THE DISTRIBUTION OF ACID-SOLUBLE PHOSPHOROUS COMPOUNDS IN TUMOR TISSUE*

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The presence of organic acid-soluble phosphorous compounds in blood and tissues has been recognized for a long time but the identification of some of these compounds and suggestions as to their possible rôle in cell metabolism are of comparatively recent date. Hexosephosphoric acid esters have been isolated from yeast (1) and from muscle tissue (2). Meyerhof (3) in his investigation of the glycolytic enzyme of muscle found that the esterification of glucose and phosphoric acid is a step in the formation of lactic acid. The glycolytic activity of tumor tissue is well known since the work of Warburg (4) but the actual mechanism involved in the splitting of glucose into lactic acid is not clear. It has not been settled with certainty whether or not an esterification of glucose precedes lactic acid formation in tumor tissue. Barr, Ronzoni and Glaser (5) reached the conclusion that phosphates are not involved in the glycolysis of tumor tissue. If this were the case, the lactic acid formation in malignant tissue would not only be quantatively but also qualitatively different from that in muscle tissue.

Recent work on muscle tissue has led to the identification of several other organic acid-soluble phosphorous compounds. Fiske and Subbarow (6) isolated phosphocreatine, a compound which is intimately connected with muscle contraction. Lohmann (7) identified pyrophosphoric acid in muscle. He suggested that this compound is in combination with adenine nucleotide. The last named compound consisting of adenine, a pentose and phosphoric acid also called adenylic acid, has been

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known to exist in muscle since Liebig. Recently it has been recognized by Parnas (8) and by Embden (9) that ammonia is split off from this substance during muscle contraction. Adenine nucleotides have also been isolated from heart (10) and from brain tissue (11). Warburg (12) observed that tumor tissue forms considerable amounts of ammonia in vitro. He suggested that this might be due to a decomposition of protein. In view of the behavior of adenlyic acid in muscle it seems possible that this compound is the source of ammonia in tumor tissue.

The facts just enumerated make it desirable to determine the distribution of phosphorous compounds in tumor tissue as a preliminary step in an investigation of their functional significance. With the exception of determinations of the content of inorganic phosphates little is found in the literature relating to this subject.

EXPERIMENTAL

Fiske and Subbarow (6) in their method of determination of phosphocreatine extracted the muscle tissue with trichloracetic acid in the cold and effected a separation of the phosphorus compounds by means of precipitation with an alkaline solution of calcium chloride. Eggleton and Eggleton (13) used barium hydroxide for the same purpose. The precipitate in both cases includes orthophosphate, adenylic acid and the second acid labile phosphorus compound (pyrophosphate), while the barium or calcium salts of phosphocreatine and hexosephosphoric acid remain in solution.

A comparison of both methods of precipitation on the same trichloracetic acid filtrate of muscle tissue showed closely agreeing values for the different fractions. Subsequently the barium precipitation was used because it is easier to remove the excess barium at a certain stage of the procedure than the calcium. This refers to the determination of total phosphorus. In estimating phosphorus the colorimetric method of Fiske and Subbarow (14) was used throughout.

The following tumors were used: (1) Jensen rat sarcoma, (2) spontaneous mammary carcinoma of the mouse and (3) transplanted mammary carcinoma of the mouse. In order to com-

pare tumor tissue with other tissues, analyses were also made on kidney, liver and muscle of rats about three months old and on the hairless skin of rats ten days old.

The tumor tissue was freed from connective tissue and as much necrotic material as possible. Only what appeared solid and of even whiteness was used for analysis. In the case of the Jensen rat sarcoma it was easy to do this; but in the mouse carcinoma, both transplanted and spontaneous, the necrosis was often distributed throughout the tumor in fine stippling so that it was impossible to remove all the necrotic material. The gastrocnemius muscle was removed under amytal anesthesia and immediately weighted in a cold room as suggested by Fiske (6).

After weighing, the tissue was placed into ice-cold N/4 trichloracetic acid, was cut into fine pieces and allowed to stand a minute. The acid was poured off, sand was added and the whole ground to a fine mud. The acid was poured back again and enough acid added to make 10 cc. for each gram of tissue used. The minced tissue was well mixed with the acid for complete extraction; then filtered.

An aliquot portion of the clear filtrate was made alkaline to phenol-pthalein with dry barium hydroxide. In this way the phosphorus compounds were separated into two fractions: (*A*) those forming soluble barium salts and (*B*) those forming insoluble barium salts. The barium precipitate was washed once with barium trichloracetate and the washings added to fraction (*A*). The barium precipitate was dissolved in a few drops of 8 N hydrochloric acid and both fractions made up to a known volume. The subsequent analysis was similar to that outlined by Eggleton and Eggleton (13).

Fraction (*A*) was analyzed for (*a*) phosphocreatine and (*b*) soluble barium esters. The phosphocreatine was split by allowing it to stand at room temperature in an acid medium. The soluble barium esters were determined by subtracting the phosphocreatine phosphorus from the total phosphorus content of the fraction.

Fraction (*B*) was analyzed for (*c*) orthophosphate, (*d*) pyrophosphate and (*e*) insoluble barium esters. Orthophosphate

was determined directly. The second acid labile phosphate or pyrophosphate was found by subtracting the orthophosphate value from the value obtained after hydrolysis with N hydrochloric acid for seven minutes in boiling water. The phosphorus of the insoluble barium esters was found by subtracting the sum of the orthophosphate plus pyrophosphate from the total phosphorus content of the fraction.

In carrying out the total phosphorus determination in fraction (*A*) and (*B*), it was found essential to remove the barium sulphate which precipitated on the addition of the sulphuric acid. Unless this precaution is observed, a loss of phosphorus occurs during the subsequent ashing with sulphuric acid. The hydrogen peroxide used in oxidizing the charred material gave no color reaction with the reagents provided the solution was boiled a minute after oxidation was completed. A total phosphorus determination was also made on the original trichloracetic acid filtrate before neutralization with barium hydroxide.

In several cases lipid phosphorus was determined. The tissue residue after separation of the acid-soluble phosphorus compounds was suspended in water and washed on a Buchner funnel to remove the last traces of the trichloracetic acid. The material was then dried over night in an oven kept at 35° C. The following day it was extracted first with alcohol and then with ether in a continuous extraction apparatus for about six hours each. The combined extracts were made up to a convenient volume. A phosphorus determination was made on an aliquot portion after ashing with sulphuric acid and oxidizing with hydrogen peroxide. The lecithin-cephalin fraction was separated from the other phospholipids in the manner described by Bloor (15) and a phosphorus determination was carried out in the same way as the total lipid phosphorus determination.

Calcium was determined in the trichloracetic acid filtrate of the tissues in the manner described by Rothwell (16) for blood filtrates.

DISCUSSION

The amount of inorganic phosphate in the three types of tumors investigated shows marked variability. Occasionally

very high values were obtained in the transplanted and spontaneous mouse carcinoma; in these cases necrotic areas were distributed throughout the tumor and could not be removed completely from the parts chosen for analysis. The fairly good parallelism between orthophosphate and calcium content makes it probable that necrotic areas contain considerable amounts of calcium phosphate. The skin of hairless young rats contains considerable amounts of both inorganic phosphate and calcium, probably in preparation for the formation of hair which is rich in calcium phosphate. The calcium content of the other tissue is low, especially that of the liver.

Phosphocreatine could not be detected in tumor tissue. Traces of a labile phosphorus fraction were found in the liver. Eggleton and Eggleton (12) found a small amount of such a fraction in heart, uterus, testicle and stomach. Gerard (17) discovered appreciable amounts of phosphocreatine in nerve tissue.

A fraction which yields orthophosphate after short hydrolysis with N hydrochloric acid at 100°C, is found in tumor tissue. It has been designated tentatively as pyrophosphate fraction, because the hydrolysis curve is identical with that of pyrophosphate and because added pyrophosphate appears quanitatively in that fraction, as Eggleton and Eggleton (13) have shown. The significance of this fraction which seems to be present in all tissue, but reaches by far the highest values in muscle, is still quite obscure. Tumor tissue contains somewhat more pyrophosphate than liver, kidney or skin.

Adenylic acid is held to constitute the largest part of the insoluble esters. Tumor tissue contains about as much of this phosphorus fraction as muscle and kidney tissue. The soluble esters are at present regarded as consisting mainly of hexosemonophosphate. In muscle this substance has been shown to be formed as an intermediary when glycogen is split into lactic acid. The presence of soluble esters in tumor tissue is therefore of special interest. The spontaneous mouse carcinoma contains decidedly more of these esters than both types of transplanted tumors and more also than was found in muscle tissue.

The calculated and determined total phosphorus content shows a satisfactory agreement in most cases.

A few words may be added regarding the different phosphorus fractions in normal tissues. By increasing the amount of trichloracetic acid per gram tissue it is possible to get rid of a liver pigment which otherwise interferes with the chlorimetric phosphorus determination. In this organ the large amount of "soluble esters" and the small amount of orthophosphate is noteworthy. In kidney too the amount of soluble esters is high as has been shown before by Eggleton and Eggleton (13). The fractionation of the acid-soluble phosphates of tumor tissue revealed no characteristic differences from normal tissue. Kidney skin and liver, as well as spleen (13) show a similar distribution of the different fractions.

The lecithin-cephalin phosphorus content in the Jensen sarcoma is higher than in the spontaneous mouse carcinoma and accordingly the total lipid phosphorus is also higher in the former tumor. Both liver and kidney contain larger amounts of lipid phosphorus than tumors.

SUMMARY

A fractionation of the acid-soluble phosphates of three types of tumors has been carried out. The orthophosphate and calcium content of the tumors was low when necrotic material was excluded from the analysis. When the necrotic areas could not be removed completely inorganic phosphates and calcium were often very high. Phosphocreatine could not be detected in tumor tissue. "Pyrophosphate," soluble and insoluble barium esters showed a similar distribution in tumor tissue as in normal tissue. The total lipid and lecithin-cephalin phosphorus content of tumor tissue was not particularly high when compared with that of liver and kidney.

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TABLE I

Phosphate Content of Transplanted Mammary Carcinoma of the Mouse
Mg. of P per 100 gm. Tumor Tissue

Orthophosphate	Phosphocreatine	Pyrophosphate	Insoluble Esters	Soluble Esters	Total P (calculated)	Total P (found)	Total Lipid P	Lecithin-Cephaline P	Calcium
90.5	—	11.5	11.5	16.5	130.0	132.0	—	—	—
101.0	—	20.0	12.0	17.5	150.0	158.0	—	—	—
95.8	—	27.2	3.0	12.5	138.5	136.0	25.4	19.0	—
44.5	—	5.0	9.5	19.0	78.0	82.5	—	—	20.4
42.9	—	6.9	16.5	17.6	82.1	91.3	—	—	30.8
33.7	—	12.5	19.6	20.6	86.4	85.0	—	—	20.4
31.4	—	11.1	22.0	20.0	83.5	81.5	—	—	21.2
27.0	—	21.1	30.5	15.5	94.0	95.0	—	—	14.4
28.2	—	9.0	9.7	10.4	56.9	65.0	—	—	15.8
Av. 54.4	—	13.8	14.9	16.6	99.7	102.9	—	—	20.5

TABLE II

Phosphate Content of Spontaneous Mammary Carcinoma of the Mouse
Mg. of P per 100 gm. Tumor Tissue

Orthophosphate	Phosphocreatine	Pyrophosphate	Insoluble Esters	Soluble Esters	Total P (calculated)	Total P (found)	Total Lipid P	Lecithin-Cephaline P	Calcium
19.1	—	16.3	12.1	37.8	85.3	85.5	47.8	26.6	—
27.9	6.5?	8.1	6.6	21.9	71.0	72.0	55.5	28.9	—
35.4	—	4.0	6.1	23.3	68.8	67.5	50.4	22.9	29.8
50.5	—	8.0	16.0	38.2	114.7	120.0	—	—	65.5
38.5	—	4.0	7.5	29.6	79.6	80.0	—	—	34.4
Av. 37.1	—	10.1	9.6	30.2	87.0	85.0	51.6	27.7	43.2

TABLE III

Phosphate Content of Jensen Rat Sarcoma

Mg. P per 100 gm. Tumor Tissue

Orthophosphate	Phosphocreatine	Pyrophosphate	Insoluble Esters	Soluble Esters	Total P (calculated)	Total P (found)	Total Lipid P	Lecithin-Cephaline P	Calcium
26.2	—	13.4	14.6	14.6	68.7	72.0	71.0	35.2	—
17.1	—	16.1	8.8	28.5	70.5	70.0	62.2	32.2	—
24.3	—	4.9	13.0	21.2	63.4	72.0	68.3	42.5	—
28.2	—	4.6	9.1	15.3	57.2	64.0	74.0	53.0	14.8
32.8	—	4.3	15.6	17.3	70.0	66.4	—	—	25.6
26.2	—	14.4	19.9	12.3	72.8	69.5	31.0	22.0	15.9
26.0	—	7.4	7.7	19.1	60.2	65.6	73.9	60.0	16.7
31.6	—	3.4	7.8	24.3	67.1	71.2	87.0	54.0	34.7
Av. 26.5	—	8.5	12.1	19.7	67.1	68.8	66.6	48.9	23.5

TABLE IV

Phosphate Content of Various Normal Tissues of the Rat

Mg. of P per 100 gm. Tissue

	Orthophosphate	Phosphocreatine	Pyrophosphate	Insoluble Esters	Soluble Esters	Total P (calculated)	Total P (found)	Total Lipid P	Lecithin-Cephaline P	Calcium
Kidney	36.2	—	4.4	18.4	31.8	90.8	98.5	96.5	53.0	19.5
	31.7	—	5.1	10.2	20.9	67.9	74.5	120.0	52.4	13.4
	26.8	—	7.0	10.7	29.8	74.3	83.0	68.7	53.5	14.1
Av.	31.5	—	5.5	13.1	27.5	77.6	85.0	95.0	52.9	14.3
Liver	18.8	10.0	3.8	9.8	45.0	87.4	94.0	86.5	57.0	4.5
	14.6	13.9	5.1	10.5	45.6	89.7	94.0	87.5	55.2	1.0
	23.5	13.1	6.7	5.6	52.3	101.2	108.2	87.2	62.0	1.0
Av.	18.9	12.3	5.2	8.6	47.6	92.6	98.7	87.0	58.0	2.1
Muscle (gastrocnemii)	31.4	42.0	54.7	17.9	15.2	161.2	165.0	—	—	—
	30.2	37.0	34.9	8.6	13.3	124.0	126.0	—	—	—
	30.0	42.0	30.6	14.4	12.8	129.6	132.0	—	—	—
Av.	30.5	40.3	40.0	13.6	13.7	138.1	141.0	—	—	—
Skin (new born rats)	40.2	—	1.8	9.5	13.5	65.0	71.6	39.2	28.6	43.3
	39.6	—	6.8	7.4	15.3	69.1	66.0	41.0	27.7	40.6
	24.5	—	5.7	7.8	12.2	50.2	51.6	41.5	29.8	25.7
Av.	34.7	—	4.7	8.2	13.6	61.2	63.1	40.5	28.7	36.5

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