	412·5	412·5	400	94·5	106·6	111·8	74	3148	3716	3650	4124	680	962·5	989·1	763
Tryptophane (100 p.p.m.) +Fumarate (100 p.p.m.)	362·5	412·5	375	387·5	76·8	95·3	72·4	62·8	3237	3784	3826	4559	686·1	874·3	738·5	738·5

TABLE XIII

Effects of Lower Concentrations of Tryptophane and Fumarate : 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 ..	475	512·5	537·5	149·6	133·2	182·8	5278	6327	8533	1663	1645	2900
Fumarate (10 p.p.m.) ..	412·5	512·5	512·5	122·3	145·1	156·3	3891	6406	7537	1153	1813	2298
Tryptophane (10 p.p.m.) ..	400	562·5	450	114·8	194	143·1	4348	7031	6000	1248	2425	1907
Fumarate (10 p.p.m.) + Tryptophane (10 p.p.m.) ..	475	512·5	525	144·9	172·7	163·3	5654	6570	7000	1725	2214	2177
Fumarate (25 p.p.m.) ..	587·5	437·5	450	165·1	136·6	113·4	4626	5029	6337	1300	1570	1597
Tryptophane (25 p.p.m.) ..	462·5	437·5	462·5	145·2	118·1	134·2	4868	4972	5710	1529	1343	1656
Fumarate (25 p.p.m.) + Tryptophane (25 p.p.m.) ..	450	462·5	450	140	146·2	138·7	4327	5929	6428	1346	1874	1980
Fumarate (50 p.p.m.) ..	575	425	425	179·5	123·2	129·6	5809	5059	6158	1812	1468	1879
Tryptophane (50 p.p.m.) ..	525	387·5	362·5	163·3	112·4	85·7	5469	4559	5493	1701	1322	1300
Fumarate (50 p.p.m.) + Tryptophane (50 p.p.m.) ..	462·5	275	375	144·3	106·9	75·1	5083	4167	3666	1586	1188	1001

as well as ornithine (1000 p.p.m.) on the fifth day. Lower concentrations of amino acids generally gave better results than higher concentrations.

The absence of any stimulatory effect due to tryptophane merited further study with lower concentrations of this amino acid; it was felt that this amino acid liberated free as a result of proteolysis during germination might have masked any effect due to further addition. In fact, low concentrations of tryptophane (Table XII) do exert a definite enhancing effect on nicotinic acid synthesis; fumarate which itself had a favourable effect (Table V) did not have much of an additive effect with tryptophane. This suggested the use of still lower concentrations of these two metabolites. From Table XIII, it may be seen that fumarate or tryptophane when used alone give higher values for nicotinic acid with increasing concentrations at earlier stages of germination; the effects are reversed at higher concentrations. When used together, they give lower values with increasing concentrations at all stages. With 100 p.p.m. of the two metabolites together, biogenesis of nicotinic acid is optimum; higher concentrations are evidently toxic. When derived metabolically, these precursors will be utilized as and when they are formed.

Effects of Certain B Vitamins

Thiamine, riboflavin, biotin and nicotinic acid were studied for any possible effects they may have on vitamin C synthesis. The values obtained with the vitamins (100 p.p.m. each) when used singly are given in Table XIV and in certain combinations in Table XV; the latter data refer to the fourth day of germination.

In general, all the B vitamins tried here accelerated the formation of ascorbic acid during germination and especially up to five days. There was no appreciable effect on ascorbic acid formation as a result of treating the seeds with the B vitamins in combination, with the exception of thiamine and nicotinic acid.

In the foregoing studies, stimulation of ascorbic acid formation was noted with certain metabolites, especially glucose, citrate, succinate and fumarate. The effects due to combinations of these with the B vitamin mixture consisting of thiamine, riboflavin and nicotinic acid (100 p.p.m. each) were studied and the values obtained for four days' old seedlings are given in Table XVI.

There was no appreciable additive effect due to metabolites (*cf.* Table XVI) and B vitamins. The enhancement with fumarate addition was essentially due to the effect of fumarate as observed earlier.

TABLE XIV

Effects of Certain B Vitamins : Ascorbic Acid

Day of germination Treatment*	Fresh basis						Dry basis					
	mg. per 100 g.			mg. per 100 seedlings			mg. per 100 g.			mg. per 100 seedlings		
	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th	3rd	4th	5th
Nil ..	34·1	30·3	19·9	8·9.	7·7	4·4	279·5	258·9	234·4	73·5	67·9	52·7
Thiamine ..	39·2	33·1	22·3	9·2	7·2	5·0	326·6	324·4	263·4	76·7	71·4	58·8
Riboflavin ..	36·8	39·0	28·1	8·3	8·6	6·4	212·9	291·0	246·4	47·8	64·5	56·8
Biotin ..	37·7	32·5	24·2	8·1	7·6	5·7	271·2	260·3	260·2	58·5	60·8	61·6
Nicotinic acid ..	37·2	38·1	27·2	7·7	9·3	5·9	229·6	273·3	245·3	47·8	67·1	53·8

* Concentrations : 100 p.p.m. each.

TABLE XV

Effects of B Vitamins in Certain Combinations

Treatment*	Ascorbic acid	Dehydro-ascorbic acid	Total ascorbic acid	Ascorbic acid oxidase activity†
	(mg. per 100 g.)			
Nil ..	161·9	11·0	172·9	2·52
Thiamine ..	163·5	14·3	177·8	2·27
Riboflavin ..	159·9	21·0	180·9	2·22
Nicotinic acid ..	149·4	24·7	174·1	2·29
Thiamine + Nicotinic acid ..	165·2	25·9	191·0	2·39
Riboflavin + Nicotinic acid ..	163·0	20·8	183·8	2·87
Thiamine + Riboflavin + Nicotinic acid ..	172·3	9·6	181·9	2·38

* Concentrations : 100 p.p.m. of each of the vitamins.

† Expressed as in (5).

TABLE XVI

Effects of Supplementation of Glucose or Its Intermediates with the B Vitamins

Treatment*	Ascorbic acid	Dehydro-ascorbic acid	Total ascorbic acid	Ascorbic acid oxidase activity†
	(mg. per 100 g.)			
Nil ..	172·1	22·2	194·3	3·86
B vitamin mixture ..	198·0	20·1	218·1	3·74
B vitamins + glucose ..	180·9	41·4	222·3	4·34
B vitamins + glucose + phosphate ..	169·5	52·1	221·6	2·97
B vitamins + citrate ..	195·4	12·7	208·1	3·66
B vitamins + succinate ..	215·1	7·3	222·4	3·62
B vitamins + fumarate ..	202·1	60·7	262·8	4·73

* Concentrations : glucose, 1% ; B vitamins consisting of thiamine, riboflavin and nicotinic acid, 100 p.p.m. each ; other salts, 100 p.p.m. each.

† Expressed as in (5).

DISCUSSION

Stimulation in ascorbic acid elaboration in plants by various cultural factors such as trace elements, fertilizers and sugars as well as environmental modifications such as germination in the dark and steeping in the cold have been observed and various hypotheses suggested as to the mode of synthesis of the vitamin. Observations such as those relating to the influence of manganese, magnesium, boron, copper, zinc, salts of potassium, nitrogen and phosphorus, light, etc., are often divergent and it is not clear in which way these factors are involved in ascorbic acid synthesis. The influence of sugars is, however, established unequivocally. Thus, in germinating pulses, glucose and mannose act as precursors. Germination in the dark stimulates vitamin C formation, also on account of the increased amounts of reducing sugars resulting from enhanced amylolysis.[1] The present work carried out with intact seedlings as well as excised embryos confirms the precursorial role of glucose and mannose (p. 34).

The favourable effect of phosphate suggests that phosphorylation may be involved during ascorbic acid formation by seedlings. It has since been ascertained that selective inhibition of phosphorylation reactions affects adversely the biogenesis of ascorbic acid (next paper).

Glucose supplemented with factors like phosphate and B vitamins has enhanced stimulatory effect on the formation of ascorbic acid; the additions, therefore, influence glucose metabolism in such a way as would result in its utilization for ascorbic acid formation to a larger degree.

Synthesis of ascorbic acid by chloretonized rats increases in presence of three-carbon compounds like glyceraldehyde and dihydroxy acetone.[20] Enhanced ascorbic acid formation in cress seedlings when treated with sodium and potassium salts of acetic, succinic, malic and aspartic acids has also been reported.[13] Working with germinating *müng* seedlings, it is observed that some of the Krebs' intermediates, particularly, fumarate and succinate, increase ascorbic acid elaboration. A stimulation in ascorbic acid formation by any substance would suggest that the substance acts as a precursor or as component or activator of the mechanism of conversion of the precursor into the product or it may act as an inhibitor of destructive mechanism. Considering the metabolic importance of the Krebs' intermediates in normal tissue metabolism, their role would seem to be as precursors rather than as activators of biosynthetic processes or as protective agents. It may, therefore, be stated that hexose transformation to vitamin C proceeds *via* these intermediates which in themselves initiate these reactions. Studies with selective inhibitors reported in the next paper also support this view.

It is interesting to note the close parallelism between the elaboration of ascorbic and nicotinic acids under a variety of experimental conditions. A similar observation, in a limited way, had also been reported earlier.[5] The role of pyridinoprotein enzymes in dehydrogenation mechanisms associated with metabolism of sugars and Krebs' intermediates suggests that a supply of the latter necessitates the formation of these enzymes for speeding up their metabolic disposal.

Treatment of seeds during soaking with certain amino acids, namely, serine, tryptophane, tyrosine, glycine and aspartic acid accelerates ascorbic acid formation during germination. These amino acids cannot obviously act as direct precursors. Their influence can only be indirect. Thus, some of the amino acids may be acting as precursors after conversion into the corresponding keto acids by deamination. Certain amino acids are glucose formers, some may also be taken up in building up the apoenzyme portions of the enzyme systems concerned in the formation of ascorbic acid. Another possibility is that amino acids may act as precursors of the coenzyme moieties of these enzyme systems; such a relation is established between tryptophane and nicotinic acid.[14, 15] Thus, the effect of tryptophane in appropriate concentrations may be through enhanced formation of nicotinic acid which may be concerned more directly in the biosynthetic processes leading to ascorbic acid.

The B group of vitamins are generally known to take part in carbohydrate metabolism. Thiamine as cocarboxylase, nicotinic acid as a component of DPN and TPN which are codehydrogenases and riboflavin as flavin-adenine dinucleotide concerned in oxygen transfer processes are all necessary in glucose oxidation. Biotin is concerned in the conversion of pyruvic to oxalacetic acid through carbon dioxide fixation. Pantothenic acid functions in the formation of citric acid from acetate and oxalacetate. The stimulation by B vitamins in ascorbic acid formation is therefore possibly ascribable to their involvement in the oxidative breakdown of glucose prior to vitamin C synthesis. It is known that lack of B vitamins in experimental diets decreases vitamin C concentrations in the lens of rats.[21] Deficiency of riboflavin and thiamine results in reduced storage of ascorbic acid in various tissues and endocrines[22] as also in the livers of mice.[23] Further proof that vitamin C biosynthesis is preceded by glucose oxidation to smaller fragments is presented in the next communication.

SUMMARY

(1) Further observations on the effects of certain cultural and environmental factors on biosynthesis of ascorbic acid in germinating seeds of *müng*

(*Phaseolus radiatus*) are reported. Seed embryos separated from the reserve food store in cotyledons and grown on a semi-solid nutrient medium have been employed in some of these studies. Concomitant changes in nicotinic acid have been also followed.

(2) In confirmation of earlier work, hexoses, particularly, glucose and mannose, have a pronounced enhancing effect on ascorbic acid formation.

(3) Certain intermediates of carbohydrate metabolism such as citrate, succinate, fumarate and malate, induce increased synthesis of vitamin C ; the effects of succinate and fumarate are noteworthy.

(4) Thiamine, riboflavin, nicotinic acid and biotin stimulate ascorbic acid elaboration.

(5) Accelerating effects are also noted with tryptophane, tyrosine, serine and glycine.

(6) A parallelism between the biogeneses of ascorbic and nicotinic acids, under various cultural treatments is observed.

(7) The possible implications of these observations have been discussed indicating probable involvement of hexose intermediates in ascorbic acid synthesis.

ACKNOWLEDGMENT

Our thanks are due to Chas. Pfizer and Co., Inc., New York, and Hoffman-La Roche, Basle, for generous gifts of chemicals. One of us (S.P.B.) is also indebted to the Lady Tata Memorial Trust, Bombay, for the award of a Fellowship.

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