

PROTEIN METABOLISM IN TUMOR CELLS AT VARIOUS STAGES OF GROWTH IN VIVO

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ABSTRACT

Protein metabolism of Yoshida ascites hepatoma cells was studied in the early phase of logarithmic proliferation and in the following stage in which cell mass remains constant (resting phase). The rate of protein synthesis was measured by a short-time incorporation of [^3H]lysine, while degradation was concurrently assessed by following the decrease of specific activity of [^{14}C]lysine-labeled proteins. Most of the labeled amino acid injected intraperitoneally into the animal was immediately available for the tumor cells, with only a minor loss towards the extra-ascitic compartment. It was thus possible to calculate the dilution of the isotope in the ascitic pool of the lysine, which increased concurrently with the ascitic plasma volume. Amino acid transport capacity did not change in the log vs. the resting cells. This fact permitted the correction of the specific activity of the proteins synthesized by tumors in the two phases, taking into account the dilution effect. Protein synthesis was found to proceed at a constant rate throughout each of the two phases, although it was 30% lower during the resting as compared to the log phase. When cell mass attained the steady-state, protein degradation occurred at such a level as to balance the synthesis. Throughout the resting phase the amount of lysine taken up by the cells and renewed from the blood remained unchanged. Protein turnover, as studied in subcellular fractions, exhibited a similar rate in nuclei and microsomes, where it proceeded at a higher level than in mitochondria. On the whole, the results encourage the use of the Yoshida ascites hepatoma as a suitable model for studying protein turnover in relation to cell growth in vivo.

INTRODUCTION

Some in vitro studies indicate that protein synthesis proceeds at similar rates throughout discrete proliferation and resting stages in normal and neoplastic cells (28, 14). On the other hand, significant protein turnover¹ seems to be confined to the resting phase (13, 17-19, 28).

The purpose of this work was an in vivo

¹ The term turnover is used in this paper to indicate the overall process of renewal of cell proteins, including both their synthesis and degradation (see reference 2).

approach to the study of protein metabolism relative to the different stages of growth of Yoshida ascites hepatoma cells. This tumor was considered a suitable model to work with, for the following reasons: (a) the tumor exhibits two well-defined stages of growth, the first being characterized by a logarithmic proliferation, the second by a long period of resting (4); (b) growth may accurately be checked by cell counting; (c) it is possible to follow the modifications of the milieu in which the cells are living.

MATERIALS AND METHODS

Male Wistar rats weighing 220–250 g were used. They were given water and a commercial complete diet (Zoofarm, Padua, Italy) *ad libitum*, up to the times indicated.

Yoshida ascites hepatoma 130 was maintained in the above-mentioned strain of rats. Experimental animals were inoculated intraperitoneally (i.p.) with 3.10^7 cells taken from the peritoneal cavity of a tumor-bearing animal, 7 days after transplantation. At established times, tumor-bearing animals were anesthetized with diethyl-ether and exsanguinated by heart puncture; the ascitic fluid (ascitic plasma plus tumor cells) was then withdrawn by a syringe, and then the peritoneal cavity was opened and thoroughly washed with known volumes of ice-cold 0.25 M sucrose.

Estimation of Tumor and Ascitic

Plasma Volumes

Tumor volume (ascitic plasma + tumor cells) was determined by subtracting the volume of sucrose washings from the total fluid recovered. The volume of the ascitic plasma was obtained by subtracting the cell volume calculated on the basis of the intracellular water (see below) from the tumor volume.

Ascitic and blood plasmas were separated from the cells by centrifugation at 1,000 g for 10 min.

Washing, Homogenization, and Fractionation of Hepatoma Cells

All manipulations were performed in a cold room at 2°C using chilled materials. Tumor cells were washed twice with cold 0.25 M sucrose. Preliminary experiments have shown that a 1% loss of cell proteins is due to the washings under these conditions. Washed cells were resuspended in 4 vol of 0.25 M sucrose. Aliquots of the cell suspensions were used for protein determination (see below). Tumor cell suspensions were homogenized with a Dounce-type stainless-steel homogenizer fitted with a Teflon ball. By centrifugation, the following cell fractions were obtained: (a) *nuclear fraction*, from the homogenate at 500 g for 10 min; (b) *mitochondrial fraction*, from the nuclear supernate at 10,000 g for 15 min; (c) *microsomal fraction*, from the mitochondrial supernate at 100,000 g for 60 min. All fractions were washed twice with ice-cold sucrose.

Intracellular Water

Total water of sedimented cells was estimated by subtracting the dry weight from the wet weight. Water trapped intercellularly was calculated from the ratio between [^3H]inulin radioactivity extracted in trichloroacetic acid (TCA) from the pellet and the radioactivity per milliliter of the supernate of an aliquot of cell suspension. Intracellular water was calculated by sub-

tracting the trapped water from the total water of the sediment.

Amino Acid Transport

Tumors were used on the 4th and 10th days after transplantation, when they reached, respectively, a 30–35 and 80–90 ml volume. For the *in vitro* experiments the cells of three tumors of the same size were harvested and pooled after centrifugation of the ascitic fluid, washed three times with Krebs-Henseleit bicarbonate buffer equilibrated with $\text{O}_2 + \text{CO}_2$ (95:5), and finally resuspended in an appropriate volume of the same buffer with or without the addition of glucose (final concentration 8 mM). The incubation mixture contained 5.10^6 cells/ml.

Initial rates of amino acid uptake were determined by incubating the cells in the presence of 1 μCi of L-[^{14}C]lysine in Krebs-Henseleit bicarbonate buffer at 37°C for 5 min. The final concentration of lysine in the mixture was 0.5 mM. The intracellular accumulation of the tracer amino acid was determined according to Guidotti et al. (11, 12).

In the *in vivo* experiments, 50 μCi of L-[^3H]lysine were injected into the animals. 1 min later, an aliquot of the ascitic fluid was withdrawn and the cells were immediately separated from the ascitic plasma by centrifugation at 500 g for 5 min at 37°C. The intracellular accumulation of the tracer amino acid was calculated from the ratio between the radioactivity per milliliter of the intracellular water and the TCA-soluble radioactivity per milliliter of the ascitic plasma (distribution ratio).

Labeling and Counting of the Cell Proteins

The labeling procedure used for studying protein synthesis and degradation was derived from the technique proposed by Arias et al. (2) for the determination of the turnover of different protein species in the same animal.

In the standard experiment, one isotopic form of an amino acid ([^{14}C]lysine) was administered i.p. to rats bearing Yoshida ascites hepatoma, and the decline of the ^{14}C radioactivity of tumor proteins was followed. At various intervals after the first isotope injection, the animals received i.p. a second administration of the same amino acid in a different isotopic form ([^3H]lysine) and were killed 80 min later. The decrease of ^{14}C radioactivity of tumor proteins was used to estimate protein degradation, while the incorporation of [^3H]lysine into tumor proteins was taken as an index of the rate of protein synthesis.

Labeling experiments were carried out with groups of rats bearing tumors that, unless otherwise indicated, had been transplanted 5 days earlier. At the beginning of the experiments, the rats were given i.p. 2 ml of physiological saline containing 6 μmol (10 μCi) of L-[^{14}C]lysine. Then, at the times indicated, the animals were given a further injection of 2 ml of physiological saline containing 6 μmol (50 μCi) of L-[^3H]lysine. Initial values of protein

radioactivity were obtained from a group of rats that, at the beginning of the experiments, received i.p. 2 ml of physiological saline containing both 10 μ Ci of [14 C]lysine and 50 μ Ci of [3 H]lysine in a total amount of 6 μ mol of the amino acid.

In all cases, the rats were killed 80 min after the administration of the [3 H]lysine. It is at this time that the highest incorporation of isotope into the tumor proteins could be detected. The animals were fasted 12 h before each isotope injection and for the 80 min immediately before killing. Radioactivity was determined on weighed aliquots of proteins precipitated by 5% (wt/vol; final concentration) TCA and purified according to Siekevitz (22). Samples were solubilized with 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) and counted in a toluene-based scintillation mixture (5 g of 2,5-diphenyloxazolone and 300 mg of 2,2'-*p*-phenylen-bis 5-phenyloxazolone in 1 liter of toluene). A Mark 1 liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.) was employed, with a setting for the double label isotope counting, the 3 H window counting 10% of 14 C counts and the 14 C window containing no 3 H counts. Counting efficiencies were 38% for 14 C and 15% for 3 H. Samples had uniform quenching as judged from internal standards.

Assays

Proteins were determined according to the method of Lowry et al. (16). L-lysine concentration in blood and ascitic plasma was estimated after deproteinization with 5% sulfosalicylic acid (3) by the method of Spackman et al. (23), using a Unichrome amino acid analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Specific radioactivity of free lysine (either 14 C or 3 H) was determined according to Cappugi et al. (6).

Radiochemicals

The radiochemicals used were L-[U- 14 C]lysine monohydrochloride (spec act 10 mCi/mmol), L-[4,5- 3 H]lysine (n) monohydrochloride (spec act 250 mCi/mmol), and [3 H]inulin (spec act 300 mCi/mmol) obtained from The Radiochemical Centre, Amersham, England.

RESULTS AND DISCUSSION

Because of a certain variability of the time-course of tumor growth, total mass was considered a better reference than the age of the tumors.

Fig. 1 shows that the ascitic plasma volume increases at a uniform rate throughout the time studied, whereas the cell mass, after a phase of logarithmic proliferation (*log phase*), ceases to increase when the tumor volume reaches 40–50 ml (6–7 days after transplantation) and thereafter it remains unchanged (*resting phase*).

This time-course is consistent with the growth

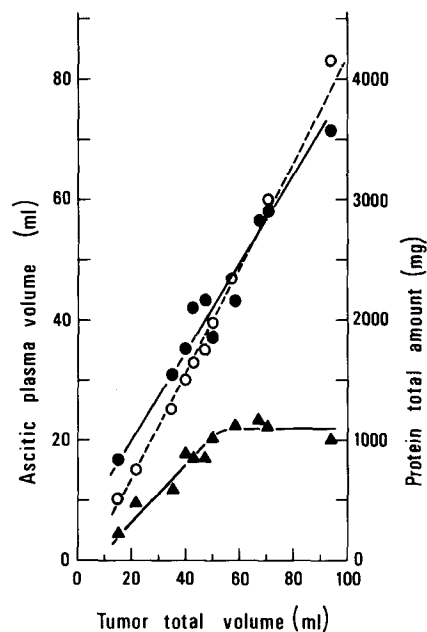


FIGURE 1 Relationships between cell mass expressed as milligrams of cell protein (\blacktriangle — \blacktriangle), ascitic plasma volume (\circ — \circ), and ascitic plasma proteins (\bullet — \bullet) in Yoshida ascites hepatomas of different total volumes. Rats were killed at various times after transplantation.

curve observed in the Yoshida ascites hepatoma by Bombara and Morabito (4).

The trypan blue exclusion test and glycogen synthesis, as studied histochemically (7), indicate that during the resting phase and up to the death of the animal all tumor cells are alive.

The analysis of protein pattern performed by gel filtration on G-200 Sephadex and by electrophoresis on polyacrylamide gel demonstrated that ascitic and blood plasma of the tumor-bearing animals have the same protein composition (Paoletti and Olivotto, manuscript in preparation). Rapid and extensive exchanges between blood and ascitic plasma have also been demonstrated for low molecular weight substances such as glucose (8).

It should be mentioned that, when proteins of ascites tumor cells are to be labeled with radioactive amino acid, lysine in our case, the concentration of this amino acid in the ascitic plasma and in the blood must be evaluated.

Whatever the tumor size, lysine concentration in the ascitic plasma is constant (0.530 μ mol/ml) and in equilibrium with the concentration in the blood (0.508 μ mol/ml).

By measuring the volume of the ascitic plasma (see Materials and Methods) it is possible to evaluate the total lysine pool in this fluid for each tumor.

At this point a tentative estimation of the initial specific activity of the labeled lysine injected, after the dilution in the ascitic pool, was carried out without taking into account for the moment the extraascitic compartment.

The formula applied was:

$$A_o = \frac{\mu\text{Ci}}{K + (C \cdot V)} \quad (1)$$

where A_o = initial specific activity of the lysine in the ascitic compartment; μCi = radioactivity of the lysine injected (a constant); K = micromoles of lysine injected (a constant); C = micromoles of lysine per milliliter of ascitic plasma (a constant); V = ascitic plasma volume (milliliters).

If the lysine transport capacity remains constant throughout the tumor phases, it is reasonable to assume that the specific activity of proteins made up at the same rate of synthesis by tumors of different sizes is proportional to the specific activity of the labeling amino acid in the ascitic compartment.

Experiments in vitro measuring the lysine transport capacity in log and in resting cells (Table I) showed no substantial differences in tumor cells harvested, respectively, at the 4th and the 10th day after transplantation.

Experiments carried out in vivo indicate that also under this condition the amino acid transport capacity is unchanged in log vs. resting phase cells.

Letting A_i and A_t represent the specific activity

of free lysine in the ascitic plasmas of animals l and i , as calculated according to Eq. 1, and P_i , P_t represent the corresponding tumor protein specific activity, then, having measured P_t , it is possible to compare the experimental value of P_i with its expected \hat{P}_i derived according to Eq. 2a:

$$\hat{P}_i = P_t \frac{A_i}{A_t} \quad (2a)$$

One may also derive from the measured value of P_i another value P_{ic} , which takes into account the dilution effect:

$$P_{ic} = P_i \frac{A_t}{A_i} \quad (2b)$$

After determining the experimental value of P_i in a tumor of 12 ml, Eq. 2a permitted the calculation of the decline of specific activity to be expected solely on the basis of the label dilution in the increasing ascitic plasma volumes, in proteins synthesized at the same rate by different tumors (Fig. 1, dotted line).

In Fig. 2 the experimental counts per minute per milligram of cellular protein labeled with [^3H]lysine are also plotted against tumor total volume. Experimental points (open circles) fall close to the calculated line while cell proteins increase at a constant rate. As the cellular mass reaches its steady-state, experimental values depart from this line although they are fitted by a curve of the same shape. A correction according to Eq. 2b of the experimental counts per minute per milligram of protein (full circles) makes it more clear that during the log phase the decline of specific activity of the cell proteins can only be accounted for by the increase of the lysine pool in the ascitic plasma. The same explanation applies to the decline of the protein specific activity measured during the resting phase, while in this stage the values are constantly 30% lower than in the log phase.

The following conclusions can be drawn from the results so far presented. When the cell mass has a uniform growth rate, protein synthesis proceeds at a constant rate. This may be deduced from the fact that at this stage radioactivity from [^3H]lysine incorporation into proteins corresponds closely to that calculated according to the dilution effect, assuming constant rates of lysine uptake and protein synthesis. This correspondence indicates that the amount of labeled amino acid escaping from the ascitic compartment is negligible in comparison with the amount remaining.

TABLE I
L-Lysine Transport Capacity in Log and Resting Phase Cells of Yoshida Ascites Hepatoma

Days after transplantation	Tumor volume <i>ml</i>	Intracellular water*	Distribution ratio†
4	30	3.89	3.68
10	90	3.22	3.28

Samples were incubated 5 min at 37°C in Krebs-Henseleit bicarbonate buffer containing L-[^{14}C]lysine.

For details see Materials and Methods.

* Microliter per milligram of cellular protein.

† dpm/milliliter of intracellular water/dpm/milliliter of extracellular water.

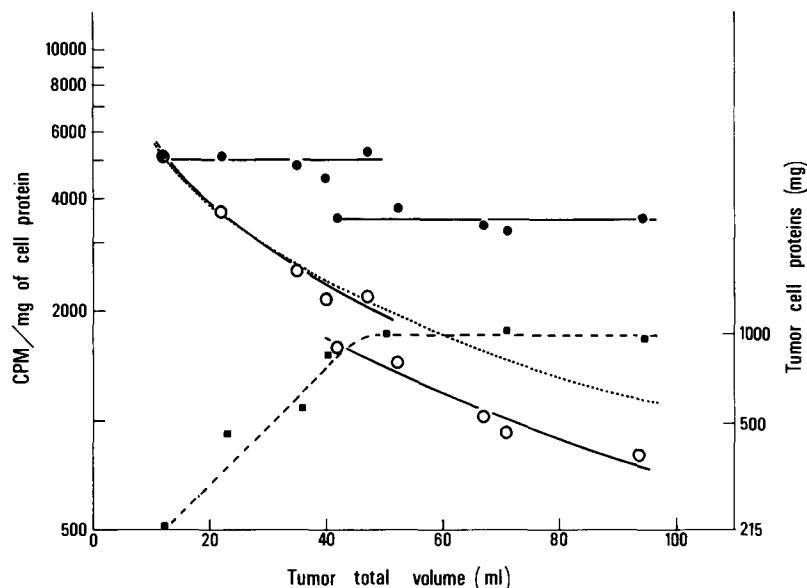


FIGURE 2 Incorporation of $[^3\text{H}]$ lysine into cell proteins at various stages of tumor growth. All values refer to 80 min after the injection of $[^3\text{H}]$ lysine and are reported as experimentally determined (O—O), as predicted according to Eq. 2a (·····) or corrected taking into account the dilution effect according to Eq. 2b (●—●). Total cell mass is expressed as milligrams of tumor cell protein (■—■). For explanation see Results and Discussion.

As the cellular mass reaches the steady-state, the rate of protein synthesis settles at a value that is 30% lower than in the log phase, but remains constant throughout the resting phase.

The last conclusion is also supported by the fact that the amount of lysine taken up by the cells and newly supplied from the blood was found to be constant during the resting phase. This finding derives from the measurements of the specific activity (At) of free lysine in the ascitic plasma withdrawn immediately after animal killing, i.e., 80 min after the injection of the labeled amino acid (see Table II). From Eq. 1 the initial specific activity (At) of free lysine in the ascitic plasma of labeled amino acid (A_0) can also be calculated. It is thus possible to estimate the amount of the initial pool which should have remained as free lysine in the ascitic compartment after the interval $0 - t$:

$$Q = L_0 \frac{At}{A_0} \quad (3)$$

where Q = amount of the initial pool (L_0) remaining in the ascitic compartment at time t (micromoles); L_0 = lysine pool (micromoles) in the ascitic plasma at time zero [initial pool; $K + (C \cdot V)$ of Eq. 1]; A_0 = specific activity (microcuries/micromole) of $[^3\text{H}]$ lysine at time zero (calculated from Eq. 1); At = specific activity (microcuries/micromole) of $[^3\text{H}]$ lysine at time t .

Let us consider, e.g., the 57-ml tumor of Table II. A_0 can be estimated as $1.46 \mu\text{Ci}/\mu\text{mol}$, whereas $At = 0.264 \mu\text{Ci}/\mu\text{mol}$, that is, 5.5 times lower. This implies that $1/5.5 (At/A_0)$ of L_0 is the amount of the initial pool remaining in the ascitic compartment at time t .

The amount of lysine taken up by the cells and replaced by unlabeled amino acid coming from the blood (R) can then be calculated:

$$R = L_0 - Q \quad (4)$$

For all the tumors considered in Table II, R is constant ($28.8 \pm 1 \mu\text{mol}$). The value of Q , on the contrary, increases concurrently with the ascitic plasma volume or, in other words, with L_0 . This makes understandable the fact that the experimental values of At rise, although A_0 decreases, when the tumor volume increases. In fact, the same amount (R) is renewed in the interval $0 - t$ in tumors having different total lysine pools. Thus, the smaller L_0 and then the higher A_0 values, the smaller the amount of L_0 remaining in the ascitic compartment (Q) and undergoing the dilution by the amino acid renewed from the blood.

TABLE II

[³H]Lysine Specific Activities in the Ascitic Plasmas of Yoshida Ascites Hepatomas Immediately and 80 min after the Injection of Isotope into Tumors having Similar Cell Masses and Different Ascites Volumes

Tumor volume	Ascitic plasma volume	Cell mass	A_o	L_o	Q	R	At
ml	ml	mg protein	$\mu\text{Ci}/\mu\text{mol}$	μmol	μmol	μmol	$\mu\text{Ci}/\mu\text{mol}$
57	53.2	1,155	1.46	34.2	6.2	28.0	0.264
67	63.3	1,165	1.26	39.6	9.3	30.3	0.298
71	67.5	1,100	1.19	41.7	11.0	30.7	0.313
94	91.1	900	0.92	54.3	28.0	26.3	0.475

For explanation see Results and Discussion.

A_o = specific activity of [³H]lysine at time zero (calculated from Eq. 1).

L_o = lysine pool at time zero in the ascitic plasma [$K - (C \cdot V)$] of Eq. 1.

Q = amount of L_o remaining in the ascitic plasma at time t (calculated from Eq. 3).

R = amount of lysine taken up by the cells and replaced from the blood in the interval $0 - t$ (calculated from Eq. 4).

At = specific activity of [³H]lysine experimentally determined at time t (80 min after the isotope injection).

After determining R for one tumor, substituting, in Eq. 4, $L_o (At/A_o)$ for Q and calculating L_o and A_o for each tumor, At can be predicted for any tumor having the same cellular mass:

$$At = A_o \frac{L_o - R}{L_o} \quad (5)$$

From the above reported data, it is also possible to estimate the rate of utilization of lysine per hour and per microgram of cell protein as 18 pmol, that is, a value of the same order as the 10.8 found by Kruse et al. for perfused Jensen sarcoma cells throughout different phases of proliferation (14).

Concurrently with the rate of synthesis studied by short-time [³H]lysine incorporation, protein degradation was followed by measuring the decline of [¹⁴C]protein radioactivity after labeling with [¹⁴C]lysine (see Materials and Methods).

Fig. 3 shows [¹⁴C]protein radioactivity of hepatomas at various times after [¹⁴C]lysine injection. Fig. 3 A and B refer to tumors, labeled while in the growing phase and Fig. 3 C and D refer to tumors labeled while already in the resting phase. Results are reported both as radioactivity per milligram of cell protein (Fig. 3 A and C) and as radioactivity of the total tumor cells (Fig. 3B and D). The latter reference is necessary when dealing with a mass that is changing during the time of the measurement.

¹⁴C radioactivity per milligram of cell protein rapidly drops as the tumor mass increases, and it continues to fall, although at a slower rate, when the cell mass settles at the steady-state (Fig. 3 A).

On the contrary, the total radioactivity keeps increasing throughout the log phase (Fig. 3 B) although the specific activity of free lysine in the ascitic plasma drops dramatically a few hours after the isotope injection (Fig. 3 B and D, dotted lines). The continuing increase of the total radioactivity of cell proteins, even when the specific activity of the labeling amino acid in the biological fluids is very low, has already been reported by LePage et al. as a peculiarity of cancer (15).

The total radioactivity increases at this stage 2.5 times, whereas the cell proteins increase 4.4 times. The decrease of counts per minute per milligram of cell protein seems, therefore, to be accounted for mostly by the increase in tumor mass. Nevertheless, it is impossible to exclude protein degradation during this stage, since any loss of radioactivity would be masked by the continuing incorporation.

During the resting phase the total radioactivity declines along with the specific one. This happens, starting from zero time, in experiments where the isotope injection is carried out when the tumor mass has already attained the steady-state (Fig. 3 C and D). In these experiments there is no initial increase of the total radioactivity, which seems therefore essentially related to the log stage of growth of these cells.

On the whole, these conclusions are suggested by the results reported in Fig. 3. During the log phase, protein catabolism, if present, is by far exceeded by the synthetic process, whereas, during the resting phase, protein degradation is counterbalancing the synthesis that is still very high throughout this period (see discussion of Fig. 2).

By cell fractionation of tumors in the steady-state, it was possible to gather some information about the protein turnover of subcellular particles. During the resting phase, the total protein half-life for microsomes (estimated from semilogarithmic plots of counts per minute per milligram of protein) was approximately 3 days. This value is close to that reported by other authors for rat liver microsomes (2, 20). For tumor mitochondria, the total protein half-life was estimated to be around 5 days, in agreement with the findings reported for liver mitochondria (25). In the nuclear fraction the half-life estimated to be around 3 days is appreciably shorter than that reported by Arias et al. (2) for nuclear proteins of rat liver.

Since sufficient information about the effect of precursor reutilization is lacking, the half-lives

reported have to be considered as possibly overestimated (10).

It should be said that the release of proteins by the cells into the surroundings (14, 28) may alter the measurement of turnover based on the decay of the specific activity of cell proteins, as the radioactivity loss may be wrongly attributed to catabolism.

Fig. 4 shows that at the time of the tumor harvesting, i.e., 80 min after the isotope injection, blood plasma proteins are highly labeled, whereas, in the ascitic plasma, labeled proteins appear only later, reaching a plateau which lasts about 3 days. The rise of the protein specific activity in the ascitic compartment is simultaneous with the drop of the specific activity in the blood, until the same value is attained in the two compartments. Even

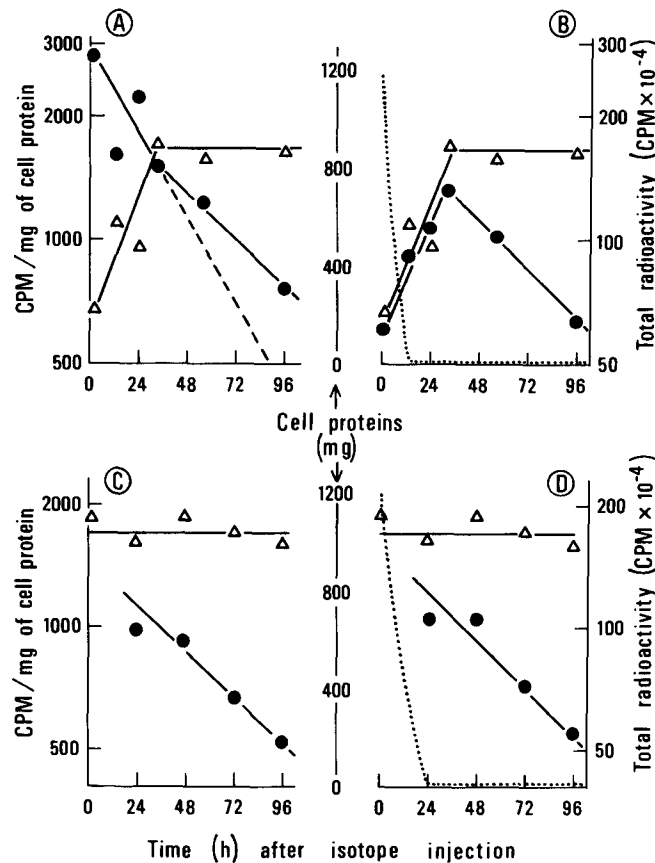


FIGURE 3 Time-course of tumor cell protein radioactivity (●—●) after labeling with [¹⁴C]lysine. At the time of the injection of [¹⁴C]lysine, the tumors were 5-days old with the cell mass (Δ—Δ) still actively increasing (Fig. 3 A and B), or 7-days old with the cell mass at the steady-state (Fig. 3 C and D). The dotted lines represent the specific activity of free [¹⁴C]lysine in the ascitic plasma.

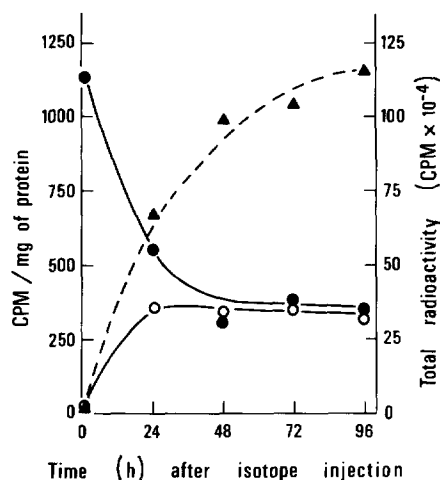


FIGURE 4 Time-course of protein labeling in the ascitic and blood plasmas. Rats at 7 days after transplantation were given i.p. $10 \mu\text{Ci}$ of [^{14}C]lysine and killed at various times thereafter. (●—●) = specific activity of blood plasma proteins; (○—○) = specific activity of ascitic plasma proteins; (▲—▲) = total radioactivity of ascitic plasma proteins.

then, the total radioactivity of the ascitic proteins continues to increase.

These findings suggest that radioactive proteins in the ascitic plasma mainly derive from the blood plasma, which continuously passes into and is retained in the ascitic compartment, accounting for the kinetics of appearance of the labeled proteins therein. Release of cellular macromolecules affecting these kinetics does not seem to take place. This is in keeping with the view that the decline of the specific activity of cell proteins reported in Fig. 3 is due to intracellular catabolism rather than to macromolecular release. Some experiments carried out in vitro indicate that macromolecules released by the tumor cells are less than 5% of the total TCA-precipitable material.

Concluding Remarks

The results so far presented and discussed encourage the use of the Yoshida ascites hepatoma as a useful model for studying protein metabolism in relation to cell growth in vivo.

The time-course of the tumor development offers the possibility of following a homogeneous cell population that, while remaining metabolically very active, undergoes a great modification of the proliferation rate. The modification of the equilib-

rium between protein synthesis and degradation and not a metabolic depression is strictly related to the arrest of the cellular mass increase attained during the resting phase.

A profitable feature of this tumor lies in the fact that most of the labeled amino acid injected i.p. into the animals is immediately available for the cells within, with only a minor loss towards the extraascitic compartment. Furthermore, the amino acid transport capacity of the cells does not change in log vs. resting phase. It is thus possible to evaluate the time-course of the protein synthesis after taking into account the dilution of the labeling amino acid in the ascitic pool.

To further stress the interest in this model, the arrest of cell proliferation cannot be ascribed to contact or density inhibition, as the cell crowding in the ascitic plasma decreases during the resting period.

It is also difficult to conceive an immunological mechanism which arrests tumor growth without leading to tumor regression.

Energy supply does not appear to be the growth-limiting factor, as it is at least sufficient to sustain amino acid transport as well as protein synthesis and degradation at similar rates in the two stages. However, within 6–7 days after tumor transplantation, glucose concentration of the ascitic plasma shows a 20-time decrease (7). The decrease of the glucose level may be related to the arrest of the cell growth, although the precise underlying mechanism remains rather speculative. In this connection, one recalls that in *Escherichia coli* the cyclic AMP level was found to be depressed by glucose (21), and that in mammalian cells the cyclic AMP concentration is inversely related to the rate of DNA synthesis (1).

Glucose availability may also be relevant in regard to the pattern and/or the metabolic rate of the carbohydrate-containing macromolecules. In fact, glucose is the source of the carbohydrates of the cell surface, whose structure and functions are important in cell cycle regulation (24, 27, 9, 5, 26).

The authors wish to express their gratitude to Professor A. Fonesu for his interest in this work and stimulating discussions. They are also indebted to Dr. P. Urbano for his advice and suggestions throughout the course of this study, and to Dr. G. Cappugi and Mr. G. Camici for the helpful collaboration in determining the lysine content and specific activity. Thanks are also due to Professor G. G. Guidotti and his coworkers for the study of the amino

acid transport. The excellent technical assistance of Mr. M. Domenichelli is gratefully acknowledged.

This work was supported by a grant from the Italian Research Council (CNR).

Received for publication 28 December 1972, and in revised form 15 March 1974.

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