85. THE ACTION OF *L*-ASCORBIC ACID ON THE *IN VITRO* RESPIRATION OF LIVER TISSUE FROM GUINEA-PIGS ON A RESTRICTED DIET. II

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IN the preceding paper [Kellie & Zilva, 1941] it was shown that the rate of respiration of liver slices from guinea-pigs maintained on a quantitatively restricted diet containing an ample supply of vitamin C was considerably increased by the addition of *L*-ascorbic acid. In this communication this phenomenon is studied in greater detail.

Technique

Determination of thickness of liver slices. Since after a long trial it was found by us that the reproducibility of results greatly depended on the thickness of the liver slices employed, special care was taken to prepare sections of suitable and uniform thickness. This was periodically measured as follows: The moist weight of all the slices used in the experiment was first obtained by weighing the lobes before and after sectioning. Their area was then rapidly determined by spreading out flat in a shallow layer of buffer solution in a Petri dish placed on a piece of photographic bromide paper and exposing to a light above the dish. A superimposed exposure was then made through a piece of translucent graph paper. After development the area of the slices was obtained by counting the number of squares in the image of each slice. The density of the liver was determined by weighing a portion in air and in water; as this value (1.09) was found to be very constant, it was unnecessary to repeat the determination in each experiment. The mean thickness was then calculated by dividing the total moist weight of the slices by the product of their area and the density.

The accuracy of this method was checked by the following two independent procedures.

(1) The mean thickness of a group of slices was obtained from measurements of the thickness of a lobe before and after sectioning by means of a micrometer gauge.

(2) Individual slices were measured by means of the focussing vernier on a microscope and the mean thickness then calculated.

Results from the three methods are compared in Table 1 and it will be seen that there is satisfactory agreement between them.

Table 1. *Slice thickness determination by various methods*

Mean thickness of slices in mm.		
Weighing	Screw gauge	Microscope
0.32	—	0.35
0.37	0.32	0.32
0.30	0.35	0.28

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Manometric technique. The manometric technique was essentially the same as that described [Kellie & Zilva, 1941] except that 1.5 ml. instead of 2.5 ml. of medium containing $10^{-3} M$ ascorbic acid were used in these experiments. As before, a freshly prepared $M/15 \text{ KH}_2\text{PO}_4$ solution adjusted to pH 7.4 with NaOH was used as a buffer. It must, however, be pointed out here that the constitution of the buffer is not without influence on the effect which ascorbate exercises on the respiration of the liver slices, as will be seen from the results obtained when 'physiological phosphate saline' [Krebs & Henseleit, 1933] was used as medium (Table 2). Whilst the respiration of the liver tissue from normal animals was

Table 2. *Respiration of guinea-pig liver slices in Krebs' physiological saline*

Volume of medium 1.5 ml. Conc. of ascorbate $10^{-3} M$ unless otherwise stated

Animals receiving restricted diet						Animals receiving normal diet		
Mean Q_{O_2} values		% increase in Q_{O_2} due to ascorbate	Mean Q_{O_2} values		% increase in Q_{O_2} due to ascorbate	Mean Q_{O_2} values		% increase in Q_{O_2} due to ascorbate
No addition	Ascorbate added		No addition	Ascorbate added		No addition	Ascorbate added	
3.44	3.51	2	1.80	2.20	22	5.97†	5.81	-3
3.25*	3.57	10	2.68	2.58	-4	4.19†	4.24	1
3.78*	4.45	18	2.08	2.48	19	7.16†	7.24	1
2.69*	2.73	1	2.34	3.20	37	4.80	4.64	-3
2.45	2.88	18	2.56	2.82	10	4.77	4.76	0

* In these exps. volume of buffer was 2.5 ml.

† In these exps. conc. of ascorbic acid was $0.5 \times 10^{-3} M$.

not affected in this case by the addition of ascorbate, as is usual in the above phosphate buffer, the increase in Q_{O_2} (the minus sign is omitted in all references to Q_{O_2} in this paper) of the tissues from the animals on the restricted diet was however neither so great nor so consistent as that obtained when the neutralized $M/15 \text{ KH}_2\text{PO}_4$ buffer solution was used. The different constitution of the buffer was in all probability responsible for the discrepancy in the results obtained by various workers [cf. Phillips *et al.* 1934 and Stotz *et al.* 1937].

The output of CO_2 was determined by the Warburg method as described by Dixon [1934], in an atmosphere of O_2 and in the same volume of phosphate buffer as that used for measurement of O_2 uptake. The CO_2 was liberated by 0.3 ml. $N \text{ HCl}$ contained in the side bulbs and the same amount of acid was also added at the end of the experiment to the flasks in which the O_2 uptake measurements were made, in order to obviate the effect of any possible change in weight of the dried tissue. Two control flasks containing buffer and buffer and tissue respectively were also included. All measurements were carried out over a period of 1 hr. since it was found by Kellie & Zilva [1941] that measurements over longer periods did not significantly alter the trend of the experiment.

Reagents. (Melting points uncorrected.)

Ascorbic acid—99–100% purity. Messrs Roche Products Ltd.

Reductic acid—was prepared from pectic acid by Reichstein & Oppenauer's method [1933]. Twice sublimed. Yellowish-white crystals. M.P. 209° (decomp.).

d-Glucoascorbic acid—sample kindly presented by Prof. E. L. Hirst, purity c. 85%. Brownish amorphous powder.

Malonic acid—commercial sample twice recryst. from ethyl acetate. White needles. M.P. 135.5° .

Hydroxymalonic acid—commercial sample recryst. from a mixture of ethyl acetate and light petroleum. White anhydrous crystals M.P. 156° . The purification is especially important because commercial samples appear to contain some impurity which acts as a substrate to liver slices.

- Iodoacetic acid—commercial sample recryst. from light petroleum. White crystals M.P. 83°.
 Maleic acid—commercial sample recryst. from ethyl acetate. White crystals M.P. 135°.
 Arsenious oxide—pure commercial sample, Kahlbaum.
 Phloridzin—commercial sample recryst. twice from water and dried *in vacuo* over H_2SO_4 ; faintly yellowish crystals M.P. 213° (decomp.).
 Sodium fluoride—Puriss. commercial sample.
 Sodium pyrophosphate—commercial sample twice recryst. from water.
 Cinnamic acid—commercial sample. M.P. 133-5°.
 Potassium cyanide—AnalaR.

Increase in respiration on addition of ascorbate to liver slices from guinea-pigs on restricted diet as compared with respiration of liver slices of the same thickness from animals on a normal diet

The texture of the livers from animals on a normal diet differs considerably from that of the livers of those maintained on a restricted diet. In order to demonstrate beyond all doubt that the differential effect of the addition of ascorbate to the liver tissue from guinea-pigs on the two diets observed by Kellie & Zilva [1941] was not due to an accidental difference between the thickness of the slices of the two groups of animals, the thickness of the slices used in this experiment was measured (Table 3). Although the thickness was of the

Table 3

Animals receiving normal diet		Animals receiving restricted diet	
Mean thickness of slices mm.	Mean % increase in Q_{O_2} due to added ascorbate	Mean thickness of slices mm.	Mean % increase in Q_{O_2} due to added ascorbate
0.33	-2	0.29	38
0.27	1	0.28	35
0.32	-4	0.31	22
0.29	-3		

same order in both cases, the liver tissue of the guinea-pigs on the restricted diet unlike that from the animals on the normal diet showed as before an increase in the Q_{O_2} on addition of ascorbate. Evidently the empirical method of cutting the liver tissue adopted by us yielded slices of similar thickness (approximately 0.3 mm.) both from guinea-pigs on a normal and on a restricted diet. This reproducibility was further confirmed by occasional measurements.

Influence of the age of animals used

As already mentioned the guinea-pigs normally used in all experiments were approximately 300 g. in weight. Animals of this weight are still growing rapidly and it was of interest to find out whether liver slices from fully grown guinea-pigs gave similar results to those from younger animals. Adult guinea-pigs about 8 months old which had been kept on a mixed diet were used. One group was kept on the normal diet previously described and another group placed on the restricted diet similar to that used for the younger animals. The loss in weight during the restriction of the diet (20-25 days) was of course relatively less. From results given in Table 4 it will be seen that liver slices from adult guinea-pigs on a restricted diet respire very slowly but the Q_{O_2} is nevertheless greatly increased by addition of ascorbate; the effect is even more marked than in the case of young guinea-pigs. It is further seen that although liver slices from adult animals on a normal diet respire more vigorously than those from animals on a restricted diet the Q_{O_2} , as in growing animals, is not increased by addition of ascorbate.

Table 4. *Respiration of liver slices from adult guinea-pigs*Medium—1.5 ml. phosphate buffer. Ascorbate concentration $10^{-3} M$

Animals receiving normal diet				Animals receiving restricted diet				
Wt. of animal g.	Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate	Wt. of animal, g.		Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate
	No addition	Ascorbate added		Before restriction	After restriction	No addition	Ascorbate added	
905	1.80	1.82	1	855	710	1.68	4.87	190
740	5.11	5.18	1	855	687	1.56	4.04	159
720	4.09	4.12	1	865	650	1.87	4.52	142

Influence of ascorbate concentration

Liver slices from guinea-pigs on a restricted diet were allowed to respire in the presence of various concentrations of ascorbate. As it was found to be impossible for us to investigate the effect of more than three or four concentrations in any one experiment it was difficult to obtain as clear a picture of the response of O_2 uptake to a wide range of concentrations of ascorbate as if the experiments were carried out in one series. However, certain deductions can be drawn from the results of a number of such experiments performed at different times (Table 5). First, it must be pointed out that the amount of ascorbate

Table 5. *Effect of varying concentrations of ascorbate on the respiration of liver slices from guinea-pigs on restricted diet*

Medium—1.5 ml. phosphate buffer

Concentration of ascorbate added mM	Mean Q_{O_2}	Increase in Q_{O_2} due to added ascorbate	Mean dry wt. of tissue mg.	Added ascorbic, $\mu g.$ Tissue dry wt., mg.	Concentration of ascorbate added mM	Mean Q_{O_2}	Increase in Q_{O_2} due to added ascorbate	Mean dry wt. of tissue mg.	Added ascorbic, $\mu g.$ Tissue dry wt., mg.
0	5.12	—	19.6	—	0	2.36	—	12.1	—
0.04	5.67	0.55	18.6	0.6	0.04	3.02	0.66	10.3	1.0
0.10	5.66	0.54	17.4	1.5	0.10	3.33	0.97	12.3	2.1
0.20	6.38	1.26	17.1	3.1	0.20	4.16	1.80	11.6	4.6
0	2.95	—	15.2	—	1.0	6.03	3.67	9.1	29
0.05	3.91	0.96	16.0	0.8	0	3.30	—	11.2	—
0.10	4.19	1.24	16.3	1.6	0.01	2.90	(-0.40)	12.6	—
0.15	4.50	1.55	14.2	2.8	0.10	4.07	0.77	12.4	2.1
0	2.46	—	19.9	—	1.0	5.59	2.29	11.0	24
0.04	2.66	0.20	18.6	0.6	0	2.76	—	10.5	—
0.10	2.91	0.45	17.2	1.5	0.2	4.16	1.40	11.8	4.5
0.20	3.38	0.92	20.2	2.6	0.6	4.91	2.15	12.7	12
1.0	4.37	1.91	17.1	15	2.0	5.41	2.65	9.9	53
0	3.04	—	9.6	—	0	3.50	—	16.4	—
0.04	4.02	0.98	9.8	1.1	1.0	6.47	2.97	13.1	20
0.10	4.81	1.77	8.1	3.3	1.5	7.10	3.60	9.9	40
0.20	5.07	2.03	10.4	5.1	2.0	6.82	3.32	11.9	44

present per unit mass of tissue is of greater significance than the actual concentration of ascorbate in the flasks. In Table 5 a column is therefore given showing the $\mu g.$ ascorbic acid per mg. dried tissue, which will be referred to as the 'relative concentration of ascorbate'.

It will be observed that even quite low relative concentrations of ascorbate produced definite increases in the O_2 uptake of the tissue. At low relative concentrations increasing amounts of ascorbate produced a more pronounced increase than at higher levels and when a relative concentration of more than 20 was

reached the Q_{O_2} almost ceased to be influenced by an increase in the amount of ascorbate present. These higher concentrations thus represent a limit to the magnitude of the increase in the O_2 uptake which may be produced by the addition of ascorbate. The curve connecting the increase in Q_{O_2} with relative vitamin concentrations is probably similar to those obtained when the effect of the concentration of a coenzyme on its rate of hydrogenation is studied [cf. Negelein & Haas, 1935; Negelein & Wulff, 1937]. In all our subsequent experiments a relative concentration of the order of 18 was employed. Under these conditions an almost maximal increase in the Q_{O_2} is obtained and small fluctuations such as occur owing to the unavoidable variation in the weight of tissue in the various flasks in any particular experiment have little effect on the results.

Influence of compounds chemically related to ascorbic acid on the O_2 uptake by liver slices from guinea-pigs on a restricted diet

Two such compounds, *d*-glucoascorbic acid, an antiscorbutically inactive homologue of *l*-ascorbic acid and reductic acid, a compound also containing a dienol group, were investigated. Both these compounds possess a reduction potential similar to that of *l*-ascorbic acid. Three sets of flasks were used in each experiment: these contained (1) no addition, (2) *l*-ascorbate and (3) an equimolar quantity of reductate or *d*-glucoascorbate. Thus there was always a direct comparison between *l*-ascorbate and the other compounds. The results in Table 6

Table 6. *Influence of ascorbic acid analogues on the respiration of liver slices from guinea-pigs on a restricted diet*

Concentration of added compounds (M)	Medium—1.5 ml. phosphate buffer							
	Mean Q_{O_2} values			% increase in Q_{O_2} due to		Loss of added substances in μ -mol./mg. dried tissue		
	No addition	<i>l</i> -Ascorbate	Reductate	<i>l</i> -Ascorbate	Reductate	<i>l</i> -Ascorbate	Reductate	
10^{-3}	2.78	4.18	3.91	50	41	0.028	0.043	
10^{-3}	3.48	4.31	4.14	24	19	0.017	0.008	
10^{-4}	2.59	4.16	3.95	61	53	—	—	
10^{-4}	2.81	4.04	3.53	44	26	—	—	
	No addition	<i>l</i> -Ascorbate	<i>d</i> -Glucoseascorbate	<i>l</i> -Ascorbate	<i>d</i> -Glucoseascorbate	<i>l</i> -Ascorbate	<i>d</i> -Glucoseascorbate	
10^{-3}	2.52	3.07	3.32	22	32	0.018	0.026	
10^{-3}	2.08	2.48	2.40	19	15	0.021	0.015	
10^{-4}	3.39	4.15	4.52	22	33	—	—	
10^{-4}	3.88	4.81	4.76	24	23	—	—	

show that both reductate and *d*-glucoascorbate bring about an increase in the Q_{O_2} of liver slices from guinea-pigs on a restricted diet. Moreover, the increases observed were of a magnitude similar to that obtained with *l*-ascorbate even when the concentration of the added substances was as low as $10^{-4}M$. The increase in the respiration is therefore not specific for *l*-ascorbic acid and is independent of the antiscorbutic activities of the compounds; it seems rather to be controlled by their reduction potentials. In this and in the following experiments the loss of the added ascorbic acid during the respiration period is given. The bearing of these figures on the general aspect of the problem will receive reference in the discussion.

The influence of 'saturation' with simultaneous food restrictions on the respiration phenomena associated with added ascorbic acid

Although the experimental guinea-pigs on the restricted diet used in this investigation received daily quantities of vitamin C several times higher than the dose necessary to protect them from macroscopic and microscopic lesions of scurvy, they were not fully saturated in respect of vitamin C. The control animals on the normal diet were of course fully saturated. It was desirable to ascertain whether the increase in the respiration of the liver tissue from restricted animals on addition of ascorbic acid might possibly have been due to the partial saturation of the animals, and with this purpose in view the following experiment was performed. One group of animals on a restricted diet received daily 30–50 mg. of ascorbic acid *per os* over a period of 10–14 days, whilst in another group 50 mg. of neutralized ascorbic acid were injected into the femoral muscle 24 hr. before the respiration of the liver tissue was studied. These conditions are known to produce full saturation [cf. Zilva, 1936]. The results in Table 7 show

Table 7. *Respiration of liver slices from guinea-pigs on a restricted diet receiving large doses of ascorbate*

Medium—1.5 ml. phosphate buffer. Ascorbate concentration $10^{-3} M$

Dose ascorbic acid (neutralized) mg./day	Method of administration	Period of dosage days	Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate	Loss of ascorbic $\mu g./mg.$ dried tissue
			No addition	Ascorbate		
30	Oral	10	3.46	5.39	56	6.1
30	"	11	3.58	5.36	50	7.1
50	"	12	6.30	7.56	20	4.7
50	"	13	5.00	5.42	8	4.6
50	"	14	6.23	5.85	-6	3.6
50	"	12	3.72	4.80	29	5.4
50	Injection	—	4.54	5.00	10	3.2
50	"	—	4.72	6.39	35	5.9
50	"	—	3.90	4.85	24	3.6
50	"	—	2.04	2.50	23	3.8
50	"	—	2.49	4.25	71	7.0
50	"	—	3.23	3.82	18	4.0

that there was such an increase in the Q_{O_2} in the majority of cases when ascorbic acid was added to the tissues that no doubt is left that the enhanced respiration which took place under these circumstances was due to a dietetic restriction as such and not to a state of partial vitamin C saturation.

The relationship between O_2 uptake and CO_2 output

It has been shown on the one hand that the livers of about 30 % of animals on a normal diet showed a significant increase in Q_{O_2} in the presence of added ascorbate [Kellie & Zilva, 1941] and that on the other hand under the same conditions only 8 % of the livers of animals on the restricted diet failed to show an increase in Q_{O_2} . Therefore the results which deviated from the majority in each group were eliminated from these as well as from the experiments in the next section in order to avoid complications.

Although the Q_{CO_2} figures (Table 8) were extremely variable in the case of the animals receiving the restricted diet, the increase in the volume of CO_2 evolved when it did occur was much lower (2/5–1/10) than the O_2 taken up. It would therefore appear that the respiration stimulated by the addition of the ascorbate

Table 8. *Effect of added ascorbate on the Q_{O_2} and Q_{CO_2} of respiring guinea-pig liver slices*

Medium—1.5 ml. phosphate buffer. Ascorbate concentration $10^{-3} M$					
Mean Q_{O_2} values		% increase in Q_{O_2} due to added ascorbate	Mean Q_{O_2} values		% increase in Q_{CO_2} due to added ascorbate
No addition	Ascorbate		No addition	Ascorbate	
Restricted diet					
3.70	5.66	53	3.16	3.36	6
2.97	6.11	106	2.27	1.64	-28
2.70	4.86	80	1.32	1.86	41
3.98	5.85	47	2.48	3.17	28
5.22	6.09	17	3.71	3.84	4
3.74	5.23	40	2.31	1.88	-19
Normal diet					
6.40	6.17	-4	4.97	4.51	-9
3.17	3.39	7	2.09	2.24	7

did not involve complete combustion. As would be expected no alteration was observed in the CO_2 output or in the O_2 uptake in the two control animals in this experiment.

The influence of inhibitors on the respiration of liver slices from guinea-pigs on a normal diet and from animals on restricted diet

In order to throw some light on the mechanism involved in the production of the increased oxidation by the addition of ascorbic acid to liver slices from animals kept on a restricted diet, the influence of a range of inhibitors on the respiration of liver slices from animals on a restricted diet as well as from animals subsisting on a mixed diet was investigated. The inhibitors chosen were substances known to modify the catabolism of carbohydrates and some of them have been shown to arrest specific reactions in the Embden-Meyerhof and other schemes of intermediary metabolism. The lowest concentrations previously found by other workers in this field to produce marked inhibition were employed. The results are given in Table 9.

Phloridzin. Phloridzin greatly reduced the Q_{O_2} of slices from animals on a restricted diet, and also largely abolished the increase in the Q_{O_2} brought about by ascorbate. The reduction in the Q_{O_2} also occurred in slices from normal animals, but in this case the addition of ascorbate had no appreciable influence either in the presence or absence of phloridzin.

Iodoacetate. The action of iodoacetate on guinea-pig liver slices caused a marked reduction in the Q_{O_2} of slices from animals on a normal diet. In the presence of ascorbate however iodoacetate was almost without influence [cf. Quastel & Wheatley, 1934]. With animals on a restricted diet quite different results were obtained. In this case in the absence of ascorbate, iodoacetate caused very little decrease in the Q_{O_2} of the slices. In the presence of ascorbate however, the addition of iodoacetate caused a rise in the Q_{O_2} higher than that obtained by the addition of ascorbate alone as will be seen from the negative values shown in Table 9 for the % inhibition. It will be seen (Fig. 1) that the typical curves for the O_2 uptake in the presence of ascorbate or ascorbate and iodoacetate in the case of the liver of guinea-pigs on a normal diet are almost coincident with those for the untreated tissue. The O_2 consumption of tissue poisoned with iodoacetate falls off much more noticeably than that of the unpoisoned slices. In the case of the animals on the restricted diet it will be

Table 9. *Action of inhibitors on the respiration of liver slices from guinea-pigs on normal and restricted diets*

O=no addition. A=ascorbate ($10^{-3}M$) added. I=inhibitor.

Medium—1.5 ml. phosphate buffer

Inhibitor	Concentration of inhibitor	Nature of diet	Mean Q_{O_2} values				% increase due to A		% inhibition due to I		Loss of ascorbate in $\mu g./mg.$ dried tissue	
			O	A	I	A+I	No I	I	No A	A	No I	I
Phloridzin	$10^{-2}M$	Normal	5.31	5.19	2.48	2.45	-2	-1	53	53	2.3	3.2
"	"	"	3.91	3.84	1.82	2.02	-2	11	53	47	2.8	2.9
Phloridzin	$10^{-2}M$	Restricted	3.66	7.18	1.59	1.99	96	25	57	72	15.9	7.3
"	"	"	3.09	6.09	1.82	2.19	97	20	41	64	10.6	5.8
"	"	"	2.77	3.40	1.33	1.32	23	-1	52	61	4.7	4.4
Iodoacetate	$10^{-3}M$	Normal	2.54	2.44	1.41	1.97	-4	40	44	19	2.7	4.3
"	"	"	6.46	6.19	4.14	6.55	-4	58	36	-6	4.0	9.9
"	"	"	3.56	3.53	2.85	3.55	-1	25	20	-1	3.5	7.2
"	"	"	5.28	5.16	3.92	5.44	-2	39	26	-5	2.6	7.0
Iodoacetate	$10^{-3}M$	Restricted	2.87	5.80	2.82	5.81	102	106	2	0	—	—
"	"	"	2.73	5.36	2.53	6.06	96	140	7	-13	11.1	12.1
"	"	"	3.47	5.38	3.49	6.62	55	90	-1	-23	8.3	11.6
"	"	"	2.65	3.60	2.40	5.98	36	149	9	-66	9.8	16.5
"	"	"	4.21	6.89	3.48	8.17	64	135	17	-19	10.9	19.0
"	"	"	3.54	4.32	2.75	5.42	22	97	22	-25	4.7	9.3
Fluoride	$2 \times 10^{-2}M$	Normal	2.60	2.65	2.25	2.48	2	10	13	6	3.7	3.8
"	"	"	5.00	5.28	2.93	3.73	6	27	41	29	3.5	4.0
"	"	"	3.44	3.32	2.47	2.63	-3	6	28	21	2.4	3.3
Fluoride	$2 \times 10^{-2}M$	Restricted	1.92	3.80	1.27	3.66	98	188	34	4	5.5	6.4
"	"	"	2.70	6.11	1.84	5.43	126	195	32	11	13.2	12.1
"	"	"	2.67	2.80	2.17	2.64	5	22	19	6	3.7	4.2
"	"	"	3.14	5.31	2.68	5.29	69	97	15	0	8.2	8.7
"	"	"	2.21	5.72	1.64	5.42	159	230	26	5	12.7	12.8
Maleate	$2 \times 10^{-2}M$	Normal	3.34	3.42	2.73	3.04	2	11	18	11	3.2	2.7
"	"	"	4.70	4.20	4.39	3.93	-11	-10	7	6	3.4	3.1
"	"	"	4.16	4.22	3.00	3.11	1	4	23	26	2.1	2.8
Maleate	$2 \times 10^{-2}M$	Restricted	2.11	4.41	1.74	4.29	109	147	18	3	8.6	9.2
"	"	"	3.34	6.14	2.75	5.50	84	100	18	10	9.5	10.2
"	"	"	2.59	5.08	2.11	4.72	96	124	19	7	7.8	8.0
Arsenite	$10^{-3}M$	Normal	2.99	3.07	1.61	2.20	3	37	46	28	2.6	4.6
"	"	"	4.99	4.91	3.24	4.21	-2	30	35	14	—	—
"	"	"	3.43	3.27	1.52	1.68	-5	11	56	49	2.2	2.7
Arsenite	$10^{-3}M$	Restricted	2.30	4.04	1.94	3.74	76	93	16	7	6.8	6.9
"	"	"	3.03	4.81	2.02	4.10	59	103	33	15	9.8	12.6
"	"	"	4.01	4.36	1.71	2.82	9	65	57	35	4.3	6.2
Malonate	$10^{-2}M$	Normal	3.36	3.42	2.65	3.14	2	18	21	8	3.8	4.8
"	"	"	4.32	4.16	3.90	3.57	-4	-8	10	14	2.2	2.1
"	"	"	2.77	2.69	2.31	2.32	-3	0	17	14	2.8	3.1
Malonate	$10^{-2}M$	Restricted	3.43	6.71	3.46	6.27	96	81	-1	7	7.3	8.1
"	"	"	2.41	5.39	2.19	4.73	124	116	9	12	9.8	8.7
"	"	"	3.48	6.62	2.89	5.98	90	107	17	10	11.3	12.5
Pyrophosphate	$2 \times 10^{-2}M$	Normal	6.32	6.00	4.61	4.91	-5	7	27	21	3.0	1.1
"	"	"	4.99	4.85	4.20	3.89	-3	-7	16	20	2.8	1.1
Pyrophosphate	$2 \times 10^{-2}M$	Restricted	2.58	4.50	1.83	1.93	74	5	29	57	6.0	2.0
"	"	"	3.20	6.87	2.23	2.63	145	18	30	62	12.4	4.0
"	"	"	2.56	4.89	1.59	1.97	91	24	38	60	9.1	3.7
Hydroxymalonate	$2 \times 10^{-2}M$	Normal	5.32	5.49	4.28	4.53	3	6	20	17	2.4	2.8
"	"	"	6.98	6.99	4.92	5.25	0	7	30	25	—	—
Hydroxymalonate	$2 \times 10^{-2}M$	Restricted	2.86	4.68	2.68	2.16	64	-19	6	54	6.9	3.2
"	"	"	3.18	4.38	2.63	2.48	38	-6	17	43	5.6	4.2
"	"	"	2.96	4.02	2.42	2.50	35	3	18	38	5.0	3.3
Cyanide	$10^{-3}M$	Normal	3.25	3.56	2.33	2.30	10	-1	28	35	2.9	2.9
"	"	"	4.25	4.17	2.49	2.66	-2	7	41	36	2.8	3.3
Cyanide	$10^{-3}M$	Restricted	2.64	4.30	1.00	1.75	63	75	62	59	7.6	5.0
"	"	"	2.72	3.24	1.38	1.62	19	17	49	50	3.8	3.3
Cinnamate	$10^{-3}M$	Restricted	2.60	3.65	2.50	3.34	40	34	4	8	6.6	6.2
"	"	"	2.69	3.44	2.62	3.47	28	32	3	-1	7.3	4.9

noticed that although after 60 min., the tissue poisoned with iodoacetate has absorbed less O_2 than the untreated material, the presence of iodoacetate produced a slight initial increase in O_2 consumption compared with the control. This effect was observed in five out of six of the experiments performed with animals on a restricted diet but with animals on a normal diet it was not once observed.

Fig. 1. Influence of iodoacetate and ascorbate on the course of respiration of liver slices from guinea-pigs maintained on normal and restricted diets. O, $\bigcirc$ — $\bigcirc$ Tissue only. I, $\triangle$ — $\triangle$ 10^{-3} M iodoacetate. A, $\square$ — $\square$ 10^{-3} M ascorbate. AI, ∇ — ∇ 10^{-3} M ascorbate + 10^{-3} M iodoacetate.

It may be added that ascorbate kept without tissue under the same experimental conditions in the presence of iodoacetate with or without glutathione remained unchanged.

Fluoride. Fluoride brought about some reduction in the Q_{O_2} of liver slices from animals on a normal diet in the presence and in the absence of ascorbate. With animals on a restricted diet the presence of ascorbate prevented the inhibition by fluoride to a large extent.

Maleate. Maleate caused some reduction in the Q_{O_2} of slices from animals on both normal and restricted diets. Ascorbate had no significant effect on the former, but in the case of the latter the extent of the inhibition was possibly somewhat reduced.

Arsenite. Arsenite brought about a marked reduction in the Q_{O_2} of slices from guinea-pigs on both normal and restricted diets. When ascorbate was also added, the inhibiting effect was somewhat diminished in the case of both groups of animals.

Malonate. The results of the effect of malonate on the liver slices from animals on normal and restricted diets show that the reduction in the Q_{O_2} was not great in either case, and the presence of ascorbate made no difference to this minor inhibiting effect.

Pyrophosphate. Pyrophosphate reduced the Q_{O_2} of liver slices from animals on a normal diet and the addition of ascorbate had no effect either in the

presence or in the absence of the inhibitor. When animals on the restricted diet were employed, an inhibitory effect was also observed but the pyrophosphate almost entirely abolished the increase in Q_{O_2} brought about by the action of ascorbate. In this respect its behaviour was similar to that of phloridzin.

Hydroxymalonate. This inhibitor reduced the respiration of liver slices of animals on both normal and restricted diets. In the latter, however, hydroxymalonate entirely eliminated the usual increase brought about by the addition of the ascorbate.

Cyanide. In experiments with cyanide, a cyanide solution was placed in the central cup for the absorption of CO_2 in order to avoid loss of HCN from the buffer medium [Krebs, 1935]. Cyanide produced large inhibitions in the Q_{O_2} of slices from animals on normal and restricted diets but the addition of ascorbate caused nevertheless the same relative increase in Q_{O_2} of the slices from animals on a restricted diet whether or not the tissue was poisoned with cyanide. It therefore did not influence the particular respiratory process which was accelerated by ascorbate.

Cinnamate. Cinnamate had no appreciable effect upon the Q_{O_2} values either in the presence or in the absence of ascorbate when liver tissue from guinea-pigs on a restricted diet was used.

DISCUSSION

The experiments described in this and the preceding communication show beyond doubt that the addition of ascorbic acid to liver slices from guinea-pigs maintained for some time on a quantitatively restricted diet increases the rate of the surviving respiration of this tissue. This occurs when the animals receive at the same time sufficient vitamin C to protect them from scurvy or even to saturate them with the vitamin and consequently the phenomenon seems to have no connexion with the development of scurvy. Furthermore, the stimulating action on the respiration is not confined to ascorbic acid alone but can be brought about by the presence of chemically related compounds such as *d*-gluco-ascorbic acid and reductic acid which possess a similar reduction potential but no antiscorbutic activity.

Provided that the necessary experimental conditions, particularly as regards the buffer and thickness of slices, are observed the results are definitely reproducible. We have a record of 106 experiments with guinea-pigs on a restricted diet and 67 experiments with animals on a normal diet. The surviving respiration of the liver tissues of 95 % of the former and of only 31 % of the latter responded to the addition of ascorbic acid by an increase in the rate of respiration. That this was not controlled by the ascorbic acid content of the livers was shown by the fact that the quantity of the vitamin in the flasks when livers of normal and consequently saturated animals were used was well below, about a tenth, the quantity of added ascorbic acid required to produce the maximum increase in O_2 uptake, and the livers of normal animals should therefore have reacted to the addition of ascorbic acid. In spite of this, only 31 % of the livers from the saturated animals on a normal diet responded to the addition of ascorbic acid. Additional point is lent to this view by the fact that, in the case of the livers of guinea-pigs saturated with vitamin C, 10 out of 12, i.e. 83 %, were found to respond to this addition. Furthermore, as was noted by Kellie & Zilva [1941], the livers from restricted animals respired on an average more vigorously after the addition of ascorbic acid than did those from normal animals.

The question arises whether the increase in the O_2 uptake was due to the oxidation of some of the added ascorbic acid or to an increase in the rate of

tissue respiration brought about by this addition. A correlation of a number of our results, selected at random from the preceding tables and arranged in the ascending order of the increase in Q_{O_2} (Table 10), does not favour the former

Table 10. *The relationship between the loss of added ascorbate and the increase in Q_{O_2} of respiring liver slices from guinea-pigs on restricted diet*

Increase in Q_{O_2} due to added ascorbate	Gross relative loss*	Corrected relative loss*	Molar ratio†	Increase in Q_{O_2} due to added ascorbate	Gross relative loss*	Corrected relative loss*	Molar ratio†
0.13	3.7	0.7	1.5	2.30	8.6	5.6	3.2
0.42	3.6	0.6	5.5	2.33	9.1	6.1	3.0
0.78	4.7	1.7	3.6	2.49	7.8	4.8	4.1
0.95	9.8	6.8	1.1	2.68	10.9	7.9	2.7
1.05	6.6	3.6	2.3	2.80	9.5	6.5	3.4
1.06	5.0	2.0	4.2	2.98	9.8	6.8	3.4
1.20	5.6	2.6	3.6	3.00	10.6	7.6	3.1
1.74	6.8	3.8	3.6	3.28	7.3	4.3	6.0
1.82	6.9	3.9	3.7	3.41	13.2	10.2	2.6
1.88	5.5	2.5	5.9	3.67	12.4	9.4	3.1

$$* \text{ 'Relative loss of ascorbate' } = \frac{\text{Loss of ascorbic acid } (\mu\text{g.})}{\text{Dry wt. of tissue (mg.)}}$$

$$\dagger \text{ 'Molar ratio' } = \frac{\text{Increase in } Q_{O_2} \times 176}{\text{Corrected loss of ascorbate} \times 22.4}$$

assumption. In correlating these results it was necessary to take into consideration two established facts, firstly, that the loss of the ascorbate was proportional to the weight of the tissue employed and secondly that a loss of ascorbate was also incurred, even when tissue from animals on a normal diet was used, although no increase in Q_{O_2} took place. The mean relative loss of ascorbate calculated as $\mu\text{g.}$ of ascorbic acid per mg. of dry liver tissue, from 21 normal animals, the respiring tissues of which showed no increase in Q_{O_2} , was 3. The observed relative loss in the case of the tissues from restricted animals was therefore corrected by subtracting this value from it. This correction was particularly justified by the fact that the relative loss of ascorbic acid in the respiration of tissues from restricted animals which showed a small increase in Q_{O_2} was not very much higher than that from normal animals. The molar ratios of (increased O_2 uptake due to ascorbate/corrected loss of ascorbate) thus obtained will be seen from Table 10 to be of the order of 3–4, which is in excess of the relative amount of O_2 required to oxidize ascorbic acid to dehydroascorbic acid ($\frac{1}{2}$ mol. O_2 per mol. ascorbic acid) or even to oxalic and *l*-threonic acids (1 mol. O_2 per 1 mol. ascorbic acid). As there was no proportional increase in the CO_2 formation (Table 8) it is hardly likely that the oxidation of the ascorbic acid proceeded to complete breakdown to CO_2 and water, which would produce 6 mol. CO_2 to 5 mol. O_2 per mol. of ascorbic acid. Even when low concentrations of ascorbate such as $10^{-4}M$ were used the molar ratio was of the same order as in the case of higher concentrations. The results rather suggest that the increase in Q_{O_2} is not due to the oxidation of the added ascorbic acid *per se* but that the compound acts catalytically on the respiration of liver tissue from the dietetically restricted animals, but does not usually do so on that of tissues from normal animals. The observed loss of the ascorbic acid is in all probability incurred whilst the acid is passing through the labile stage as dehydroascorbic acid. The observation that the greater the increase in respiration, the greater the destruction of the added vitamin (Table 10) might according to this view have been expected. It is to be noted that the loss of the ascorbic acid analogues

when added to respiring liver slices from animals on a restricted diet was of a similar magnitude to the loss of ascorbate.

There is no evidence so far to show whether ascorbic acid also stimulates the respiration of the liver of the intact guinea-pig on a restricted diet. The gas metabolism of scorbutic guinea-pigs, but not of guinea-pigs on a deficient diet containing vitamin C, has been studied by a number of workers and their results are contradictory. Abderhalden [1921] maintains that there is no difference between respiration of normal and scorbutic guinea-pigs. Jarussowa [1926], Soderström & Törnbloom [1933] and Scoz *et al.* [1937] found that the O_2 consumption of scorbutic guinea-pigs was less than that of healthy animals, whilst Calcinai & Galigani [1934] and Mosonyi & Rigó [1933] assert the contrary. Baucke [1938] concludes that the O_2 uptake of guinea-pigs on a scorbutic diet fluctuates greatly from day to day. The stimulating action of ascorbic acid on the liver respiration of underfed guinea-pigs *in vivo* can with our present knowledge be viewed only as a theoretical possibility. The observations made *in vitro*, nevertheless, lay bare some potentialities for future research.

As a preliminary effort an attempt was made in this investigation to ascertain whether a connexion exists between the above phenomenon and some of the links in schemes of carbohydrate metabolism. The influence of inhibitors, known to interfere with some of the stages postulated to occur in these schemes, was accordingly investigated on the accelerating action produced by added ascorbic acid on the respiration of liver slices from dietetically restricted animals as well as on that of liver slices from normal animals.

The failure of cyanide to influence the relative increase of the O_2 uptake produced by the addition of ascorbate in liver slices from animals on a restricted diet, excludes the possibility of this reaction being associated with the ultimate stages of the aerobic oxidation of carbohydrates. The great reduction in Q_{O_2} increase brought about by the action of phloridzin, which is known to inhibit the phosphorylation of carbohydrates, the first stage in the breakdown of glycogen, suggests a possible connexion of the accelerated respiration produced by ascorbate in the liver tissue from underfed animals with any one of the subsequent stages in the degradation of this carbohydrate. It does not however throw any light on the actual stage involved. The further fact that iodoacetate does not prevent the accelerating action of added ascorbate rather indicates that the increased respiration may be connected either with any of the stages concerned in the anaerobic formation of triosephosphate from glycogen or in the aerobic degradation of the intermediary products, but not with the compounds formed by the anaerobic breakdown of triosephosphate. The results obtained with fluoride point to the same conclusion but in a rather less striking manner. The ineffectiveness of malonate as an inhibitor excludes the possibility of the association of succinic dehydrogenase with the phenomenon under discussion. The results obtained with pyrophosphate and hydroxymalonate are difficult to interpret. Although pyrophosphate, which inhibits the action of succinic dehydrogenase, has also a retarding action on the accelerating effect of the added ascorbate, a connexion between the two must be ruled out in view of the results obtained with malonate. The possibility that the inhibitory action of pyrophosphate on the increase in the Q_{O_2} brought about by the addition of ascorbate is due to its precipitating effect on certain divalent catalytically active cations [Ochoa, 1939] cannot be excluded. It is unlikely that the action of hydroxymalonate has any connexion with the oxidation of lactate [cf. Jowett & Quastel, 1937] since iodoacetate which prevents the formation of lactate does not inhibit the stimulating effect of the added ascorbate. The significance of the negative

results obtained with maleate and arsenite cannot at present be interpreted. The observation that cinnamate does not influence the phenomenon under observation excludes its being connected with the oxidation of fats, or at least of crotonic and butyric acids, since Jowett & Quastel [1935] found that cinnamate inhibited their oxidation to acetoacetic acid.

The results so far obtained by us in our effort to link up the accelerating action of ascorbic acid and its analogues on the respiration of the liver tissue from dietetically restricted guinea-pigs with known reactions in animal metabolism can only be considered as pointers, and further work is now in progress.

SUMMARY

The addition of low concentrations of *l*-ascorbic acid increased the rate of respiration of the surviving liver tissue of about 95 % of guinea-pigs maintained on a quantitatively restricted diet containing vitamin C at levels more than sufficient to protect these animals from macroscopic and microscopic lesions of scurvy. Even the respiration of liver tissue from guinea-pigs on a restricted diet fully saturated in respect to vitamin C behaved in this way. The liver tissue of only about 30 % of animals on a normal diet responded to the addition of *l*-ascorbic acid. The liver tissue from both growing and adult guinea-pigs reacted alike in this respect.

The addition of compounds chemically related to *l*-ascorbic acid such as *d*-glucoascorbic acid and reductic acid, which possess a reduction potential of the same order as *l*-ascorbic acid but no antiscorbutic activity, also stimulated the respiration of liver tissue from guinea-pigs on a restricted diet.

Some added *l*-ascorbic acid is lost during the process of respiration, but the loss is insufficient to explain the increase in Q_{O_2} on the assumption that this compound is oxidized to oxalic and *l*-threonic acids, nor do measurements of CO_2 production suggest complete breakdown of the acid. The loss of the added *l*-ascorbic acid analogues during respiration was of a similar magnitude to that of *l*-ascorbic acid. At low concentrations increasing amounts of *l*-ascorbic acid produced a more pronounced increase in respiration than at higher levels, until a level was reached when further addition ceased to influence the rate of respiration.

The increase in respiration was largely abolished by phloridzin, pyrophosphate and hydroxymalonnate; it was not affected by maleate, arsenite, malonnate or cinnamate. Iodoacetate depressed the Q_{O_2} value of liver tissues from normal animals but the addition of *l*-ascorbic acid prevented this inhibition. In the case of animals on a restricted diet the simultaneous addition of iodoacetate and *l*-ascorbic acid brought about a higher O_2 uptake than the addition of *l*-ascorbic acid alone. Fluoride decreased the respiration of the liver tissues from animals on a restricted diet in the absence, but not in the presence, of *l*-ascorbic acid. The relative increase in Q_{O_2} produced by added *l*-ascorbic acid in slices from animals on a restricted diet was the same in the presence of cyanide as in its absence, in spite of the great reduction in respiration produced by this reagent. The bearing of these results on the possible connexion between the accelerating effect of added *l*-ascorbic acid on the respiration of liver tissue from guinea-pigs on a restricted diet and their carbohydrate metabolism is discussed.

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