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CREATINE AND PHOSPHORUS COMPOUNDS IN MALIGNANT TUMOURS.

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Except in a few isolated cases, creatine has been found solely in vertebrate animals. The ordinary forms of malignant tumour also seem to be limited to vertebrates, and so it appeared conceivable that the occurrence of creatine could have some relation to malignant disease. Edlbacher and Kutscher [1931], however, found no creatine phosphate nor arginine phosphate in tumours. The type of tumour used in their experiments is not stated and no units are given for any of their results. Since then Franks [1932] has shown that the Crocker Institute mouse tumour 180 (a sarcoma) contains a small amount of phosphagen as estimated by the method of Eggleton and Eggleton [1929]. The experiments described below, which were completed before the appearance of Franks' work, show that some phosphagen occurs in tumours. The phosphagen content of tumours is indeed only one-twentieth that of normal resting voluntary muscle, while the creatine content is about a tenth of the amount found in such muscle. The low phosphagen content, however, does not necessarily mean that this substance is of no significance in tumour metabolism. Clark, Eggleton and Eggleton [1931] have shown that normal hearts contain only a tenth as much phosphagen as skeletal muscle, yet these authors [1932] have shown that the amount of phosphagen, small though it is, is essential for cardiac metabolism.

Perhaps the most striking feature in the distribution of the phosphorus compounds in tumours is the large amount of total phosphorus present. Although there is less acid-soluble phosphorus present than in muscle, the amount of nucleic acid and nucleoprotein is three or four times as great. It would appear that malignant tissue would form as good a starting material as thymus gland for the preparation of nucleic acid.

Edlbacher and Kutscher [1931] found an active nucleotide phosphatase in tumours, and this is confirmed by Franks' [1932] findings that on incubation the total acid-soluble phosphorus of tumour tissue in-

creased. In the latter experiments some of the increase in acid-soluble phosphorus would be accounted for by autolysis of nucleic acid. Experiments described below show that on incubation in bicarbonate solution without carbohydrate there is no phosphate esterification, but a considerable production of free orthophosphate. Edlbacher and Kutscher also found this production of free phosphate, and state further that this process continued even in the presence of fluoride and carbohydrate. Franks, however, found some esterification when glucose alone was added. The glycolysis of tumours in the presence of glucose is much greater than the esterification. In an hour, twenty to sixty molecules of lactic acid are formed for each atom of phosphorus esterified, but even this esterification is stopped by the presence of fluoride [Edlbacher and Kutscher, 1931], which has been shown to inhibit glycolysis of malignant tissue [Dickens and Šimer, 1929]. Edlbacher and Kutscher, and Franks, found pyrophosphate in tumours. Edlbacher and Kutscher apparently found as much pyrophosphate as free inorganic phosphate. Franks' highest figure for pyrophosphate is equal to about half the amount of orthophosphate and that is the order of the amount found in the present investigation. From the results given below it is obvious that the increase in free phosphate on incubation is largely at the expense of this pyrophosphate fraction, and this would appear to be the case in the experiments of Edlbacher and Kutscher, and of Franks (though in neither paper is this stated). The part of the phosphorus metabolism of incubated tumour tissue which can be observed is therefore mainly a hydrolysis of adenylpyrophosphoric acid to give free orthophosphate and inosinic or adenylic acid. At the same time there is an increase in acid-soluble phosphorus due to hydrolysis of nucleic acid. Even in the presence of carbohydrate when rapid glycolysis occurs the free orthophosphate tends to increase. This fact could be accounted for either by the extraordinary activity of the phosphatases in the system, or by the fact that the free orthophosphate in the trichloroacetic acid extract is not present as such in the tissue, but is bound in some labile compound in which condition it is incapable of undergoing esterification.

Estimation of creatine.

Creatine and creatinine were estimated colorimetrically by Folin's [1915] method in which the colour given with alkaline picrate is compared with the colour given by a standard creatinine solution. Tumours immediately after removal were divided into two parts, one of which was

ground with sand and picric acid and used for the creatinine estimation, while the other was heated at 125° C. for half an hour with N H_2SO_4 to convert creatine into creatinine. After the first estimations it was found that the creatinine content (1·5, 1·8, 2·5, 1·2 mg. per 100 g. tissue of Jensen rat sarcoma) was as small as that in blood or muscle and could be neglected, and that hydrolysis of the whole tissue with sulphuric acid gave brown solutions which reduced the accuracy of the creatine estimation. In most of the experiments, therefore, creatine was estimated on the trichloroacetic acid extract of the tissue which was prepared as for the estimation of phosphorus compounds. The first results (Table I) were obtained from human tumours, which were generally analysed within 1 or 2 hours after their removal from the subject.

TABLE I. Creatine content of human tumours.

				Normal tissue of same person		
Case	Type of tumour	Weight of tissue (g.)	Creatine (mg. per 100 g.)	Type	Weight of tissue (g.)	Creatine (mg. per 100 g.)
a	Carcinoma of breast	20·5	11	Breast	27·1	5·2
	Metastases in glands	2·78	104	Pectoral muscle	9·7	390
b	Carcinoma of breast	19·6	50·5	Breast	16·1	Under 5
c	Carcinoma of rectum	21·4	64	Rectum (mucous membrane and muscular coats)	11·9	121
d	Carcinoma of breast	15·7	14	—	—	—
e	Carcinoma of rectum	17·4	11·5	Mucous membrane of rectum	11·5	15

It will be seen that there is great variation in the creatine content of the tumours examined. This is probably due to the fact that such samples may be contaminated with surrounding tissue, which masks the true content in different types of tumours. They serve at any rate to show that human tumours contain creatine. The highest figure (104 mg. per 100 g.) is given by the glandular metastases from a breast cancer; in these there would be very little admixture with other tissues.

In Table II is shown the creatine content of some tumours of the rat and mouse, namely (*a*) a primary spindle-celled tumour of the rat produced experimentally by subcutaneous injections of 1:2:5:6-dibenzanthracene in a fatty medium [L.R. 66: Burrows, Hieger and Kennaway, 1932]; (*b*) grafted tumours of the rat derived from another primary spindle-celled tumour produced in the same way as (*a*); these grafted tumours, which are denoted throughout this paper by the reference number L.R. 10, were obtained from the ninth and twelfth

transplanted generation; (*c*) the Jensen rat sarcoma; and (*d*) spontaneous mammary carcinomas of the mouse.

The primary tumour L.R. 66, and the tumours of the same type in the ninth and twelfth grafted generations (L.R. 10, Tables II and IV) contain amounts of creatine of the same order. Two of the tumours examined contained more creatine than has been found in numerous other estimates which have been made on similar tumours. Some determinations were made on the necrotic tissue of large tumours, and in these the creatine content was lower than in whole tumours or in their growing edge, showing that the creatine must be contained largely in the living tissue.

TABLE II. Creatine content of rat and mouse tumours.

			Normal skeletal muscle from same animal	
Type of tumour	Tumour (g.)	Creatine (mg. per 100 g.)	Muscle (g.)	Creatine (mg. per (100 g.)
Rat				
Grafted L.R.10 tumour	5·0	59	3·0	536
,, ,,	5·0	57	—	—
Jensen sarcoma	30·0	105	5·0	560
,,	18·35	90	5·0	580
			5·0	630*
			5·0	590*
,,	6·0	45	—	—
,,	7·2	41	—	—
Primary 1:2:5:6-dibenzanthracene-lard tumour. L.R. 66	10·0	31	—	—
Jensen sarcoma, necrotic material	8·0	14	—	—
,, growing tissue	48	46	—	—
,, necrotic material	4·8	11	—	—
,, two whole tumours	54	27	—	—
Mouse				
Spontaneous mammary carcinoma	6·6	36	—	—
Spontaneous mammary carcinoma (two tumours pooled)	4·8	44	—	—

* Normal rat muscle.

Most of the animal tumours examined contained 30–60 mg. creatine per 100 g., and this amount is greater than that occurring in any normal tissue except skeletal muscle, heart, nerve and testis, which contain about 500, 250, 100 and 100 mg. per 100 g. respectively. It is less than the free creatine of muscle (*ca.* 95 mg. in 100 g.) as found by Walpole's method, but it is much larger than the blood creatine (*ca.* 5 mg. per 100 g.), and may therefore be of significance. Further experiments are to be made on this subject.

Phosphorus compounds in tumours.

Rats weighing 150–250 g., with large grafted tumours of connective tissue (10–40 g.), were killed by a blow on the head and the tumour rapidly removed, weighed and well ground in 10 p.c. trichloroacetic acid containing ice. After filtration the protein precipitate was well pressed on the filter and the filtrate immediately used for estimation of the phosphorus fractions. Phosphorus was estimated colorimetrically after Martland and Robison [1926]. In a preliminary orientating experiment in this way seven tumours (Jensen rat sarcoma) weighing together 104 g. were extracted with 250 g. of ice and trichloroacetic acid. Various phosphorus fractions were estimated in the acid extract and in the precipitate from which nucleic acid was extracted by warming with dilute caustic soda. The results obtained are given in Table III.

Table III. Phosphorus compounds in rat tumours and frog muscle, expressed as mg. P per 100 g. tissue.

	L.R.10 tumour	Jensen rat sarcoma	Frog muscle
Total phosphorus	254	235	182
Total acid-soluble phosphorus	52	56	134
Free orthophosphate	25	22	15*
Phosphagen (Meyerhof and Lohmann, 1928, precipitation method)	2·5	—	—
Phosphagen (Eggleton and Eggleton, 1929, extrapolation method)	1·7	1·2	65*
Pyrophosphate (P hydrolysed in *N* HCl at 100° in 7 min.)	11·5	12·2	25*
Hexose diphosphate (calculated from P hydrolysed in *N* HCl at 100° in 30 min.)	0·5	—	Trace*
Hexose monophosphate (P sol. as barium salt)	8·0	—	8*
Other acid-soluble P (nucleic acid and adenylic acid)	7·0	—	30*
Total acid-insoluble phosphorus	202	179	48
Total acid-insoluble phosphorus soluble in NaOH (nucleic acid and nucleoprotein)	196	152	43

* From Eggleton [1929].

The amount of phosphorus of tumours which is not soluble in acid is remarkably high. It would seem that tumour tissue contains three or four times as much nucleic acid as a normal tissue, such as muscle. The difference in nucleic acid content between tumour and other tissue is enormously greater and probably more significant than the difference in lecithin content found by Jowett [1931].

As stated in the introduction the free orthophosphate content is sometimes high, being greater than that present in resting muscle. In Franks' experiments incubation with carbohydrate did not remove this free phosphate, and it is possible that it is actually in some labile combination

which is rapidly hydrolysed even when removal and treatment with trichloroacetic acid containing excess of ice are carried out rapidly. Experiments were made in which the tissue was frozen with carbon dioxide snow in some cases and liquid air in others before extracting with trichloroacetic acid, but even under such conditions the free orthophosphate was often more than 20 mg. per 100 g. tissue. The extract made from frozen tissue was always very cloudy, so that the results were not so accurate as those obtained by the usual procedure.

Next to the free orthophosphate, the pyrophosphate is the most plentiful of the acid-soluble fractions. Lohmann [1931] has shown that pyrophosphate is really combined with adenylic acid in the form of adenyltriphosphoric acid in skeletal muscle (with 2 atoms of labile P to each atom of "organic" P); and in the form of adenyldiphosphoric acid (with 1 atom of labile P to each atom of "organic" P) in heart muscle. In the presence of magnesium salts these compounds act as co-enzyme in lactic acid production. It was of interest therefore to isolate and examine the pyrophosphate fraction of tumours. This was done by Lohmann's method, precipitating as barium salt, then as mercury salt and finally removing the inorganic phosphate by precipitation with ammoniacal magnesia mixture. The resulting compound precipitated as barium salt contained 1·9 atoms of labile P to each of organic P in one preparation from grafted L.R.10 tumour, and 2·08 atoms of labile P to one atom of organic P in a sample prepared from Jensen rat sarcoma. The co-enzyme or adenylpyrophosphoric acid of malignant tissue would therefore appear to be the same as that obtained from skeletal muscle.

The effect of survival on the acid-soluble phosphorus compounds including phosphagen was next determined. Large grafted rat tumours were rapidly excised and cut in half. One half was immediately ground with ice and trichloroacetic acid while the other half was first allowed to stand in Ringer-bicarbonate for 1 or 2 hours. Phosphagen was estimated by the extrapolation method of Eggleton and Eggleton [1927] and by Lohmann's method [1928] in which orthophosphate is precipitated with ammoniacal magnesium citrate[1]. There are some discrepancies in the results obtained for phosphagen as estimated by the precipitation and extrapolation methods. This may be due to a small amount of arginine phosphate which is not estimated by the extrapolation method. On the other hand the extrapolation method is much less accurate when applied

[1] Estimations made by the method of barium precipitation after Eggleton and Eggleton [1929] gave results comparable with those obtained by Meyerhof's method, *e.g.* four consecutive experiments gave 2·7, 2·6, 4·1 and 3·5 phosphagen P per 100 g.

to small amounts. According to both methods of estimation there is an autolysis of phosphagen on incubation. Typical results are shown in Table IV.

TABLE IV. Creatine and acid-soluble phosphorus compounds in grafted rat tumours expressed as mg. P per 100 g. moist tissue.

Rat No.	Type	Condition	Creatine content	Free ortho-phosphate	Phosphagen P estimated by: Pre-cipitation	Phosphagen P estimated by: Eggleton extra-polation	Pyro phos-phate	P after ½ hour hydrolysis in *N* HCl (hexose di-phosphate)	Total acid-soluble phos-phorus
1	J.R.S.	Freshly excised	29	20·0	—	1·8	—	—	50·8
2	J.R.S.	Stood 1 hour in Ringer	27	27·2	—	0·2	—	—	52·9
3	J.R.S.	Stood 2 hours in Ringer	33	32·4	—	0·7	—	—	55·0
4*a*	L.R. 10	Freshly excised	31	16·5	3·2	0·6	12·3	0·5	52·0
4*b*	„	Stood 1 hour in Ringer	33	22·0	2·4	0·7	8·9	0·7	55·4
5*a*	„	Freshly excised	31	24·4	3·9	1·0	11·2	1·1	56·4
5*b*	„	Stood 2 hours in Ringer	34	34·7	0·7	Nil	4·0	0·6	60·2
6*a*	„	Freshly excised	30	18·5	2·3	2·4	16·2	0·8	54·3
6*b*	„	Stood 1 hour in Ringer	31	26·8	1·1	0·4	11·1	0·8	59·0

It can be seen that survival leads to a rise in orthophosphate content and a smaller rise in the total acid-soluble phosphorus. It is evident that this free orthophosphate is derived partly from phosphagen and the acid-insoluble fraction. This latter accounts for the increase in total acid-soluble phosphorus. The effect must be partly due to the active nuclease found by Edlbacher and Kutscher, but it must also be in part due to the high concentration of substrate present upon which such an enzyme can act. Ashford and Holmes [1931] have recently found a similar increase of acid-soluble phosphorus on incubation in brain tissue. The largest part of increase in orthophosphate is, however, due to hydrolysis of adenylpyrophosphate; the occurrence of this hydrolysis shows that tumours contain pyrophosphatase. These results explain those of Franks, in which under similar conditions free phosphate was formed. In the presence of glucose this free phosphate would be esterified to hexose monophosphate which, as Robison and Morgan [1930] showed, is precipitated to a considerable extent by 10 p.c. alcohol when inorganic phosphate is present. Franks found in muscle a change in the opposite direction (*i.e.* a decrease in the phosphorus precipitated as barium salt by 10 p.c. alcohol), and this appears to be due to decomposition of creatine phosphate. The important change in the phosphorus

compounds of tumours on incubation is therefore due to pyrophosphatase hydrolysing adenylpyrophosphoric acid, which is the co-enzyme of lactic acid formation.

SUMMARY.

1. Human tumours contain creatine. The wide range in the amount found is probably due to varying admixture with other tissues.

2. The majority of the tumours from rat and mouse examined contained very little creatinine but 30–60 mg. creatine per 100 g. tissue. In freshly excised tumour, and hence probably *in situ*, a quarter to a third of the creatine is combined with phosphate as judged by phosphagen estimations. The phosphagen breaks down on standing.

3. The amount of acid-insoluble phosphorus in malignant tumours is very high (200 mg. per 100 g.), being about four times as great as in skeletal muscle.

4. Tumours contain practically as much adenylpyrophosphoric acid as does skeletal muscle and the compound would appear to be the same as in skeletal muscle. This compound is hydrolysed on incubation of the tumour; hence a pyrophosphatase must be present.

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