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STUDIES ON THE METABOLISM OF PLASMA PROTEINS IN TUMOR-BEARING RATS‡

INTRODUCTION

The "autonomy" of neoplastic tissue is intimately related to its ability to carry out net synthesis of protein regardless of the demands of the host. Clinically, this is manifest in the failure of the tumor to exhibit the wasting of other tissues of the cachectic host. Accordingly, Mider and others[13] have referred to tumors as "nitrogen traps." In experiments of LePage *et al.*[12] on this subject, the weight of most nontumor tissues of fasted tumor-bearing rates diminished to 30-50% of values for control tissues. On the other hand, the increase in weight of tumors was not significantly different in fasted animals and those fed *ad libitum*. Moreover, the uptake of C^{14} in proteins of the tumors was equal in rate and quantity in fed and fasted tumor-bearing rats following injection of glycine-2-C^{14}.

Among the possible explanations for these clinical and laboratory findings, two have been investigated recently, namely, the possibility that tumors might have a peculiarly great avidity for amino acids of the blood and the possibility that tumors might take up plasma proteins directly. Initial evidence that tumors exhibited a greater uptake of amino acids came from the studies of Zamecnik *et al.*[23] who reported that hepatoma slices incorporated isotope of labeled alanine into protein at a rate eight times that of slices of normal liver or liver of the hepatoma-bearing rat. These findings have been confirmed by Winnick *et al.*,[22] but they are in sharp contrast to the results obtained after injection of labeled amino acids into tumor-bearing rats. In 1944 Shemin and Rittenberg[17] found that uptake of N^{15}-labeled amino acids into protein *in vivo* was not greater in tumors than in other tissues and their findings were subsequently confirmed for a variety of tumors by Zamecnik *et al.*,[24] Norberg and Greenberg,[14] and others; the

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‡ This investigation was supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research and an Institutional Grant from the American Cancer Society.
Received for publication January 21, 1955.

present studies provide further confirmation of the results of Shemin and Rittenberg.

Utilization of plasma protein as a source of amino acids was demonstrated by Whipple and Madden who infused plasma into fasted dogs and maintained their nitrogen balance.[21] In the normal animal, the rate of turnover of plasma proteins is rapid, as is indicated by the turnover time of 8.2 days for albumin in the rat.[19] While these findings have indicated that plasma proteins are utilized by nontumor tissues, evidence has only recently been presented for concentration of plasma protein in tumors. Korngold and Pressman[11] reported localization of antibodies labeled with I^{131} in the Murphy-Sturm lymphosarcoma and Wagner[20] has found that virus in tumors is destroyed as the antibody level of the host rises. Babson and Winnick[1] noted a greater uptake of isotope of biosynthetically-labeled plasma proteins by tumors than by liver and kidney. They found that the relative specific activities of two labeled amino acids was the same in plasma proteins and proteins of tumors and, further, the labeling of tumor protein was not affected by injection of large quantities of non-radioactive amino acids.

Our interest in this subject has developed from two lines of investigation. In studies on the metabolism of acetate-1-C^{14} and pyruvate-2-C^{14} in tissues of tumor-bearing rats, it was found that nontumor tissues rapidly transferred the isotope to amino acids. In experiments with acetate-1-C^{14}, the isotope in nontumor tissues was found chiefly in glutamic acid and in experiments with pyruvate-2-C^{14}, the isotope in nontumor tissues was found chiefly in glutamic acid and alanine.[2,3,4] On the other hand, the tumors studied did not metabolize acetate-1-C^{14} and transferred the isotope of pyruvate-2-C^{14} primarily to lactate. These experiments suggested that the tumors might be dependent upon other tissues for their supply of "non-essential amino acids" for protein synthesis.

Another suggestive line of investigation[9] concerned transplantation experiments involving the Shope papilloma. It was found that adult rabbit skin infected *in vitro* with the Shope virus and transplanted to the brains of immune rabbits developed papillomas containing infectious virus. In like manner, the infectivity of the virus remained unchanged in domestic rabbit papillomas reinfected *in vitro,* despite growth in immune animals. In contrast, no evidence of virus could be found in the V2 carcinoma (the malignant tumor derived from the Shope papilloma) after similar treatment although infectious virus could be recovered after growth in normal nonimmune rabbits. These results suggest that antibody failed to penetrate the cells of normal skin or of benign papilloma but did penetrate the cells of

the carcinoma and indicate that the transformation to cancer was associated with an increased permeability of cell walls.

The experimental questions which arose from these studies were: (i) could tumors take up plasma protein to a greater extent than other tissues, and (ii) could the tumors utilize these proteins for growth?

MATERIALS AND METHODS

The animals used in these experiments were male Sprague-Dawley rats weighing 150-200 grams. They were obtained from the Charles River Laboratories in Boston, Massachusetts, and were fed *ad libitum* on Purina Laboratory Chow. The Walker 256 carcinosarcoma and Jensen sarcoma used in these experiments were transplanted by Mrs. Rose K. Busch. The tumors were transplanted 7-9 days prior to the experiment and weighed 3-5 grams in the aggregates of 2-4 sites of transplantation.

Glycine-2-C^{14} and DL-lysine-2-C^{14}, with specific activities of 19 microcuries per mg. and 3.3 microcuries per mg. respectively, were obtained from Tracerlab, Inc. In studies on the uptake of radioactive amino acids, 0.5 mg. of labeled glycine or 1 mg. of labeled lysine* was injected intraperitoneally into tumor-bearing rats. For preparation of the radioactive plasma, 2 mg. of labeled glycine or 10 mg. of labeled lysine were injected intraperitoneally into normal rats weighing 250-350 grams. At the end of the desired interval, generally three hours, the rats were anesthetized with nembutal (0.5 mg./100 grams body weight) and exsanguinated by arterial puncture at the bifurcation of the aorta by means of a 20 ml. syringe and a 19-gauge needle; 0.25 ml. of heparin (5000 U./ml.) were added to the syringe. The heparinized whole blood was centrifuged at 1500 r.p.m. for 10 minutes and the supernatant plasma was dialyzed for a minimum of two hours against a buffer of 0.1 M glycine or DL-lysine and 0.01 M sodium phosphate buffer at pH 7.4. An average of 375,000 c.p.m. (counts per minute) was found in 5 ml. of plasma and the average specific activity of the plasma protein was 1100 c.p.m./mg. At three hours after intravenous injection of 3-5 ml. of the radioactive plasma into tumor-bearing rats, the average specific activity of plasma proteins of the recipient rats was 280 c.p.m./mg.

At the selected time, the tumor-bearing rats receiving the radioactive tracer were anesthetized with nembutal and exsanguinated as noted above. The tissues were excised and placed in liquid air[6] except in experiments in which the cellular particulates were isolated by differential centrifugation. Following dispersion of the tissues in perchloric acid, the precipitated proteins were treated with 5% trichloroacetic acid, 95% ethanol, ethyl ether, chloroform-methanol (2:1) twice,[18] petroleum ether-acetone (1:1), and butanol. The suspension of protein in butanol was placed on an alundum disk in a separated glass funnel on a filter flask connected to a vacuum line; suction was applied until the plate was dry. Radioactivity was determined in a preflush flow counter.

In experiments involving differential centrifugation of the homogenates, the excised tissues were placed in ice-cold saline and then dispersed in 0.3 M sucrose. The procedures used in preparation of nuclear and mitochondrial fractions were essentially

* The authors are indebted to Dr. D. M. Bonner for initial samples of these amino acids.

those of Schneider[16] and were carried out in a model PR-2 International refrigerated centrifuge. The microsomal fraction was prepared by centrifugation of the sample in a Spinco preparative centrifuge in an average gravitational field of 60,000 g for one hour.[10]*

RESULTS

Figure 1 presents the rates of labeling of proteins of liver, Walker 256 carcinosarcoma, kidney, Jensen sarcoma, and skeletal muscle following injection of glycine-2-C^{14}. These data are in agreement with those of previous reports[14, 17, 24] which have indicated that uptake of isotope of amino acids

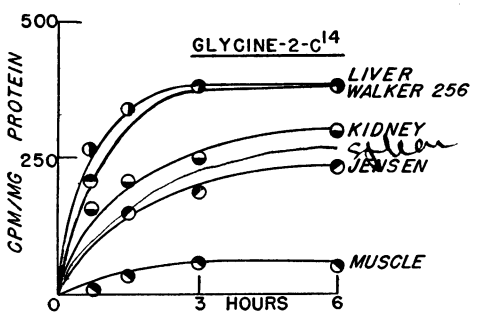

FIG. 1.

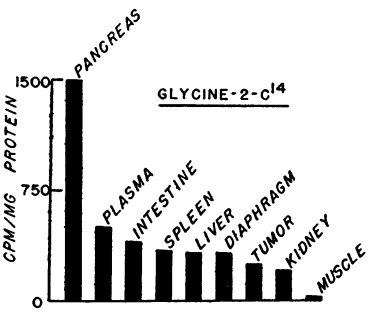

FIG. 2.

FIG. 1. Rates of labeling of tissue protein following injection of glycine-2-C^{14}. Data are averages of two to four experiments carried out at each time point.

FIG. 2. Specific activities of protein of tissues three hours after intraperitoneal injection of glycine-2-C^{14}. Values for the Jensen sarcoma and Walker 256 carcinosarcoma are pooled. Specific activities of protein of heart, testis, thymus, and brain ranged between those of muscle and kidney.

by protein of tumors is not significantly different from that of nontumor tissues. While the increase in specific activity of protein of the rapidly growing Walker 256 carcinosarcoma was equal to that of liver, values for protein of the Jensen sarcoma, a more slowly growing tumor, were lower than those of liver or kidney. The curves reached a plateau in the 3-6-hour interval. The specific activity of proteins of skeletal muscle was very low throughout the study. Figure 2 presents specific activities of protein of various tissues three hours after injection of glycine-2-C^{14}. It is apparent that the values for the tumors are within the range for those of other tissues. The specific activities of protein of the spleen, plasma, whole intestine and pancreas were all significantly greater than those of the

* We are indebted to Drs. D. M. Bonner and J. H. Milstone for the use of their Spinco ultracentrifuges.

tumors at this time. It is of interest that Friedberg *et al.*[8] have shown that values for intestinal mucosa were much higher than those of kidney, liver, or plasma, and slightly higher than pancreas. The specific activities of proteins of the heart, testis, thymus, and brain were determined at three hours after injection of the labeled glycine and these values ranged between those of muscle and kidney (Fig. 2). In studies with DL-lysine-2-C^{14}, essentially the same findings were obtained three hours after injection of the tracer. The specific activity of protein of the Walker 256 carcinosarcoma approximated that of liver or spleen (Fig. 1), while the specific activity of protein

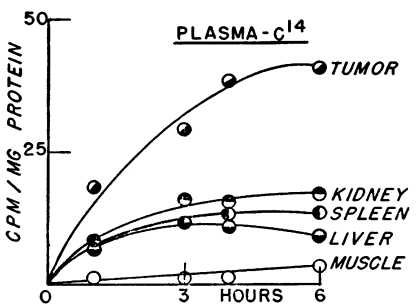

FIG. 3.

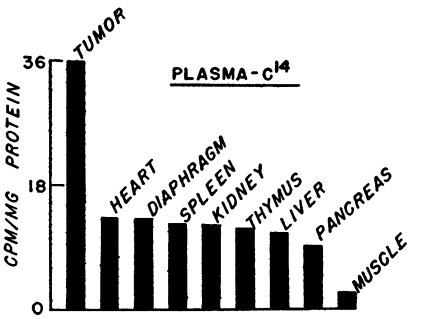

FIG. 4.

FIG. 3. Rates of labeling of tissue protein following injection of labeled plasma. Values for the Jensen sarcoma and Walker 256 carcinosarcoma were pooled inasmuch as significant differences were not found. The specific activity of the plasma proteins of the recipient rats varied from 180-330 c.p.m./mg. with an average value of 280 c.p.m./mg.

FIG. 4. Specific activity of protein of tissues three hours after injection of plasma labeled with glycine-2-C^{14}. Values for Walker 256 carcinosarcoma and Jensen sarcoma are pooled. Specific activities of protein of intestine, brain, testis, and lung ranged from 1.7-8.2 c.p.m./mg. and fall between those of muscle and pancreas.

of the pancreas was three to four times that of protein of the spleen (Fig. 2). Values for lung, kidney, and muscle decreased in the same order as with glycine-2-C^{14}.

PLASMA PROTEIN

The rates of uptake of isotope of plasma protein by protein of tissues is indicated in Figure 3. The specific activity of protein of the tumors increased steadily in the first four hours after injection of the labeled plasma; no significant differences were found in the data for the Jensen sarcoma and the Walker 256 carcinosarcoma. The rate of increase of the specific activity of protein of the tumors was 2½-10 times that of other tissues (Fig. 3).

The curves for kidney and spleen reached a plateau in the 3-6-hour period, while that for liver began to decline after the third hour. The specific activity of protein of skeletal muscle was very low at each time point studied. At three hours after injection of the labeled protein, values for the specific activity of protein of the tumor were 35-41 c.p.m./mg. (Fig. 4) compared with a range of 11-14 c.p.m./mg. for heart, diaphragm, spleen, kidney, thymus, and liver. Values for intestine, brain, testis, and lung ranged from 1.7-8.2 c.p.m./mg. and would appear in Figure 4 between pancreas and

TABLE 1. LABELING OF INTRACELLULAR PARTICULATES FOLLOWING INJECTION OF LABELED PLASMA

Values are c.p.m./mg. protein of the particulates and supernatant solutions. Methods of preparation of microsomal and mitochondrial fractions are in the text. Each value in the table is the average of four experiments, two with glycine-2-C^{14} labeled plasma and two with DL-lysine-2-C^{14} labeled plasma. Values are pooled inasmuch as no significant differences were found for the experiments with the two labeled amino acids. Ranges are presented in parentheses.

Tissue	*Whole homogenate*	*Mitochondrial fraction*	*Microsomal fraction*	*Supernatant fraction*
Walker 256 carcinosarcoma	32.4 (21.8-43.6)	26 (20.2-36.9)	29.5 (18.4-46.0)	54 (22.4-77.0)
Liver	11.2 (10.0-11.8)	11.5 (7.4-17.6)	10.5 (9.3-11.5)	25 (8.7-49.2)
Kidney	13.1 (10.5-14.7)	8.6 (6.4-9.7)	9.1 (6.8-10.2)	41 (14.1-39.7)
Lung	58.8 (27.4-95)	26 (9.2-45)	11.1 (6.7-18)	99 (52.4-146)

muscle. The specific activities of protein of the lung varied markedly, i.e., from 11 c.p.m./mg. to 75 c.p.m./mg., and were occasionally highest of the tissues studied. This variation may be due to the relatively large amount of blood or plasma pooled in the very small lung tissue mass or the uptake of protein aggregates by lung. In agreement with the values obtained for plasma labeled with glycine-2-C^{14}, the specific activity of protein of the Walker 256 carcinosarcoma was two to three times that of liver or kidney, three hours after injection of plasma protein labeled with DL-lysine-2-C^{14}.[1]

LABELING OF CELLULAR PARTICULATES *IN VIVO*

The possibility that isotope of plasma protein entered the protein of intracellular particulates was investigated by determination of the specific activity of proteins of these particulates after tissue homogenates had been

subjected to differential centrifugation.[10, 16] Table 1 presents the specific activities of proteins of cellular particulates of Walker 256 carcinosarcoma, kidney, liver, and lung. Values for the nuclear fractions are not presented inasmuch as they closely reflected values of the whole homogenates; in these experiments no special precautions were taken for purification of the nuclei. The specific activities of proteins of the mitochondrial, microsomal, and supernatant fractions were sufficiently similar in experiments with either labeled glycine or lysine that the data were pooled in Table 1. The

TABLE 2. LABELING OF INTRACELLULAR PARTICULATES BY INCUBATION WITH LABELED PLASMA

In these experiments, 0.5 and 1.0 ml. of labeled plasma were incubated with homogenates of tissues over a range of times with no significant changes in the labeling. Values presented are specific activities of proteins of particulates and supernatant solutions.

Tissue	*Ml. labeled plasma*	*Whole homogenate*	*Mitochondrial fraction*	*Microsomal fraction*	*Supernatant fraction*
Tumor	0.5	106	39	70	350
	1.0	264	78	154	508
Liver	0.5	66	38	37	166
	1.0	161	82	103	340
Kidney	0.5	210	49	59	818
	1.0	345	90	103	...
Lung	0.5	390	119	43	930
	1.0	660	95	207	880

data for mitochondria reflect the same trends as those for microsomes with the exception that the average value for lung was equal to that for tumor, although the variation was very great. The control experiments for these studies are presented in Table 2. Inasmuch as the labeling of protein of the particulates could have occurred by adherence of plasma proteins to mitochondrial and microsomal preparations or by precipitation of particles, such as small clumps of fibrin, from plasma in the same gravitational field as the cellular particulates, it was necessary to determine the extent of these processes before a conclusion could be reached on the incorporation of plasma proteins or their degradation products into the particulates.* Accordingly, varying amounts of labeled plasma were incubated with homogenates of liver, kidney, lung, and tumor for varying intervals, and

* We are indebted to Dr. G. A. LePage for helpful suggestions on this point.

the specific activity of the cellular particulates was determined as in the *in vivo* experiments (Table 2). From these studies, ratios could be obtained for the relative specific activities of proteins of the particulates and those of the supernatant fraction. In tumors, the ratio of the specific activity of protein of the mitochondrial fraction to that of the supernatant fraction varied from 1 : 7 to 1 : 9, and the corresponding values for the microsomal fraction were 1 : 3.5 to 1 : 5 in the *in vitro* studies. Since the specific activity of protein of the supernatant fraction of the tumor was 54 c.p.m./mg. in the *in vivo* experiments (Table 1), the maximal *in vitro* labeling which could occur if all the isotope were in plasma protein would be 6.7 c.p.m./mg. for protein of the mitochondria and 13.5 c.p.m./mg. for protein of the microsomes. Accordingly, the minimal extent of labeling *in vivo* was 19.3 c.p.m./mg. for protein of tumor mitochondria and 16 c.p.m./mg. for protein of tumor microsomes. For the mitochondria of liver, kidney, and lung, the corresponding values were 5.7, 4.6, and 14.4 c.p.m./mg., respectively. For the microsomes, the corresponding values were 4.8, 4.1, and 3.5 c.p.m./mg. for liver, kidney, and lung, respectively. It would thus appear that labeling of the protein of the mitochondria of tumors was 3.5 and 4.2 times that of liver and kidney, respectively, while the labeling of microsomes of tumor was 3.5 to 4.8 times that of liver, kidney, and lung. These data suggest that the tumor is not only more effective in trapping radioactive plasma, but is also more effective than other tissues in its utilization.

DISCUSSION

These initial experiments indicate a greater labeling of "tumor protein" than proteins of other tissues following injection of radioactive "plasma." It is clear that both "tumor protein" and "plasma" represent complex mixtures of proteins and other compounds. From the studies of Cohn and his co-workers,[6] it is evident that there are many kinds of proteins in plasma and undoubtedly even the number of different antibodies is large. In experiments now in progress, radioactive plasma proteins are being separated by the methods developed by Cohn *et al.*[6] and the isolated fractions are being reinjected into tumor-bearing rats. It remains to be determined whether one or many protein fractions contribute to the labeling of proteins of the tumor. On the other hand, "tumor protein" is a mixture of compounds which share the properties of insolubility in protein precipitants and organic solvents used for extraction of lipids. The nature of the proteins which are the recipients of the isotope of radioactive plasma has not been studied, but it seems possible that only a part of the proteins of the tumor can be synthesized from products of plasma proteins. In this case, an extensive study

of the labeling of individual proteins of the tumor would be justified. Before an understanding of the mechanism of utilization of the plasma proteins can be achieved, specific proteins of tumors will have to be isolated and degraded. These studies do not permit evaluation of the extent of degradation of plasma protein before the fragments are incorporated into tumor protein.

While the possibility exists that the avidity displayed by the tumors for plasma protein reflects a permeability difference between tumors and other tissues,[7] either at the cellular level or at the level of tissue structure, the evidence presented indicates that there is transfer of isotope to cellular particulates *in vivo*. Moreover, it is possible to calculate that 1000 mg. of tumor protein could take up 1030 mg. of plasma protein in 24 hours, an amount sufficient to account for the known doubling in weight of the tumor in this time interval.[12] Thus, in three hours, the specific activity of protein of the tumor was 36 c.p.m./mg. so that one gram of tumor protein would take up 3.6×10^4 c.p.m. in three hours. Since the specific activity of plasma protein of recipient rats averaged 280 c.p.m./mg., 3.6×10^4 c.p.m. represents an uptake of 128 mg. of plasma protein in three hours. If this rate were constant, one gram of tumor protein would take up 1030 mg. of plasma protein in 24 hours. These considerations are based on values for the whole homogenates. Although similar calculations for the uptake of amino acids are difficult because of the unknown size of the pool diluting the radioactive precursor, it is possible to calculate the percentage of isotope of glycine-2-C^{14} or lysine-2-C^{14} injected which enters the tumor protein. Of the 11,000,000 c.p.m. of labeled amino acid injected, 200,000 c.p.m. can be accounted for in 500 mg. of tumor protein (average specific activity equals 400 c.p.m./mg.). This value represents 1.8% of the total amino acid injected. On the other hand, 18,000 c.p.m. of 375,000 c.p.m. in the injected plasma protein were found in the tumor. At three hours, an average of 245,000 c.p.m. remain in the plasma and accordingly the tumor has removed 12.8% of the isotope lost from the plasma.

These experiments may be of significance in three aspects of the study of neoplasia. First, the growth of the tumor in the starving animal and cachectic human may be related to its ability to trap plasma protein and utilize the degradation products for synthesis of tumor protein. Secondly, the ability of the tumor to invade and metastasize may reflect its more favorable nutritional requirements or conversely, the ability of the tumor to take up label of plasma protein may reflect cellular changes important to invasion and metastasis. Finally, the factors involved in concentration of

the plasma protein may be related to differences between benign and malignant tumors in that the former may not effectively take up plasma proteins.

SUMMARY

1. The rate of uptake of isotope of glycine-2-C^{14} and DL-lysine-2-C^{14} by proteins of tumors was in the range for nontumor tissues over the period of 45 minutes to 6 hours following intraperitoneal injection of the isotope.

2. The rate of uptake of isotope of plasma proteins labeled with glycine-2-C^{14} or DL-lysine-2-C^{14} by tumor protein was 2½ to 10 times that of other tissues studied, with the exception of lung which gave variable results.

3. Labeling of microsomal fraction of tumor was 3.5 to 4.8 times greater than that of kidney, liver, and lung three hours after intravenous injection of labeled plasma protein.

4. Labeling of the mitochondrial fraction of tumors was 3.5 to 4.2 times greater than that of kidney and liver respectively three hours after intravenous injection of labeled plasma protein.

5. It can be calculated from the data on labeling of tumor protein that uptake of plasma protein could account for the doubling of the weight of tumors in 24 hours.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Miss Catherine Joan Even.

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