CCLXXXV. GLUTATHIONE AND ASCORBIC ACID IN TISSUES OF NORMAL AND TUMOUR-BEARING ALBINO RATS.

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A POWERFUL reducing substance giving the indophenol reaction for ascorbic acid was shown by Birch and Dann [1933] to be present in a wide variety of animal tissues in amount comparable with the glutathione present. It then became apparent that values for tissue glutathione determined by methods depending upon its reducing capacity might include both glutathione and ascorbic acid. In tumour tissue, using a colorimetric nitroprusside method, Boyland [1933] showed that only about one-third of the total iodine value was due to glutathione whilst the remainder could be accounted for by the indophenol titration as ascorbic acid. It should be pointed out however that it is not yet certain that the material in all tissues reacting in this method is actually ascorbic acid.

Recently the author [1935] has demonstrated a method which appears to be absolutely specific for glutathione. Using this method, which depends upon the degree of activation of glyoxalase by glutathione, further investigation has been made of the glutathione contents of tumour tissue and other tissues of the cancer-bearing animal along with ascorbic acid titrations of the same tissues. Since tumours contained a relatively high amount of this ascorbic acid-like material, experiments were likewise carried out to see whether the concentration of this substance could be changed experimentally in the animal with resultant effect upon growth of the tumours.

METHODS.

Animals. Albino rats of the Germantown strain, covering a weight range of 75–250 g., were used. The tumour-bearing animals had received transplanted tumours of the Philadelphia No. 1 sarcoma and Walker No. 256 carcinoma strains. A histological description of these tumours has been given by Waldschmidt-Leitz *et al.* [1933]. The rats were fed on a standard diet consisting of corn, oats, bread and lettuce.

Tissue extracts. These were prepared by a method similar to that proposed by Okuda and Ogawa [1933]. The tissues were removed immediately from the stunned and decapitated animal. After weighing each tissue, it was placed in a mortar, covered by one volume of 0·25 *M* salicylsulphonic acid and then ground in the absence of sand or other extraneous agent. The tissue readily disintegrated to a fine pulp. The pulp was then washed into a graduated cylinder or tube by means of 0·125 *M* salicylsulphonic acid and made up to a volume corresponding to five times the weight of the tissue used, or 1:5 dilution. In the case of liver, a 1:10 filtrate was used, making up to volume with 0·1 *M* salicylsulphonic acid instead of 0·125 *M*. In the case of adrenal, where only 20–30 mg. were available per animal, a 1:50 filtrate was used. The adrenals were ground with 0·3 ml. of

0·1 *M* salicylsulphonic acid and made up to volume with the same strength acid. With the last two tissues, where deviation was made from the usual 1:5 dilution, the strength of acid used had to be varied as indicated in order to bring the resulting filtrates to the same degree of acidity. After standing for about 15 min. and thorough mixing, the extracts were filtered through Whatman No. 30 filter-paper.

Glutathione estimation. The manometric glyoxalase method previously described by the author [1935] was used throughout.

Ascorbic acid estimation. This was based upon the principle of Birch *et al.* [1933] of addition of the ascorbic acid solution to a definite amount of standardised 2:6-dichlorophenolindophenol. In these experiments however the salicylsulphonic acid extracts of the tissues were used instead of trichloroacetic acid extracts. The superiority of the former acid for indophenol titrations of ascorbic acid was pointed out in the previous paper [Woodward, 1935].

Experimental.

Normal animals.

The values for normal animals, fasting 24 hours, are given in Table I. A few values were also determined on animals not fasting. The only differences observed were in the liver and kidney glutathione values, which were higher in the non-fasting animals. It was also noted that the ascorbic acid value remained constant for a much longer time in the case of the filtrates from the fasting animals. Only the fasting values were used therefore for comparisons. In some of the tissues studied, there seems to be a surprisingly small range of values. This is particularly true of the glutathione in adrenal, kidney and spleen, and the ascorbic acid in liver and kidney.

Table I. *Normal rats.*

Rat			Glutathione (mg. per 100 g.)				Ascorbic acid (mg. per 100 g.)			
No.	Wt. g.	Sex	Adrenal	Liver	Kidney	Spleen	Adrenal	Liver	Kidney	Spleen
1	81	M	85	160	69	88	400	27	18	53
2	83	M	110	148	70	95	343	30	22	51
3	145	F	125	164	80	102	292	22	16	31
4	147	M	95	160	106	—	345	18	14	—
5	156	M	90	140	54	98	333	29	15	29
6	160	F	160	198	96	92	329	19	15	19
7	172	M	125	192	76	86	385	23	15	34
8	174	M	100	192	72	87	331	29	18	23
9	211	M	90	190	70	92	437	32	21	27
	Average		109	172	77	92	355	25	17	33

Tumour-bearing animals, untreated.

Table II gives the results on rats with Walker No. 256 carcinoma and with Philadelphia No. 1 sarcoma. Both tumours were found to contain large amounts of glutathione and of a material which titrates as ascorbic acid. The concentration of the latter is higher in tumour tissue than in any other tissue studied with the exception of adrenal. Brain and thymus were investigated in a few cases and found to contain amounts of ascorbic acid in the vicinity of 35 mg. per 100 g. each, the glutathione amounting to 45 and 50 mg. per 100 g. respectively. In no tissue does there seem to be a significant increase or decrease in the concentration of either glutathione or ascorbic acid when the tumour-bearing animals are compared with the normals.

Table II. *Tumour-bearing rats. Untreated.*

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	75	17	3	4	92	—	114	60	—	50	—	22	13	—
2	124	18	2	2	73	100	152	76	—	47	518	25	20	—
3	151	18	10	7	76	—	—	—	—	45	—	—	—	—
4	153	18	5	3	88	—	—	—	—	35	—	—	—	—
5	153	19	9	6	90	95	170	62	—	56	438	26	15	—
6	178	20	11	6	83	110	—	—	—	53	543	—	—	—
7	198	18	4	2	94	85	156	79	91	44	383	23	13	25
8	215	21	15	7	100	110	176	72	100	52	364	23	17	41
9	220	16	5	2	88	100	204	74	89	38	346	24	14	35
10	260	17	10	4	111	90	110	79	108	56	360	24	15	32
			Average		90	99	155	72	97	48	422	24	15	33
Philadelphia No. 1 sarcoma:														
1	80	22	5	6	64	80	102	51	—	72	464	25	17	—
2	90	35	12	13	94	105	154	69	86	65	333	18	14	21
3	100	28	9	9	79	70	—	64	86	73	342	27	20	27
4	135	32	16	12	90	—	136	94	82	80	415	20	15	39
5	139	26	9	6	101	95	158	72	106	73	486	24	17	32
6	204	31	23	11	61	88	118	68	—	45	375	14	11	—
7	293	40	36	12	82	—	—	—	—	45	—	—	—	—
			Average		82	88	134	70	90	65	403	21	16	30

A characteristic difference between the two tumours studied is noted in the relative glutathione and ascorbic acid contents of each. The Walker No. 256 carcinoma is characterised by a higher glutathione content and a lower ascorbic acid content than the Philadelphia No. 1 sarcoma. Thus the ratio of glutathione to ascorbic acid in the carcinoma is usually over 1·6 with an average of 1·9, whilst in the sarcoma it is usually under 1·4 with an average of 1·3.

It should be pointed out that the above-mentioned figures for tumour tissue were obtained only on that part of the tumour which was healthy growing tissue. Comparative analyses on necrotic parts from some of the tumours, Table III,

Table III. *Comparison of healthy and necrotic tumour tissue.*

	Glutathione		Ascorbic acid	
	Healthy mg./100 g.	Necrotic mg./100 g.	Healthy mg./100 g.	Necrotic mg./100 g.
Philadelphia No. 1 sarcoma	82	9	45	8
	104	5	52	Trace
	82	8	45	9
Walker No. 256 carcinoma	76	12	25	5

showed a very small amount of both reducing substances in these areas. Since it is not possible entirely to separate the growing cells from the necrotic, it seems quite probable that, in purely necrotic cells, none of either of the reducing materials is present. Edlbacher and Jung [1934] had previously reported that ascorbic acid was 10 times lower in necrotic tissue of Jensen rat sarcoma, and that the total iodine value, representing both glutathione and ascorbic acid, was 4–5 times lower. The fact that these materials are non-existent or low in necrotic tissue may explain discrepancies which have been reported in the literature with regard to the ascorbic acid value and particularly the glutathione content of tumours. It has been the experience of the author that in the Ehrlich mouse carcinoma the necrotic cells are scattered throughout to such an extent that it is practically impossible to obtain a representative sample of healthy tissue for analysis. This probably accounts for the fact that the values for

Ehrlich carcinoma are lower than those for the other two tumours. Analyses on the best material available gave for glutathione 22, 30 and 43 mg. per 100 g. and for ascorbic acid 9, 13 and 11 mg. per 100 g. Similar values for ascorbic acid in this tumour were reported by Galigani [1934].

Tumour-bearing animals, treated with ascorbic acid.

An extremely large stimulation of tumour growth has been reported by Fodor and Kunos [1934] in mice with Ehrlich carcinoma which were fed or injected with ascorbic acid. An attempt to duplicate their results with a series of animals injected subcutaneously has failed here. There was no increased growth of the tumours. On the whole the growth of these tumours was so erratic even in the controls as to allow of no definite conclusions. The experiment was therefore repeated on a series of rats with Walker No. 256 carcinoma and with Philadelphia No. 1 sarcoma. With both these tumours the rates of growth were practically the same as in the controls. At the end of the experiment, the tissues were subjected to the same analyses as those of the untreated tumour-bearing animals. The results, Table IV, show that the ascorbic acid concentration has not been significantly increased in any tissue with the possible exception of tumour tissue, and here the significance of the apparent slight increase is doubtful.

Table IV. *Tumour-bearing rats after ascorbic acid injections.**

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	153	17	10	7	—	165	200	72	106	—	206	30	22	44
2	142	19	4	3	128	120	176	80	—	53	303	20	14	—
3	155	20	6	4	104	115	138	91	—	50	242	20	13	—
4	160	18	3	2	105	105	178	68	—	67	495	29	18	—
5	152	19	10	7	94	85	108	50	—	69	570	27	15	—
6	164	20	9	5	88	105	—	—	—	56	543	—	—	—
			Average		104	116	160	72	—	59	393	25	16	—
Philadelphia No. 1 sarcoma:														
1	81	33	11	14	81	63	86	44	66	77	300	26	17	26
2	97	48	12	12	86	80	104	50	80	83	318	29	20	34
3	111	28	13	12	96	75	—	65	—	79	461	25	17	—
4	126	26	9	7	83	85	132	60	—	78	425	23	17	—
5	137	32	12	9	102	85	168	74	—	77	354	25	16	—
			Average		90	78	123	59	73	79	372	26	17	30

* In the carcinoma group, rats Nos. 1, 2 and 3 had received subcutaneously 0·5 ml. containing 10 mg. for 8, 10 and 11 days respectively; Nos. 4, 5 and 6, 0·5 ml. containing 20 mg. for 10, 11 and 12 days respectively.
In the sarcoma group, rats Nos. 1 and 2 had received subcutaneously 0·6 ml. containing 4·5 mg. for 11 and 25 days respectively; Nos. 3, 4 and 5 had received 0·5 ml. containing 10 mg. for 16, 14 and 20 days respectively.
All analyses were made 24 hours after the last injection.

Tumour-bearing animals, treated with mannose and glucose.

The origin of ascorbic acid in plant tissues was investigated by Ray [1934] whose results indicated that ascorbic acid could be formed naturally from the hexoses, particularly mannose. The association of mannose in plant tissues rich in ascorbic acid had previously been noted by Euler and Klussmann [1933, 1]. Evidence was also offered [Guha and Ghosh, 1934] that mannose, alone among the sugars, could be converted into ascorbic acid *in vitro* by certain tissues of the rat. As a substance which might possibly be transformed into ascorbic acid by tumours *in vivo* and thus stimulate their growth, mannose was injected

subcutaneously into a series of tumour-bearing rats. From analyses of tissues of rats under such treatment, Table V, it is seen that no such synthesis occurred. Neither the concentration of ascorbic acid in the tumours and other tissues nor the rate of growth of the tumours was affected in any way.

Rats with Philadelphia No. 1 sarcoma were likewise treated with glucose without any effect upon the tumours or other tissues (see Table VI).

Table V. *Tumour-bearing rats after mannose injections.**

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Walker No. 256 carcinoma:														
1	196	15	4	2	112	100	208	75	116	58	418	22	14	18
2	186	16	10	5	115	100	192	82	164	41	281	17	13	41
3	208	16	7	3	109	—	—	—	—	43	—	—	—	—
4	219	19	15	7	85	70	168	68	104	40	214	23	19	35
5	224	19	8	4	119	70	132	62	108	71	400	24	17	33
6	130	18	12	9	118	—	—	—	—	54	—	—	—	—
7	158	18	5	3	84	—	—	—	—	45	—	—	—	—
			Average		106	85	175	77	123	50	328	22	16	32
Philadelphia No. 1 sarcoma:														
1	97	46	15	15	110	—	108	54	—	67	—	23	14	—
2	107	46	21	20	94	—	108	51	—	77	—	27	14	—
			Average		102	—	108	53	—	72	—	25	14	—

* In the carcinoma group, all the rats received subcutaneously 0·5 ml. 50% mannose daily, No. 1 for 7 days, Nos. 2, 3, 4 and 5 for 8 days, and Nos. 6 and 7 for 9 days. In the sarcoma group, the rats received subcutaneously 0·25 ml. 50% mannose daily for 24 days. Analyses were made 24 hours after the last injection.

Table VI. *Tumour-bearing rats after glucose injections.**

Rat		Tumour			Glutathione (mg. per 100 g.)					Ascorbic acid (mg. per 100 g.)				
No.	Wt. g.	Age days	Wt. g.	%	Tumour	Adrenal	Liver	Kidney	Spleen	Tumour	Adrenal	Liver	Kidney	Spleen
Philadelphia No. 1 sarcoma:														
1	201	26	8	4	71	68	162	68	76	48	361	19	15	28
2	221	33	23	10	—	90	144	68	—	—	247	25	16	—
3	245	39	43	18	104	85	202	78	100	52	286	20	13	21
4	260	40	30	12	82	—	—	—	—	45	—	—	—	—
			Average		86	81	169	71	88	48	298	21	15	25

* All the rats received subcutaneously 1 ml. 50% glucose twice daily for the first 11 days, then 1 ml. daily for 14, 21, 27 and 28 days respectively. Analyses were made 24 hours after the last injection.

Tumour-bearing animals, treated with oxidation-reduction dyes.

In an attempt to decrease the ascorbic acid content of the tumours, tumour-bearing animals have been injected with oxidation-reduction dyes known to react with ascorbic acid, that is, dyes with a more positive potential than that of ascorbic acid. 2:6-Dichlorophenolindophenol, toluylene blue and the dye prune have been used in a series of 10 rats for each dye with tumours about 0·5 cm. in diameter, half of each group receiving the dye subcutaneously near the site of the tumours and half into the tumours. There was no marked difference in the growth of any of the tumours as compared with a control group. The tumours which had been injected with prune were however among the largest developed. Analyses of a few of the tissues are reported in Table VII. Here also there has been no definite effect on the concentrations of ascorbic acid and glutathione in the tumour or other tissues. Euler and Klussmann [1933, 2] had been able to reduce the ascorbic acid content of guinea-pig adrenal by half in

4 hours by a single subcutaneous injection of methylene blue, but it must be borne in mind that guinea-pig tissues are much more susceptible to vitamin C (ascorbic acid) variations than rat tissues.

Table VII. *Philadelphia No. 1 sarcoma rats after injection of oxidation-reduction dyes.**

Dye	Site of injection	Rat wt. g.	Tumour Age days	Tumour Wt. g.	Tumour %	Glutathione (mg. per 100 g.) Tumour	Adrenal	Liver	Kidney	Ascorbic acid (mg. per 100 g.) Tumour	Adrenal	Liver	Kidney
Prune	Subcut.	103	37	15	15	94	105	150	58	72	355	31	18
	,,	87	33	20	23	115	—	—	—	36	—	—	—
	Tumour	80	33	17	21	96	—	—	—	36	—	—	—
	,,	99	39	18	18	91	—	—	—	61	—	—	—
Toluylene blue	Subcut.	110	38	13	12	96	90	94	52	67	440	21	17
	Tumour	94	33	12	13	120	—	—	—	64	—	—	—
	,,	108	39	22	20	91	—	—	—	61	—	—	—
2:6-Dichloro-phenol-indophenol	Subcut.	96	37	16	17	108	100	132	66	59	367	20	17
	Tumour	100	33	13	13	87	—	—	—	57	—	—	—
	,,	99	39	18	18	90	—	—	—	58	—	—	—

* 0·2 ml. 0·1 % dye daily at first, increasing to 0·4 ml. as the tumours became larger. Analyses were made 24 hours after the last injection.

The question also arises as to whether the dyes were able to penetrate into the tumour cells. Prune and the indophenol in their oxidised (coloured) forms were quite apparent in the necrotic areas of the tumours where the analytical figures have shown a lack of reducing substances. In the healthy growing parts of the tumours no evidence was found that any dye, even in its reduced (colourless) form, was present, for, when tissue slices or tissue extracts were treated with ferricyanide or with hydrogen peroxide, no colour developed. It is therefore possible that the dyes did not penetrate into the tumour cells and for this reason could not react with the ascorbic acid present.

Tumour-bearing animals treated with X-rays.

Having failed to change materially the concentration of ascorbic acid or glutathione in tumours by any of the injection experiments, it was thought of interest to investigate the concentration of these substances in a tumour whose growth had been checked by X-rays. Such tumours were kindly supplied by Dr George Bancroft of this laboratory. Using twin tumours on a rat and applying X-rays to one tumour, the other being shielded, he had been able to produce varying degrees of retardation of growth in the treated tumour as compared with the control tumour on the same rat. Analyses of such pairs of tumours are recorded in Table VIII. Where the X-ray treatment was effective, a distinct diminution of glutathione in the tumours was found (Nos. 3, 4, 5, 9, 10 and 11). Ascorbic acid was likewise distinctly decreased in the Philadelphia No. 1 sarcomas, but not in the Walker No. 256 carcinomas. In tumours resistant to X-rays, the decreases in these values were not noted (Nos. 6 and 7). Furthermore, in a tumour which had started to grow again after X-ray treatments were stopped (No. 8), both reducing substances were found in an amount as high as in the control tumour.

It seems very likely that the decreased glutathione content found in X-ray-treated tumours is not a direct effect of the X-rays on glutathione since it was shown by the author [1933] that glutathione is extremely resistant to large doses of X-rays. These doses were 7–10 times larger than those applied to the rat tumours by Dr Bancroft. It has however been shown by Kinsey [1935] using softer X-rays than those used in this laboratory that glutathione in pure solution

Table VIII. *Twin tumours after X-ray treatment of one.*

No.	Tumour strain	Treatment	Tumour wt. g.	Glutathione mg. per 100 g.	Ascorbic acid mg. per 100 g.	Remarks
1	Philadelphia No. 1 sarcoma	Both untreated	11·6 12·8	109 112	59 61	Both growing about the same
2	,,	,,	26·2 30·2	96 106	50 53	,,
3	,,	X-ray Untreated	0·7 2·5	56 82	29 42	Irradiated tumour regressing slowly
4	,,	X-ray Untreated	1·8 30·0	16 74	16 53	Growth of irradiated tumour inhibited, slight growth after X-ray treatment stopped
5	,,	X-ray Untreated	3·8 11·3	81 111	50 62	Growth of irradiated tumour considerably retarded. No regression
6	,,	X-ray Untreated	7·1 12·2	80 85	65 61	Growth of irradiated tumour retarded slightly. Fairly resistant to X-rays
7	,,	X-ray Untreated	15·5 30·0	86 91	52 55	,, ,,
8	,,	X-ray Untreated	4·0 37·5	76 72	58 64	Growth of irradiated tumour inhibited at first. Tumour has started to grow since treatment stopped
9	Walker No. 256 carcinoma	X-ray Untreated	0·4 4·2	28 68	23 29	Irradiated tumour regressing rapidly
10	,,	X-ray Untreated	4·3 16·9	69 88	26 31	Growth of irradiated tumour inhibited. No regression
11	,,	X-ray Untreated	2·0 22·5	50 78	26 28	Irradiated tumour regressing slowly

may be somewhat destroyed. The decrease in glutathione may find an explanation as a secondary effect, being an expression of the reduced metabolism of the treated tumours.

Summary.

1. Walker No. 256 carcinoma and Philadelphia No. 1 sarcoma were found to contain glutathione in amount comparable with other body tissues of the rat and ascorbic acid-like material in amount higher than any other tissue studied except adrenal. These reducing substances were present only in the growing parts of the tumour, there being practically none in the necrotic part. Ehrlich mouse carcinoma is too necrotic throughout to make a good separation and values obtained with this tumour are therefore low. Glutathione is slightly higher in the carcinoma than in the sarcoma, whilst ascorbic acid is somewhat lower in the former. Thus the ratio of glutathione to ascorbic acid in the carcinoma is usually over 1·6 and in the sarcoma under 1·4.

2. Corresponding tissues in the normal and tumour-bearing rats showed no significant differences in their glutathione and ascorbic acid contents.

3. Long-continued injections of ascorbic acid, mannose, glucose or oxidation-reduction dyes into tumour-bearing rats did not materially affect the concentrations of ascorbic acid or glutathione in the tumour tissue or other tissues of the body. The growth of the tumours was likewise not affected.

4. X-ray treatment caused a decrease in the glutathione values of the tumours provided that the treatment was effective in retarding the growth of the tumours; the ascorbic acid value was reduced only in the Philadelphia No. 1 sarcoma. In tumours resistant to X-rays, no decrease in the values was noted.

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