Cell Proliferation and Metabolism of Ehrlich Ascites Tumor Cells Grown in Chemically Defined Albumin Medium for Several Passages

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Albumin Medium, Cell Growth, Ehrlich Ascites Tumor Cells, Metabolism

Cell proliferation, cell cycle distribution, viability, uptake of α-aminoisobutytic acid, cyclo leucine and 2-deoxyglucose as well as DNA, RNA and protein synthesis of Ehrlich ascites tumor cells grown in suspension culture over three passages in serum free nutrient medium supplemented with 1–2% bovine serum albumin (Cohn fraction V, Serva) were measured and compared to cells grown in medium supplemented with 15% horse serum. At the end of the fourth passage *in vitro*, that is the third passage in serum free albumin medium, the growth of the cells is reduced to about 10% of the controls, accumulation of the cells in G 2 is delayed and the number of dead cells rises to 30%. The transport rates of α-aminoisobutyric acid and of 2-deoxyglucose slowly decrease while the uptake of cycloleucine shows a more complex behaviour in albumin medium. Incorporation of thymidine and leucine into the cells corresponds to the growth rate, while the incorporation of uridine in normal as well as in albumin medium exhibits different pattern. The present experiments demonstrate, that it may be possible to preserve this cells in a fairly "physiological" state during two passages in albumin medium.

In a recent publication we have presented the results of our experiments on the proliferation, viability, synthesis of macromolecules and transport of amino acids of Ehrlich ascites tumor cells grown in serum free medium supplemented with albumin [1]. These investigations were performed with cells cultured over a period of 24 h and have shown that optimal cell growth is obtained in nutrient medium supplemented with 1% bovine serum albumin (Cohn fraction V, Serva); cell proliferation under these conditions is reduced to 50% as compared to controls in normal medium and a marked trend of the cells to attach to glass has been observed.

Our further studies on the synthesis of membrane components of the cells in serum depleted nutrient medium have revealed [2] that the uptake and incorporation of ethanolamine but especially of choline increases drastically in albumin supplemented medium.

In the present communication we report the results of studies with Ehrlich ascites cells grown for several passages in serum depleted medium containing albumin. These experiments give some information on the question, how long it may be possible to maintain macromolecular synthesis and cell proliferation of Ehrlich ascites cells in a chemically defined buffered medium without addition of specific growth factors.

Reprint requests to Prof. Dr. Fr. Schneider.

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Materials and Methods

All chemicals used were of "p. a. grade" or for "biochemical purposes" from Merck, Darmstadt, Serva, Heidelberg or Roth, Karlsruhe. Biochemicals were from Boehringer, Mannheim and Sigma, München. Radiochemicals were purchased from Amersham, Braunschweig or Schwarz/Mann, Heidelberg. Horse serum was a gift from Behringwerke, Marburg. Bovine serum albumin was purchased from Serva, Heidelberg. Microcillin was a gift from Bayer, Leverkusen.

Cells and culture media

Hyperdiploid Ehrlich ascites tumor cells, serially grown in the peritoneal cavity of female NMRI mice were transferred to modified Eagle medium [3] supplemented with 15% horse serum, 30 mg/l streptomycin and 575 mg/l Microcillin Bayer. Unsiliconized glass flasks were used for suspension cultures. For this line of EAT cells the nutrients of the medium were sufficient for a 24 h culture period. After this period the cells were collected by centrifugation and resuspended in fresh medium giving rise to a cell density of about 6.5×10^5 cells/ml. For further details see ref. [4]. Growth was estimated by turbidity measurements of cell suspension at 578 nm [5]. An appropriate calibration curve was obtained by enumeration of cells with a hemocytometer. Viability of cells was assessed by staining with 0.1% nigrosin.

Albumin containing media were prepared by dissolving bovine serum albumin in serum free medium. 20 mM HEPES was added to enhance the buffer capacity of the culture medium. This buffer substance has no effect on the proliferation rate and number of dead cells.

Protein-, RNA- and DNA-synthesis

The relative rates of protein-, RNA and DNA-synthesis were measured by the incorporation into the acid insoluble material of [L-U-^{14}C]leucine (spec. act. 330–348 mCi/mmol) [2-^{14}C]uridine (spec. act. 60 mCi/mmol) and [2-^{14}C]thymidine (53–61 mCi/mmol); for further details see ref. [6].

Amino acid transport

Rates of uptake of amino acids were measured in the appropriate intervals by incubation of the cells with [2-^{14}C-α]aminoisobutyric acid (spec. act. 51 mCi/mmol) or [^{14}C-cyclo]leucine (spec. act. 60 mCi/mmol) and determination of the radioactivity in the acid soluble fraction. The amino acids were diluted to a final activity of 2 μCi/ml; 0.1 ml were incubated with 1 ml cell suspension for 10 min at 37 °C. The uptake of label was measured in 0.5 ml supernatant in Rotiszint 11 (Roth, Karlsruhe) in a Packard Tricarb scintillation spectrometer after destroying the cells with 1 ml 5% trichloroacetic acid.

Uptake of [2- deoxy-D-1-^{3}H] glucose

Rates of uptake of [2-deoxy-D-1-^{3}H]glucose were measured with samples of 1 ml cell culture. The cells were packed by centrifugation, washed with 10 ml of glucose free medium, and resuspended in 1 ml normal medium or albumin medium containing 2.5×10^{-4} M D-glucose and 0.5 μCi 2-deoxyglucose (spec. act. 51 mCi/mmol), after 10 min incubation with shaking, the cells were collected by centrifugation, washed two times with Hanks solution and extracted with 1 ml 10% trichloroacetic acid. Radioactivity was measured in 0.5 ml of the extract in 10 ml R 22 in a Tri Carb 3003.

DNA-Histograms

DNA-histograms of the cells were obtained with the flow cytometer ICP 11 from Phywe, Göttingen. The cells were prepared for the measurements as described by Schumann and Göhde [7]. As a simple parameter for the description of the relative cell cycle distribution we used the G 2 in % of G 1 value (= G 2/G 1 × 100) [8].

Results and Discussion

Growth and viability

All experiments described were performed in serum free culture medium supplemented with 1–2% bovine serum albumin (Cohn-fraction V) from Serva. As we have shown in a previous communication [1], albumin of this source was most suitable for our purposes because optimum growth rates were observed and adhesion of the cells to glass was less than with other albumin preparations tested. Growth rates and number of dead cells in 1% albumin medium and in normal medium over several passages *in vitro* are illustrated in Table I. The parameters were measured in each case at the end of the passages. Fig. 1 demonstrates the corresponding

Medium	2nd passage *in vitro* 1st passage in albumin medium		3rd passage *in vitro* 2nd passage in albumin medium		4th passage *in vitro* 3rd passage in albumin medium	
	Growth $\frac{dN}{dt} \cdot \frac{100}{N}$	% of dead cells	Growth $\frac{dN}{dt} \cdot \frac{100}{N}$	% of dead cells	Growth $\frac{dN}{dt} \cdot \frac{100}{N}$	% of dead cells
Normal medium	68 ± 15 n = 26	2 ± 1	51 ± 19 n = 26	4 ± 1	53 ± 19 n = 26	4 ± 1
1% albumin medium	30 ± 7 n = 20	3 ± 1	15 ± 9 n = 20	10 ± 4	8 ± 6.0 n = 20	30 ± 7
% of control	44		29		15	

Table I. Growth rates and number of dead cells in 1% albumin medium and in normal medium over several passages *in vitro*, measured at the end of each passage of 24 hours.

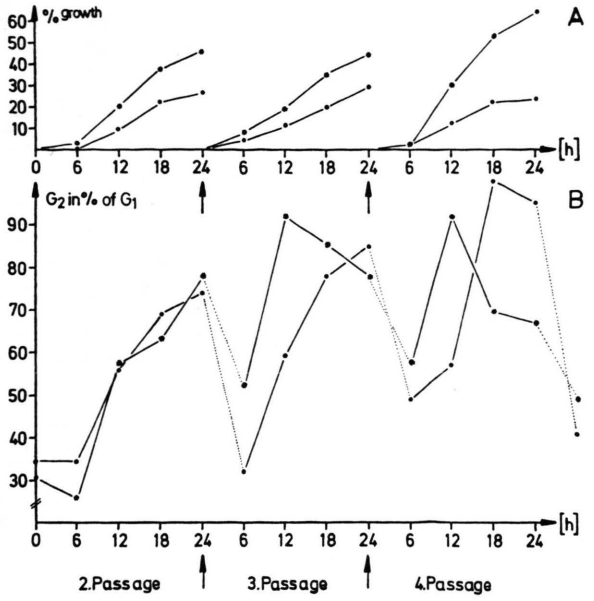

Fig. 1. A. Growth of EAT cells in the 2. to 4. passage in normal medium * —— * and serum free medium supplemented with albumin ● —— ●. B. G 2/G 1 ratio of the cells; the values were calculated from DNA-histograms.

growth curves and the G 2/G 1 ratios, which were calculated from DNA histograms of the cells at the appropriate intervals.

While in the first passage in albumin medium, the growth rate of the cells is still about 50% of the controls in normal medium, it decreases drastically in the 3rd and 4th passage with a tenfold increase of the number of dead cells in the 4th compared to the 2nd passage. Obviously serum albumin alone can not even preserve the viability for several passages. It should be noted however, that growth of these cells in normal medium also decreases always in the 3rd passage and then remains constant. The reason for this decline of cell growth is discussed extensively by Woo *et al.* [9] and Barford *et al.* [10]; variations in cell cycle time, loss of cells or accumulation of cells in G 1 are suggested to be responsible for the reduced proliferation rate. Beginning with the 2. passage in albumin medium, aggregation of the cells and irregularities in their size were observed. Similar impairments of cells in serum free cultures are described by Knutson [11] with mouse sarcoma cells. Some informations on the cell cycle distribution of cells grown in albumin medium were obtained from DNA-histograms. From Fig. 1 it can be seen, that in the 3rd and 4th passage a delayed rise of the G 2/G 1 ratio takes place which may be a consequence of a prolongation of the cell cycle time. Typical DNA histograms which were used for the calculation of the G 2/G 1 ratios are shown in Fig. 2.

Respiration and glycolysis of the cells were not significantly affected (data not shown).

Transport processes

It is generally accepted that albumin has a stabilizing effect on the membranes of cultured mammalian cells [12]. Since normal transport activities

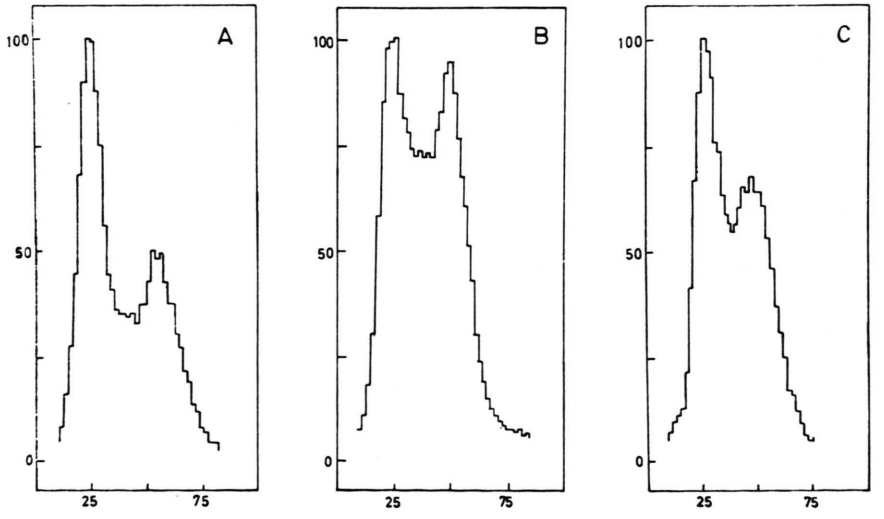

Fig. 2. DNA-histograms of Ehrlich ascites tumor cells from the 3rd *in vitro* passage. The first peak represents cells with 2 c DNA = G 1, the second those cells with 4 c DNA = G 2. A at the beginning of the passage; B after 10 h in normal medium; C after 10 h in albumin medium. Ordinate: Counts/Channel (relative units); Abscissa: Channel number.

are closely related to the integrity of the membranes, we have studied the uptake of α-aminoisobutyric acid, cyclo-leucine and 2-deoxy-D-glucose by EAT cells cultured in albumin medium over several passages. These amino acids were chosen to measure the activity of the Na^+ dependent A-system for the transport of neutral and the Na^+ independent L-system for the transport of branched and cyclic amino acids [13]. The uptake of α-aminoisobutyric acid and cyclo-leucine by the cells in the second to fourth passage is demonstrated in Figs 3 and 4. The transport characteristics of the two amino acids in normal medium are very similar over the three passages *in vitro*. In albumin medium, a slow decrease of the uptake of α-aminoisobutyric acid is observed which amounts to 50% of the controls at the end of the fourth passage *in vitro*. Somewhat more complex is the uptake of cyclo-leucine in albumin medium. In the first passage in albumin medium (= 2nd passage *in vitro*) the uptake of cyclo leucine by the cells is much higher than in normal medium and passes through a maximum; this is in agreement with the enhanced utilization by the cells of leucine and isoleucine previously described for the first passage in albumin medium [1]. In the 3rd and 4th passage the uptake of cyclo leucine follows a minimum curve; but even at the end of the 4th passage the control values are found again. Obviously the A-system and L-system for the transport of amino acids are differently affected by the exchange of

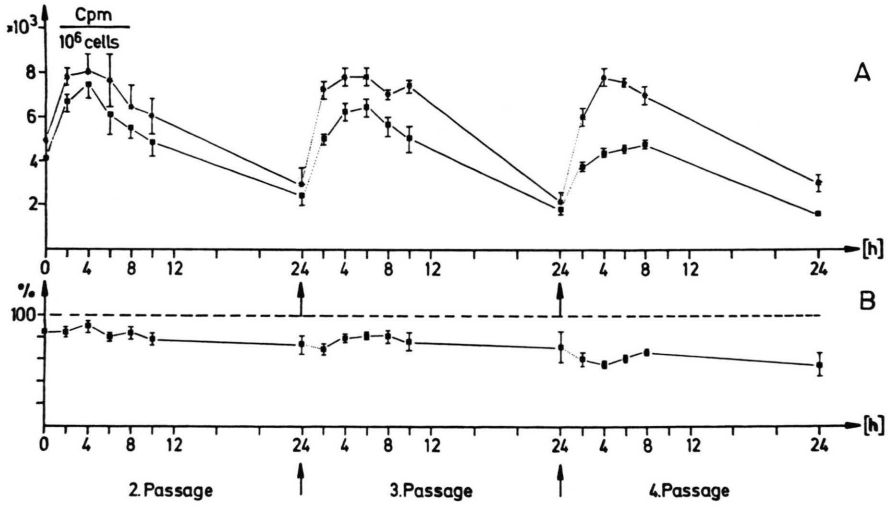

Fig. 3. A. Uptake of α-aminoisobutyric acid by the EAT cells grown in normal medium *——* and serum free albumin medium ■——■. B. Relative uptake of α-aminoisobutyric acid by cells grown in albumin medium in percent of the controls.

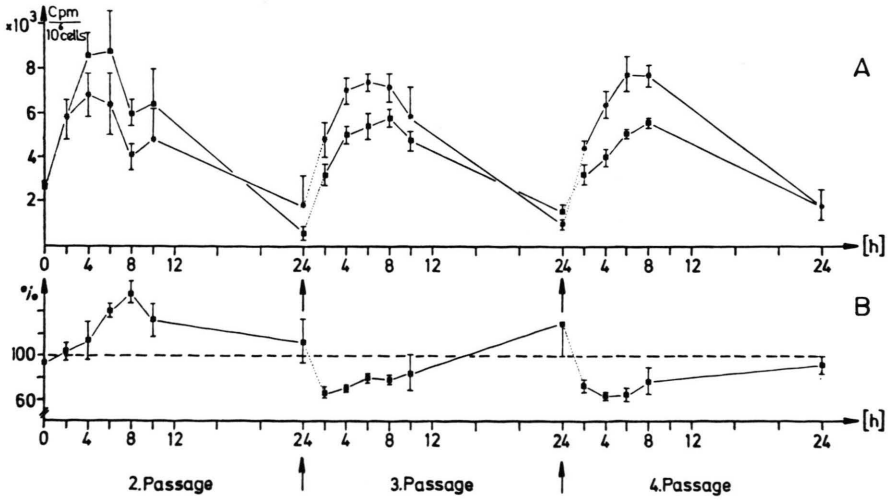

Fig. 4. A. Uptake of cyclo leucine (see under 3 A). B. Relative uptake of cyclo leucine (see under 3 B).

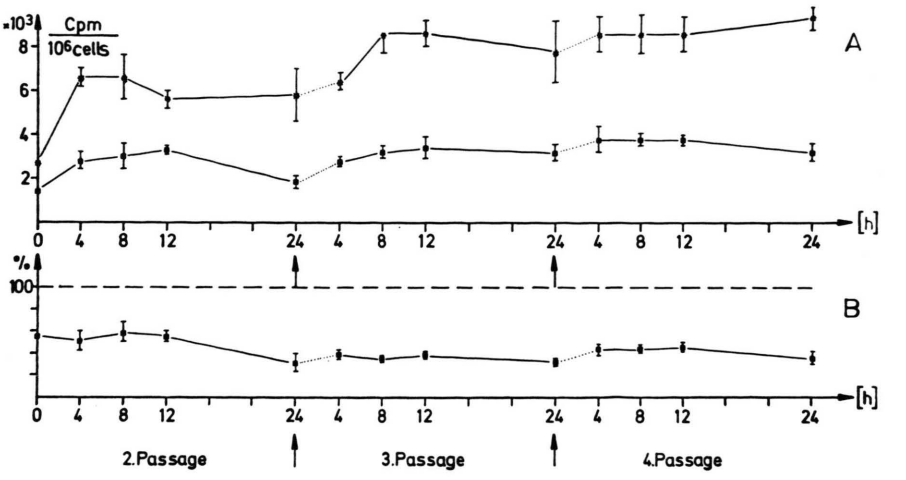

Fig. 5. A. Uptake of 2-deoxy-D-glucose (see under 3 A). B. Relative uptake of 2-deoxy-D-glucose (see under 3 B).

serum for albumin in the nutrient medium. These results demonstrate further, that no simple correlation between the uptake of amino acids and the growth rate and protein synthesis (see below) in albumin medium exists. Therefore, it seems unlikely that the level of intracellular amino acids regulates growth. No experiments have yet demonstrated that transport is a primary regulator of cell growth and evidence is accumulating that contradicts this hypothesis [14].

The uptake of 2-deoxyglucose (Fig. 5) by cells grown in normal medium exhibits a slight increase over several passages; at the end of the 4th passage it is about 30% higher than in the 2nd passage. Growth of the cells in serum free medium supplemented with 2% albumin produced decreased 2-deoxyglucose uptake, which was determined to be 30–60% of the controls. As was shown by Cunningham [15], hexose uptake seems not to be causally linked with the proliferative state of cultured cells. Our experiments are in agreement with this observation.

Macromolecular synthesis

DNA-, RNA- and protein synthesis of cells grown in normal and serum free medium supplemented with 2% albumin measured by the incorporation of labelled precursors are illustrated in Figs 6, 7 and 8.

In normal medium the incorporation of thymidine and leucine into the acid insoluble fraction of the cells corresponds to the proliferation rate; the net rate of protein accumulation is directly proportional to the growth rate. The uptake of thymidine, the activation of thymidine kinase and the incorporation of the nucleotides into DNA are closely coupled processes and take place only during the synthesis of DNA [16]. The thymidine nucleotide pool during the S-phase is therefore nearly constant [17] and the radioactivity of the DNA is therefore a fairly reliable

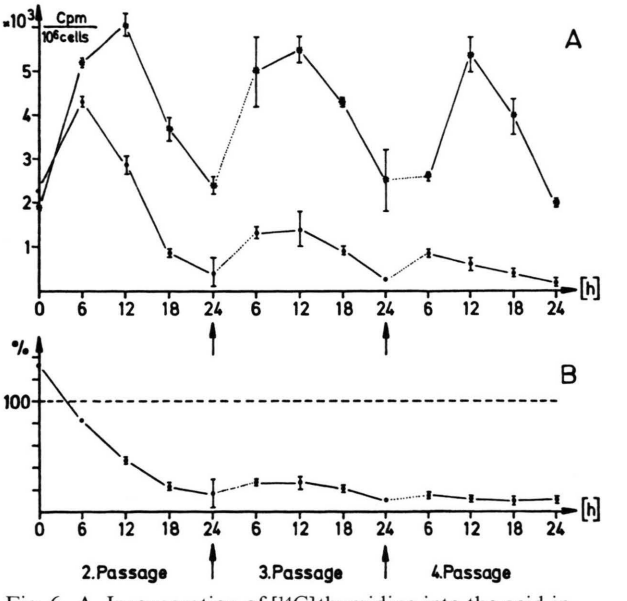

Fig. 6. A. Incorporation of [^{14}C]thymidine into the acid insoluble fraction of EAT cells grown in normal medium *——* and serum free albumin medium ●——●. B. Relative rate of [^{14}C]thymidine incorporation into the acid insoluble fraction of EAT cells grown in albumin medium (controls = 100%).

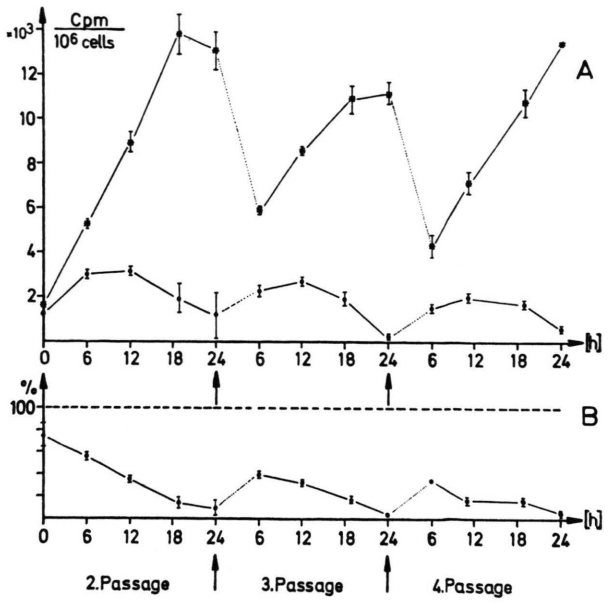

Fig. 7. A. Incorporation of [^{14}C]leucine (see under 6 A). B. Relative incorporation of [^{14}C]leucine (see under 6 B).

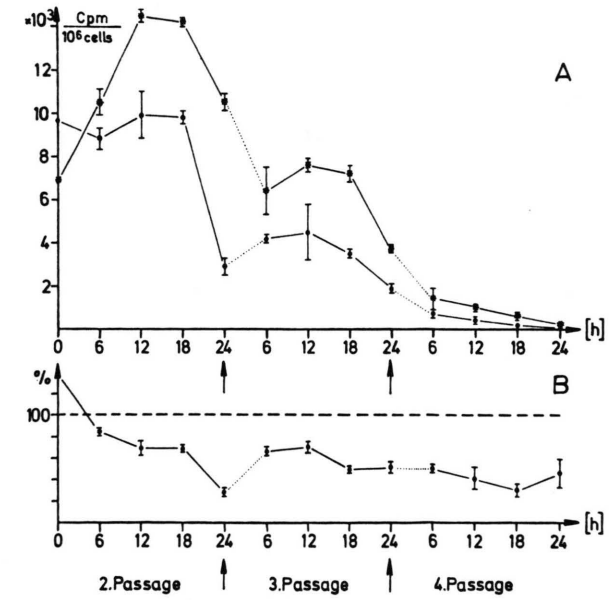

Fig.8. A. Incorporation of [^{14}C]uridine (see under 6 A). B. Relative incorporation of [^{14}C]uridine (see under 6 B).

measure of the rate of DNA-synthesis. However, a different incorporation pattern is found for uridine; even in normal medium a countinuous decrease of radioactivity in the acid precipitate after incubation of the cells with labelled uridine is observed. Stimulation of the endogenous nucleoside synthesis by glucose as is described in ref. [18] accounts presumably for the incorporation rate decreasing over several passages. This stimulation of nucleoside synthesis may cause a dilution of the labelled precursor and thereby simulates a decrease in RNA synthesis.

DNA and protein synthesis are strongest affected in serum free medium supplemented with albumin. A rapid and considerable decrease of thymidine and leucine incorporation is already observed in the first passage in serum depleted medium. This confirmes the well-known dependence of DNA synthesis on concomitant protein synthesis [14]. Concerning the incorporation of [^{14}C]uridine into the cells in albumin medium an unexpectedly high radioactivity in the acid insoluble fraction is observed even in the 4th passage. As is well known from the literature [19, 20] the incorporation of labelled uridine into acid insoluble fraction of cells does not always give a reliable measure of the RNA synthesis. We therefore do not assume that in albumin medium the rate of RNA synthesis is really greater than under normal conditions.

Referring to the question, how long it may be possible to maintain viability, cell proliferation and macromolecular synthesis of Ehrlich ascites tumor cells in a chemically defined medium, the present experiments demonstrate, that this cell line may be preserved in a fairly "physiological" state during two passages in albumin medium. There is no doubt that many problems can be studied using this cells in albumin medium and that by far more reliable results will be obtained in the presence of albumin than in buffer solutions only, which are frequently employed. This seems to be especially important for investigations regarding transport processes and membrane properties. At the present time, however, all experiments concerning proliferation kinetics and cell cycle of Ehrlich ascites tumor cells can only be performed in serum containing media.

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