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

## II. Labeling of Intracellular Particulates of Tissues by Radioactive Albumin and Globulin§

Evidence has been accumulating for the selective localization of radioactive plasma proteins in tumors. A number of clinical studies have shown that plasma proteins labeled with $I^{131}$ can be utilized for localization of hepatic metastases as well as primary cerebral neoplasms.[1, 10, 11] Studies on localization of radioactive plasma proteins in animal tumors have been carried out by Babson and Winnick,[1] Bauer *et al.*,[2] Gavrilova,[7] and Busch and Greene.[8] The last studies[8] indicated that the specific activity of tumor proteins increased at a significantly greater rate and to a greater extent than proteins of other tissues following injection of plasma biosynthetically labeled with radioactive amino acids, L-lysine or glycine-$C^{14}$. Several questions arose from these studies which are germane to most of the studies carried out thus far.[1, 2, 3, 5, 6] First, plasma was used as the tracer material and, accordingly, the rôle of the specific plasma proteins in labeling tumor proteins was not determined. Secondly, there was no assurance that the labeling of the tumor proteins was not due to a non-dialyzable constituent of plasma other than plasma proteins. Finally, the values for the specific activity of the proteins of the lung varied markedly from considerably lower to considerably greater than the corresponding values for the tumor; the possibility was suggested that the lung might be concentrating denatured proteins or protein aggregates. In the present studies, some of these questions were approached by fractionation of the radioactive plasma with the techniques of Cohn *et al.*,[7]; the uptake of isotope was then determined in intracellular particulate fractions prepared by differential centrifugation of homogenates of tissues. The most significant difference found between the Walker 256

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carcinosarcoma and other tissues of the tumor-bearing rat was the markedly greater uptake of the isotope of labeled albumin by the microsomal fraction of the Walker tumor.

## MATERIALS AND METHODS

The tracer amino acid utilized in these studies was L-lysine-U-$C^{14}$ obtained from the Nuclear Instruments and Chemicals Company; the specific activity was 9.1 millicuries per millimole. The donor rat received 30 microcuries of L-lysine-U-$C^{14}$ and was exsanguinated four to six hours later. The radioactive plasma was separated from the red cells by centrifugation and was immediately separated into three fractions by the method of Cohn *et al.*[7] The first of these fractions, the proteins of fractions I, II, and III of Cohn *et al.*, contains the bulk of the globulins of the plasma and will be designated as the globulin fraction in this paper. The second fraction, comprising the proteins designated as fractions IV and V by Cohn *et al.*,[7] contains the plasma albumin and will be referred to as the albumin fraction in this paper. Fraction VI of Cohn *et al.*[7] contained so little protein and radioactivity that it was not reinjected into tumor-bearing rats. Following the fractionation, the proteins were dissolved in 2-5 ml. of saline solution and dialyzed overnight against a phosphate buffer containing one gram of nonradioactive L-lysine per liter. With the exception of the experiments of long duration, i.e., 24 and 48 hours, the experiments were terminated three hours after intravenous injection of the radioactive proteins. Differential centrifugation of the sucrose homogenates of tissues was carried out as previously described.[8] In each case, the isotope in the proteins of the plasma and the tissue was determined by an improved plating technique.[4] The protein was first precipitated with 0.6 *N* $HClO_4$ and then was homogenized in turn with 1.5 *N* trichloracetic acid, 95% ethanol, 100% ethanol, 2:1 chloroform:methanol,[7] benzene, and finally ethyl ether in a ratio of 5-10 ml. of each solution per gram of tissue. After each addition, the sample was dispersed in the solvent, and then centrifuged in a field of 2,000 times gravity; the supernatant solvent was discarded.

For complete sedimentation of the protein, three to five minutes of centrifugation were required when the sample was treated with benzene and ethyl ether and five to eight minutes were required after dispersion in aqueous media or ethanol. Following extraction with ethyl ether, the samples were permitted to dry at room temperature overnight. For more rapid drying, the mouths of the homogenizer tubes were covered with a single thickness of filter paper and the tubes placed in a polyethylene container in a desiccator which was evacuated with a water pump. Evaporation was promoted by an infra-red lamp one and one-half feet over the desiccator, and was generally complete in one to two hours after desiccation was initiated. The resulting precipitate was either a fine white powder or a hard white pellet which could be easily broken into a fine white powder with the aid of a metal spatula. The apparatus consisted of a stainless steel pestle (Fig. 1), designed to fit snugly into the well of a stainless steel planchet which was essentially a small mortar (Fig. 1). The over-all diameter of the planchet and pestle was one inch. For large samples, i.e., 15 mg. or greater, the diameter of the well of the planchet was ¾ inch, and the width of the raised edge was ⅛ inch. For plating of smaller samples, a similar apparatus was made; in this case, the diameter of the well of the planchet as well as the corresponding end of the pestle

was ⅜ inch. For complete adherence of the sample, the planchet with the larger well required a sample of at least 15 mg., while the planchet with the smaller well required a sample of at least one to two mg. The powdered protein sample was evenly spread over the well of the planchet, the pestle was gently placed on the powder, and the apparatus was placed in a vise for compression of the sample. Striking the pestle several sharp blows with a hammer was also satisfactory for compression of the

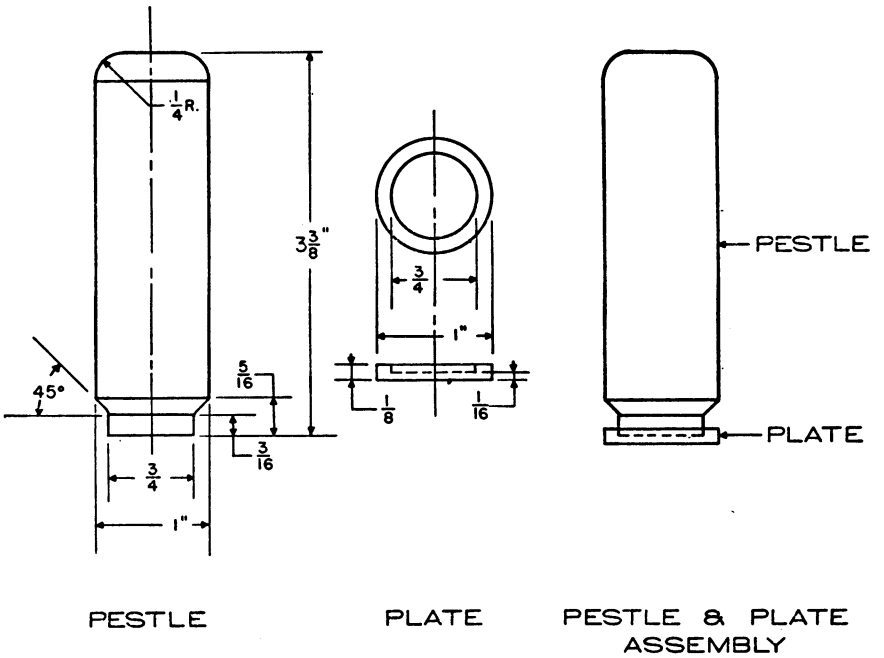


FIG. 1. Diagram of apparatus used for compression of powdered tissue proteins. The stainless steel planchet (plate) is essentially a small mortar into which the stainless steel pestle can be snugly fitted. The powdered protein is compressed in the mortar and the result is a plated sample with a smooth surface.

sample, but inasmuch as a small spray of radioactive protein was occasionally observed, the sample was prepared in a hood under a plastic "tent."

The resulting plate contained the sample in a compressed form with a smooth surface. Any particles on the edges could be brushed away without disturbing the surface. The plated sample was stable in shape, form, and color for at least two days. Following the use of these samples, no contamination of the flow counters was noted. In the calculations of the data, variations in the specific activity of the plasma proteins of the receptor rat were encountered. The data are corrected to a specific activity of plasma protein of 100 cpm./mg. to eliminate discrepancies between individual experiments and between the albumin and globulin experiments which otherwise would have been difficult to compare.

## RESULTS

### UPTAKE OF PLASMA PROTEINS

*Whole Homogenates*

Figure 2 presents the specific activities of the proteins of the whole homogenates of tissues of animals injected with the globulin or albumin fractions. In both cases, the specific activity of the protein of the tumor was greater than that of the other tissues studied. Moreover, the values for the specific

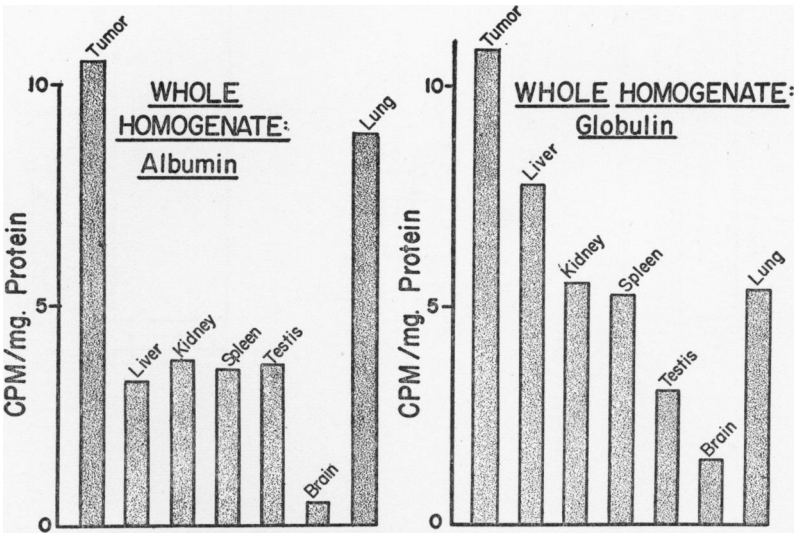


FIG. 2. Specific activities of the proteins of the whole sucrose homogenate of tissues following injection of either albumin or globulin labeled with L-lysine-U-$C^{14}$. Each bar is the average value for three experiments for tumor, lung, liver and kidney, two for spleen and one for testis. Each experiment was terminated three hours after the radioactive plasma protein was injected. The values are corrected to a plasma protein specific activity of 100 cpm./mg. in the receptor rat.

activities of the tumor proteins were approximately the same following injection of each type of plasma protein. In previous studies,[8] the values for the specific activities of proteins of the whole homogenates of the lung were closer to those of the tumor than were the corresponding values for other tissues. In the albumin-treated animals this result was obtained again. The specific activity of the protein of the tumor of the albumin-treated animals was approximately three times that of the testis, liver, kidney, and spleen and ten times that of the brain. In animals injected with radioactive globulin, the specific activity of the protein of the tumor was not so markedly different from that of the other tissues. Although the specific activities of

the proteins of the whole homogenates of the kidney and spleen were significantly greater in the animals injected with the radioactive globulin, the specific activity of proteins of the liver of globulin-treated rats was two to three times that of the corresponding proteins of albumin-treated rats.

The apparent lack of the uptake of albumin by the liver may reflect a dilution of the radioactive albumin with albumin being formed in the liver.* The values for lung in the globulin series were approximately one-half those

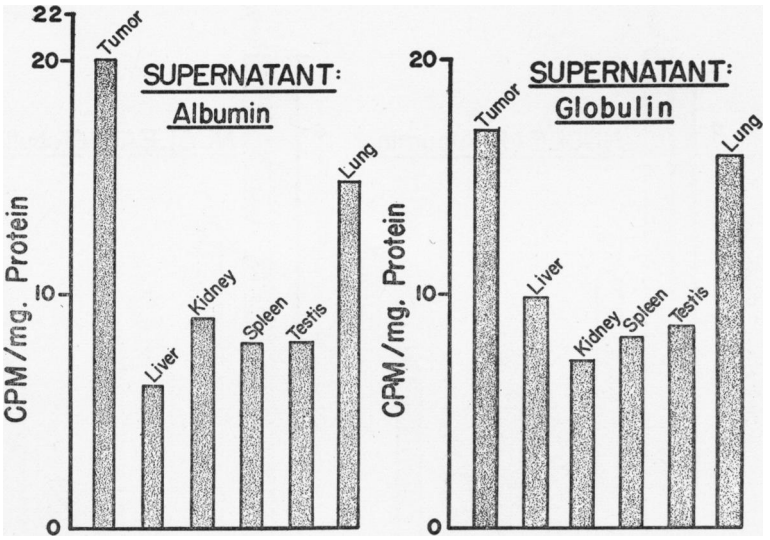


FIG. 3. Specific activities of the proteins of the supernatant fractions of tissues of tumor-bearing rats three hours after injection of either albumin or globulin labeled with L-lysine-U-$C^{14}$.

in the albumin series. It was of particular interest that the wide variations in the specific activities of lung proteins found with the whole plasma proteins were not obtained in these experiments with purified plasma proteins.

*Supernatant Fraction*

The specific activities of the proteins of the supernatant fractions are presented in Figure 3. Although these values are uniformly higher than those of the whole homogenates, they mirror the values for the whole homogenates to a considerable extent. Based on the values for the whole plasma

* If the albumin formed by the liver were emerging from the cells at the same sites at which the radioactive plasma albumin was being absorbed, the labeled plasma albumin would be locally diluted by the newly formed albumin.

proteins of 100, it can be noted that the value for the specific activity of proteins of the tumor of the albumin-treated animals was one-fifth for the circulating plasma proteins. In view of the very pale appearance of the tumor and the small blood vessels which supply its needs, this value suggests that the tumors concentrate plasma proteins more effectively than other tissues. The values for the specific activity of the protein of the su-

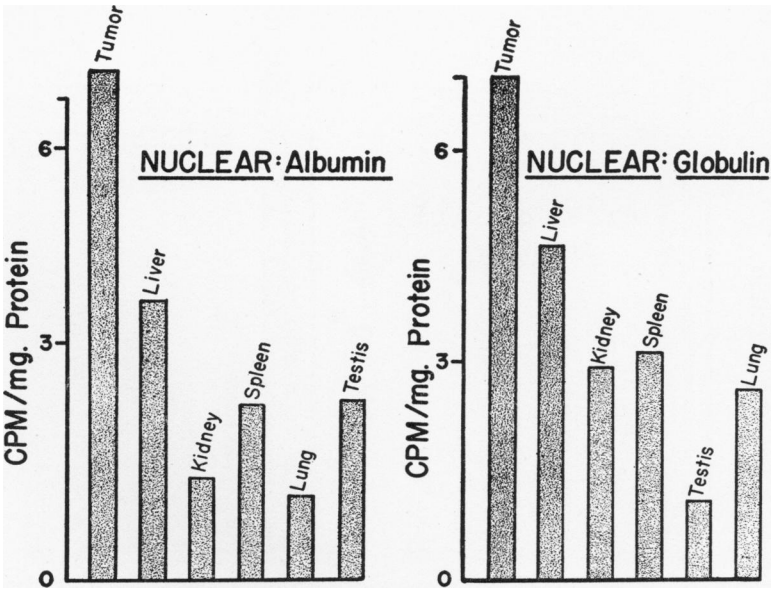


FIG. 4. Specific activities of the proteins of the nuclear fractions of tissues three hours after injection of either albumin or globulin labeled with L-lysine-U-$C^{14}$.

pernatant fractions of lung were significantly higher than those for other non-tumor tissues. The specific activities of the proteins of the supernatant fractions of the livers were proportional to the values for the whole homogenates. The values for the specific activities of the proteins in the supernatant fraction for kidney, spleen, and testis were approximately the same in both the albumin and the globulin experiments.

## LABELING OF INTRACELLULAR PARTICLES

### *Nuclear Fraction*

As these experiments were carried out, the nuclear fraction includes considerable cellular debris as well as cell nuclei. Although the values reflect those of the whole homogenates (Fig. 4), the removal of the supernatant

fractions resulted in lower values in all tissues. In the albumin series (Fig. 4) the value for the specific activity of the proteins of the tumor was twice that for the liver and three to five times the values for other tissues. It is particularly notable that the proteins of the lung exhibited a very low specific activity in the nuclear fraction as well as the other cellular particulates as will be noted below. In the albumin series, the specific activity of the protein of the nuclear fraction of the kidney was also very low. In

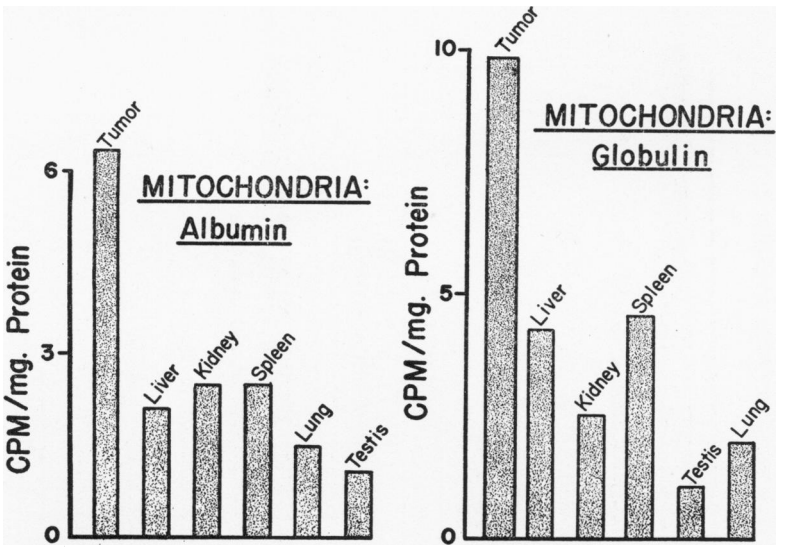


FIG. 5. Specific activities of the proteins of the mitochondrial fraction of tissues three hours after injection of either albumin or globulin labeled with L-lysine-U-$C^{14}$.

the experiments with radioactive globulin the values for the lung were also very much lower than would have been anticipated from the data for the supernatant fraction, but the other values were in the same proportion as those of the whole homogenates.

*Mitochondrial Fraction*

The values for the specific activities of the proteins of the mitochondrial fraction are presented in Figure 5. Following injection of radioactive albumin the specific activity of the proteins of the mitochondrial fraction of the tumor was two and one-half to three times those of the liver, kidney, spleen, and lung. The specific activity of the protein of the mitochondria of the tumor was less in the albumin than in the globulin experiment. This result was also found for liver and spleen. In the globulin experiment the

specific activity of the protein of the mitochondrial fraction of the tumor was also significantly greater than that of the non-tumor tissues; inasmuch as the values for both liver and spleen were greater than those of the albumin series, the ratio was only two for the specific activities of the proteins of the tumor mitochondria to the corresponding values for the liver and spleen.

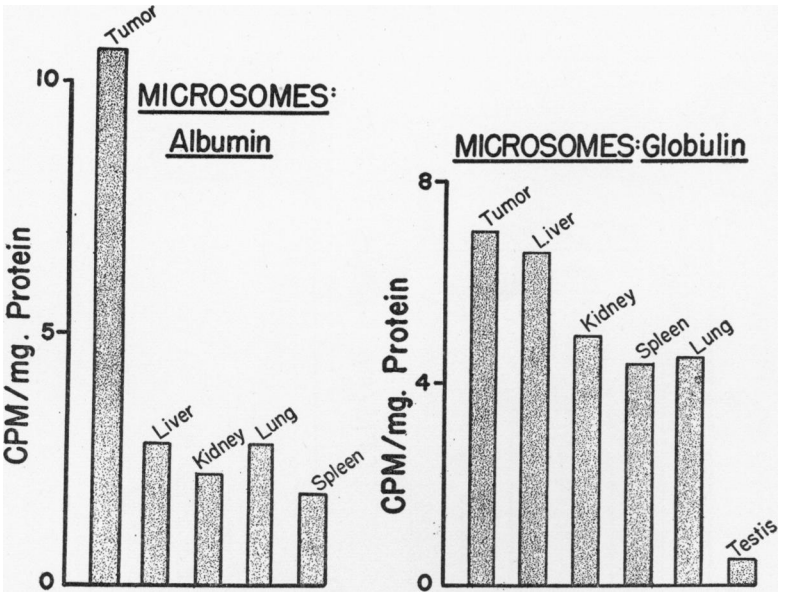


FIG. 6. Specific activities of the proteins of the microsomal fractions of tumors and other tissues three hours after intravenous injection of either albumin or globulin labeled with L-lysine-U-$C^{14}$.

*Microsomal Fraction*

The most significant difference in the labeling of cellular particles was found in the microsomal fraction (Fig. 6) of the albumin series. In this series, the specific activity of the proteins of the tumors was uniformly four to six times greater than those of the other tissues studied.

The value for the lung was approximately that found in the other tissues, despite the high specific activity of the protein in both the whole homogenate and the supernatant fraction of the lung. The specific activity of the proteins of the microsomal fraction of the tumor was approximately that of the whole homogenate of the tumor, while the values for liver, kidney, lung, and spleen were one-third to two-thirds those of whole homogenate. The specific activities of the proteins of microsomal fractions of the globulin

series were significantly greater for most tissues than were the corresponding specific activities in the albumin series. These values ranged from 5-7 cpm./mg. protein and although the values for the liver and tumor were greater than those for kidney, spleen, and lung, the significant differences in the values for the individual tissues noted in the albumin series were not apparent. In the albumin series the values for the testis were too low to record on the graph.

TABLE 1. SPECIFIC ACTIVITIES OF PROTEINS OF TUMORS AND OTHER TISSUES OF RATS BEARING JENSEN OR WALKER TUMOR AT TIME PERIODS OF 24 AND 48 HOURS AFTER INTRAVENOUS INJECTION OF DIALYZED PLASMA LABELED WITH L-LYSINE-U-$C^{14}$. VALUES ARE IN CPM./MG. PROTEIN.

| | *24 Hours* | | *48 Hours* | |
|---|---|---|---|---|
| | *Walker* | *Jensen* | *Walker* | *Jensen* |
| Kidney | 6.2 | 5.6 | 11 | 9.6 |
| Liver | 7.1 | 6.2 | 13 | 10.7 |
| Spleen | 12.3 | 10.0 | 17 | 9.4 |
| Lung | 12.3 | 4.5 | .. | 20.3 |
| Walker tumor | 20.2 | | 27 | ... |
| Jensen tumor | | 16.7 | | 22.1 |
| Plasma | 113.0 | 58.3 | 70 | 100 |

#### DURATION OF THE UPTAKE OF THE RADIOACTIVE PROTEINS

The experiments of Table 1 were designed to test the duration of the difference in the specific activities of the proteins of the tissues containing the proteins of highest specific activity and the proteins of the Walker 256 and Jensen tumors. The specific activities of the proteins of the tumors were consistently greater than those of liver, kidney, spleen, and lung for a period 48 hours after the radioactive plasma was injected into the tumor-bearing rat. However, the specific activity of the protein of the spleen as well as liver and kidney increases with time. From the standpoint of the total radioactive protein taken up, the tumor would appear to be taking up far greater quantities of the radioactive plasma proteins than the other tissues during the 48-hour time period studied, inasmuch as it doubles in size daily at the times of these experiments.

#### IN VITRO LABELING OF CELLULAR PARTICULATES

The experiments of Table 2 were designed to study the possibility that factors in the supernatant fraction of the tumor might selectively precipitate plasma proteins or that the blood or plasma remaining in the tissue might

influence the labeling of the particles. The data of the albumin series indicate that very little labeling of the microsomal fractions of any of the tissues occurred *in vitro* even with a concentration of isotope in the supernatant fractions two to ten times that of the *in vivo* experiments. Similar results were obtained for the nuclear fractions and the mitochondrial fractions of tumor, liver, and kidney. On the other hand, in the globulin series, appreciable labeling of the particles was noted, but the differences between

TABLE 2. LABELING OF INTRACELLULAR PARTICLES "IN VITRO"
Tissues of tumor-bearing animals were homogenized and subjected to differential centrifugation as noted in the text. After the initial homogenization in sucrose, an aliquot of radioactive albumin or globulin was added to each homogenate. The radioactivity in the particles and whole homogenate was determined as noted in the ext. The values which are mostly averages for two experiments are presented in cpm./mg. protein.

| | *Whole homogenate* | *Nuclear fraction* | *Mitochondrial fraction* | *Microsomal fraction* | *Supernatant fraction* |
|---|---|---|---|---|---|
| | | | *Albumin series* | | |
| Tumor | 20.5 | 0.4 | 0.3 | 1.6 | 51.3 |
| Liver | 22.5 | 0.2 | 0.4 | 1.2 | 31.0 |
| Kidney | 13.3 | 1.9 | 0.7 | 1.2 | 36.2 |
| Spleen | 19.2 | 0.5 | 3.3 | 1.4 | 57.2 |
| Lung | 21.1 | 0.5 | 3.5 | 0.9 | 62.5 |
| | | | *Globulin series* | | |
| Tumor | 19.1 | 8.0 | 10.5 | 27.7 | 46.5 |
| Liver | 18.2 | 6.1 | 13.9 | 17.5 | 31.3 |
| Kidney | 16.2 | 6.7 | 9.7 | 15.6 | 28.7 |
| Spleen | 23.7 | 6.7 | 8.9 | 15.2 | 33.3 |
| Lung | 12.9 | 8.2 | 6.1 | 13.7 | 30.7 |

tumors and other tissues were not very great. Only the microsomal fraction of the tumor was more extensively labeled than that of the other tissues and this may be related to the higher isotope concentration in the supernatant fraction of the tumor.

## DISCUSSION

The concentration of plasma proteins by tumors is approached only by the lung, as indicated by the data for the specific activities of the protein of the whole homogenate and supernatant fractions of the tissues. However, the tumor of the albumin-treated animal exhibits a significantly greater

labeling of the proteins of the microsomes than other tissues, including the lung. The same effect, to a lesser degree, was noted in the labeling of the proteins of the mitochondrial fraction. Inasmuch as the lung has a smaller mass of microsomes and mitochondria than the tumor, these data indicate that the labeling of the microsomal fraction is not due simply to simultaneous sedimentation of contaminants of the albumin fraction along with tumor microsomes or mitochondria, for in that case the values for the lung should have been consistently greater than those for the tumor. It should be noted that although these data suggest that fractions of plasma proteins and particularly albumin, are selectively incorporated into microsomes of the tumors, the considerations noted previously[8] with regard to the complexity of the proteins of the tumor cells also apply to the complexity of the proteins of the microsomal fraction which is yet undefined. It is apparent from the lack of specificity of the methods for obtaining these particles that the possibility exists that the tumors may have a higher concentration of a selected particle. The recent studies of Littlefield *et al.* have furnished additional evidence which suggests that the microsomal fraction may be a site of synthesis of protein.[9] The possibility exists that greater labeling of the tumor microsomes in these experiments is related to their findings. It does not seem likely that free lysine liberated in other tissues from the plasma protein can account for the selective labeling of tumors and their particles of previous data[8] on the uptake of radioactive amino acids by tumors and other tissues. These data do not provide information on the state of the lysine when incorporated into proteins of the tumor particles.

Although these data are related to the problems of cachexia and differentiation of benign and malignant tumors as already noted,[8] these studies suggest the possibility of chemotherapeutic approaches to tumors via large protein molecules or toxic compounds of similar structure mimicking or linked to proteins. If the plasma proteins are partially incorporated into the endoplasmic reticulum,[10] it would seem possible either to link radioactive atoms to albumin molecules or to incorporate amino acid analogues such as ethionine into these molecules. If such molecules were selectively taken up by tumor microsomes, they might interfere with synthesis of new proteins. While these considerations are very early ones, they represent an approach to the problem of cancer chemotherapy which has not been extensively investigated.

## SUMMARY

1. The labeling of the proteins of whole homogenates and particulate fractions of Walker 256 carcinosarcomas and other tissues of the rat was

determined following intravenous injection of labeled albumin and globulin fractions.

2. Three hours after injection of labeled albumin the specific activity of the proteins of the whole homogenate of tumors was three times those of liver, kidney, spleen, and testis, but not significantly greater than that of lung. However, the specific activity of the microsomal proteins of the tumor was four to six times that of other tissues including the lung.

3. Three hours after injection of radioactive globulin the specific activity of the whole homogenates of the tumors was 1.5-2.0 times greater than those of the liver, kidney, spleen, and lung. The specific activities of the proteins of the non-tumor tissues were greater in animals treated with radioactive globulin than in those injected with radioactive albumin. In contrast to the results following injection of radioactive albumin, there was little difference in the specific activity of the proteins of the microsomal fractions of the tumor and other tissues.

4. The differences in the specific activity of the proteins of the tumor and other tissues persisted for 48 hours after injection of radioactive plasma.

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