

# Proliferation and Metabolic Activities of Ehrlich Ascites Tumor Cells in Chemically Defined Albumin Media

Regina Teschner, Stefan Postius, Monika Löffler, and Friedhelm Schneider

Physiologisch-Chemisches Institut der Universität Marburg, Lahnberge, D-3550 Marburg

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Cell proliferation, viability, DNA-, RNA-, protein synthesis, amino acid transport, respiration and lactate/glucose quotient of Ehrlich ascites tumor cells grown in suspension culture in serum free medium supplemented with albumin charges of different origin were studied. Optimal cell growth was obtained in nutrient medium supplemented with 1% bovine serum albumin (Cohn-fraction V, Serva). Cell proliferation under these culture conditions was delayed to 50% as compared to controls in normal medium; the rate of synthesis of macromolecules was reduced; energy metabolism was not significantly impaired. The trend of the cells in albumin medium to attach to glass was independent from the pH of the cultures between 7.2 and 8.0; it was enhanced by fatty acid deprivation of the albumin.

It is now well established, that most cell cultures require the addition of serum to synthetic media for their maintenance and growth [1]. The primary significance of the serum is not clear but it seems to provide necessary growth factors, perhaps hormones and growth promoting polypeptides [2] or it may protect the cells by interaction of the serum proteins itself, especially of albumin with the cell membrane [3].

Since serum is a very complex mixture of numerous biologically active compounds [4], the chemical composition of nutrient media containing serum is not defined.

Recent reports in the literature [2, 5] strongly suggest, that it may be possible to eliminate serum from culture medium and that perhaps any cell culture line can be grown in synthetic medium supplemented with the "appropriate" growth factors. Most thoroughly studied among the main components of the serum was the growth promoting activity of serum albumin from different species.

We therefore have extended our investigations on relations between energy metabolism and cell proliferation of in suspension culture grown Ehrlich ascites tumor cells [6–9] on studies of the growth and metabolic activities of these cells in chemically defined media supplemented with serum albumin of different origin. In the present communication we report the results of these experiments, which demon-

strate that it is possible to culture this cell line under conditions, which allow the investigation of problems that require serum free nutrient medium.

## 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 obtained from Amersham, Braunschweig or Schwarz/Mann, Heidelberg. Horse serum and the different human albumin species were a gift from Behringwerke, Marburg. 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's medium [10], supplemented with 15% horse serum, 30 mg/l streptomycin and 575 mg/l Microcillin Bayer. 600 ml un-siliconized 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. For details see ref. [11]. Growth was estimated by measurement of the turbidity of cell suspensions at 578 nm [12]. An appropriate calibration curve was obtained by numeration of cells with a hemocytometer. Viability of cells was assessed by staining with 0.1% nigrosin [13].

Reprint requests to Prof. Dr. Fr. Schneider.

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A most sensitive test for leaky cells is the measurement of lactate dehydrogenase in the supernatant of cell cultures: 5 ml cell suspension were freed from cells by centrifugation at  $550 \times g$  for 2 min; after centrifuging the supernatant at  $2000 \times g$  for 5 min, 2 ml were used for the measurement of lactate dehydrogenase [14].

Serum and albumin free medium (SAFM) has the same composition as normal medium without addition of horse serum.

Albumin containing media were prepared by dissolving bovine or human serum albumin in serum free medium. 20 mM HEPES was added to the albumin media to enhance the buffer capacity of the culture medium. The buffer substance had no effect on the proliferation rate and number of dead cells within 24 hours. For experiments in lipid deprived nutrient medium, albumin was freed from fatty acids by charcoal treatment as described in ref. [15].

#### *Glycolysis and respiration*

L-lactate production of the EAT-cells was assayed as described by Bergmeyer [16]; respiration was determined amperometrically [17]. D-Glucose was measured with the test combination GOD-Perid from Boehringer, Mannheim. Protein concentration was determined according to a modified method of Wolf [18].

#### *Protein-, RNA- and DNA-synthesis*

The relative rates of protein-, RNA- and DNA-synthesis were measured by L-[U- $^{14}\text{C}$ ]leucine-, [2- $^{14}\text{C}$ ]uridine and [2- $^{14}\text{C}$ ]thymidine incorporation into acid insoluble material as described previously [19]. The spec. act. of leucine was 330 – 348 mCi/mmol, of uridine 60 mCi/mmol, of thymidine 53 – 61 mCi/mmol.

#### *Amino acid transport.*

Rates of amino acid uptake were measured in the desired intervals by incubation of the cells with [2- $^{14}\text{C}$ ]- $\alpha$ -aminoisobutyric acid (spec. act. 51 mCi/mmol) or [ $^{14}\text{C}$ ]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  $\mu\text{Ci/ml}$ ; 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 scin-

tillation spectrometer after destroying the cells with 1 ml 5% trichloroacetic acid.

#### *Amino acid analyses*

Amino acid analyses of culture media and cells were performed with a Multichrom B Analyzer from Beckman. The samples were deproteinized with perchloric acid, neutralized with KOH and prepared for the analysis as described in ref. [14].

## **Results**

#### *Growth and metabolic activities of Ehrlich ascites cells in the first passage in albumin medium (second passage in vitro).*

Growth and diverse metabolic activities of EAT cells were studied in serum free media supplemented with albumin. The following species, charges and concentrations of albumin were used:

0.5, 1.0 and 2.0% bovine serum albumin (Cohn-fraction V) from Serva, Heidelberg (0.5-AMS; 1-AMS; 2-AMS).

1% bovine serum albumin (purest grade) from Behringwerke, Marburg (1-AMB).

The optimum growth rates were observed in 1-AMS medium with an increase of cell number within 24 hours of about 38% and of protein of 52%, while in parallel control cultures in the presence of serum the corresponding values were 79% and 97%, respectively. By way of contrast, the highest incorporation rates of [ $^{14}\text{C}$ ]thymidine, -uridine, -leucine and -lysine were found in 1-AMB medium. The growth rate in this medium could, however, not be estimated because the cells began soon to attach to the surface of the glass flasks. This adhesion of cells to glass occurred more or less in all albumin containing media tested. The different albumin media were therefore essentially compared on the basis of incorporation rates of precursors of macromolecular synthesis into the acid insoluble material of the cells. This incorporation rates were evaluated from the data of short term incubation experiments, which allowed an exact determination of the cell density. The number of dead cells at the end of the first passage in 1-AMS- and 1-AMB media was found to be 3%, in parallel control cultures 2%. Removal of fatty acids from serum albumin by charcoal treatment resulted in a slight increase of attachment of the cells to glass.

pH at the beginning	7.2	7.5	7.8	8.0
pH at the end	6.85	7.2	7.5	7.8
Growth in %	18	23	17	11
% of dead cells	2	1	1	2
Lactate production ( $\mu\text{mol}/10^6$ cells)	5.52	6.00	6.52	6.41
Glucose uptake ( $\mu\text{mol}/10^6$ cells)	3.93	5.40	6.69	5.77
Lactate/glucose quotient	1.40	1.11	1.15	1.11
Thymidine incorporation in % of the controls at pH 7.5	47	100	51	50

Table I. Growth and metabolic activities of EAT cells in 1-AMS at different pH values within a period of 12 hours.

Studies on the effect of modification of the pH (7.2; 7.5; 7.8; 8.0) of the serum deprived, albumin containing media on growth and DNA-synthesis have shown a maximal cell proliferation and [ $^{14}\text{C}$ ]thymidine incorporation and lowest number of dead cells at physiological pH. The attachment of the cells to the glass was not significantly influenced by changes of the pH of the medium. Further details of the results of these experiments are summarized in Table I.

With regard to the trend of the cells in albumin medium to attach to the glass, we have examined the effect of permanent rotation of the flasks (0.25 rpm) on cultures grown in normal and modified media. In normal medium the rate of proliferation was not significantly influenced by rotation of the culture vessels, the number of dead cells increased from 2 to 4%. The attachment of the cells to glass observed in albumin media could not be prevented by rotation; they began to coagulate after a period of 6–8 hours.

Lactate production and lactate/glucose quotient in normal and 1-AMS medium were not significantly different within 12 hours:  $0.730 \pm 0.133 \mu\text{mol}$  ( $n=30$ ) and  $0.710 \pm 0.160 \mu\text{mol}$  ( $n=8$ ) lactate per  $10^6$  cells were produced in normal or 1-AMS medium; the corresponding values for the metabolism of glucose were  $0.592 \pm 0.098 \mu\text{mol}$  and  $0.557 \pm 0.153 \mu\text{mol}$

per  $10^6$  cells; the lactate/glucose quotient was calculated to be 1.23 and 1.27, respectively. Respiration was not significantly impaired in albumin medium (data not shown).

The incorporation of [ $^{14}\text{C}$ ]thymidine, [ $^{14}\text{C}$ ]uridine, [ $^{14}\text{C}$ ]leucine, and the uptake of [ $\alpha\text{-}^{14}\text{C}$ ]aminoisobutyric acid into the cells within the first passage (= 2. passage *in vitro*) in 1-AMS-, 1-AMB and in serum and albumin free medium (SAFM) is illustrated in Fig. 1–3: In the first seven hours DNA synthesis in 1-AMB is normal; then it declines to 40% of the controls after further 17 hours, while in 1-AMS from the beginning a continuous decrease of [ $^{14}\text{C}$ ]thymidine incorporation to about 20% at the end of the passage is observed (Fig. 1 a). The incorporation of [ $^{14}\text{C}$ ]uridine (Fig. 1 b) is far less impaired by the change of the medium than the incorporation of [ $^{14}\text{C}$ ]thymidine. The protein synthesis of the cells in 1-AMB passes through a maximum and declines only in the second half of the passage below the control values (Fig. 1 c). In principle the same trend appeared with [ $^{14}\text{C}$ ]lysine (not shown). In 1-AMS, however, the rate of incorporation of leucine does not differ significantly from the values in SAFM.

Though the protein synthesis in albumin media generally declines continuously in the second part of the passage, the consumption of leucine and isoleu-

	Consumption in nmol/ml cell suspension					% Growth $\frac{dN}{dt} \cdot \frac{1}{N} \cdot 100$
	Met	Ile	Leu	Tyr	Phe	
Normal medium	$18 \pm 4^*$	$170 \pm 10$	$166 \pm 10$	$21 \pm 3$	$23 \pm 3$	43
1-AMS	$16 \pm 1$	$220 \pm 22$	$223 \pm 25$	$22 \pm 2$	$23 \pm 8$	26
per cent of control	89	128	135	106	106	61

\* S.E.

Table II. Consumption of some amino acids by EAT cells in normal and 1-AMS within 24 hours. Cell density at the beginning was  $7.2 \times 10^5$  cells/ml ( $n=4$ ).

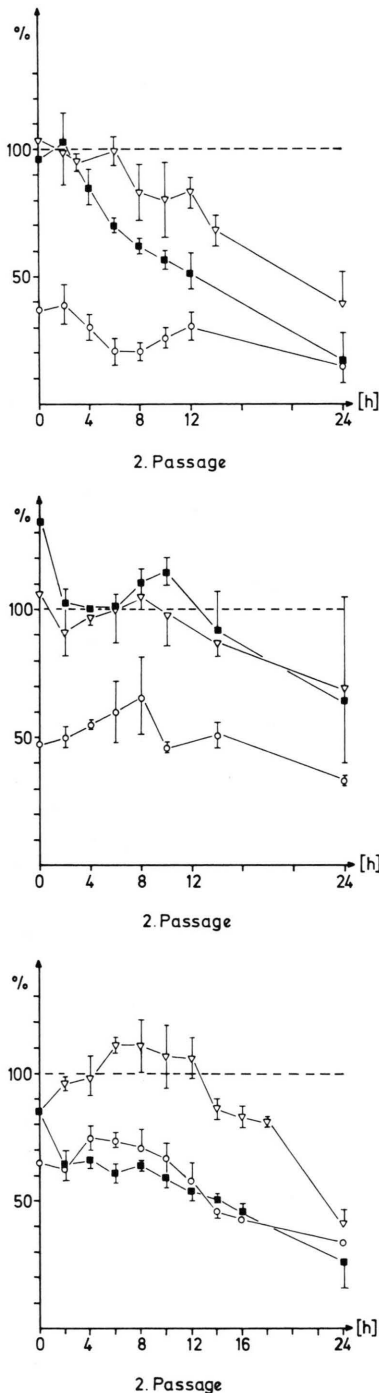


Fig. 1. Relative incorporation of label into acid insoluble fractions of EAT cells a) from [ $^{14}\text{C}$ ]thymidine, b) [ $^{14}\text{C}$ ]uridine, c) [ $^{14}\text{C}$ ]leucine,  $\circ$ — $\circ$  in serum and albumin free medium (SAFM),  $\blacksquare$ — $\blacksquare$  in medium supplemented with 1% bovine serum, albumin from Serva (1-AMS),  $\nabla$ — $\nabla$  in medium supplemented with 1% bovine serum albumin from Behring (1-AMB).

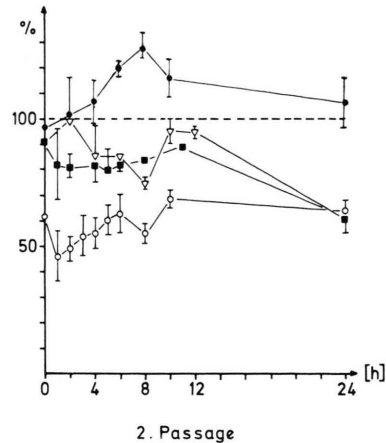


Fig. 2. Uptake of [ $\alpha$ - $^{14}\text{C}$ ]aminoisobutyric acid into acid soluble fraction of EAT cells.  $\circ$ — $\circ$  Cells grown in serum and albumin free medium (SAFM);  $\blacksquare$ — $\blacksquare$  cells grown in medium containing 1% albumin from Serva (1-AMS);  $\triangle$ — $\triangle$  cells grown in medium containing 1% albumin from Behring (1-AMB);  $\bullet$ — $\bullet$  uptake of cyclo-leucine by cells grown in medium with 1% albumin from Serva (1-AMS).

cine increases significantly as is revealed by the amino acid analyses of the media. From Table II it becomes evident that cells in 1-AMS take up 35% more leucine and 28% more isoleucine within 24 hours than in normal medium.

Obviously the cells in albumin media do not only use these amino acids for their protein synthesis but metabolize a considerable quantity of them.

The relative uptake of [ $\alpha$ - $^{14}\text{C}$ ]aminoisobutyric acid and [ $^{14}\text{C}$ ]cyclo-leucine by the cells grown in the modified media is depicted in Fig. 2. Within the first 12 hours the transport of [ $^{14}\text{C}$ ]aminoisobutyric acid is only weakly affected in 1-AMB and 1-AMS. An unexpectedly high transport of [ $^{14}\text{C}$ ]cyclo-leucine in the first passage was found with cells cultured in 1-AMS, values up to 150% of the controls were observed.

The behavior of the cells grown in serum and albumin free medium (SAFM) is characterized by a diminished transport activity for amino acids and a reduced biosynthetic activity even at the beginning of the cultures. Cells in this medium begin rapidly to attach to the glass and on shaking prone to coagulate. Their viability is impaired as becomes evident from the rise in the number of dead cells and an increase of LDH activity in the medium. The LDH activity over a 24 h period in albumin medium is twice and in SAFM three times as high as in normal nutrient medium containing 15% horse serum.



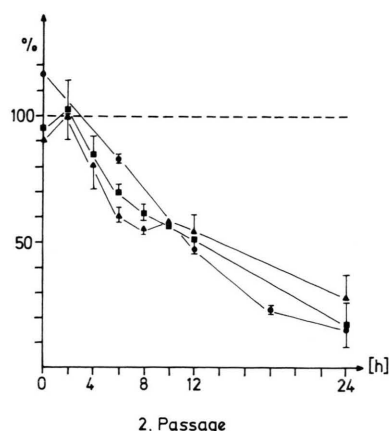


Fig. 3. [ $^{14}\text{C}$ ]thymidine incorporation of cells grown in media with different albumin concentrations of the same origin (Serva):  $\blacktriangle$ — $\blacktriangle$  0.5% (0.5-AMS);  $\blacksquare$ — $\blacksquare$  1% (1-AMS);  $\bullet$ — $\bullet$  2% (2-AMS).

#### Relationship between growth rate, metabolic activities and albumin concentration

It is well known, that the rate of proliferation of certain cells depends on the albumin concentration of the culture medium [20]. We therefore have compared cell growth and metabolic activities of EAT cells in 0.1-, 1.0- and 2-AMS. Table III shows that there were no significant differences in the proliferation rate of the cells in serum free media with different albumin concentrations. In 1-AMS and 2-AMS the number of dead cells was found to be 2–3%, in 0.5-AMS about 6.5%. The lactate/glucose quotients after a period of 12 hours were identical in all experiments. In good agreement with the proliferation kinetics of the cells, no significant differences of the [ $^{14}\text{C}$ ]thymidine incorporation pattern were found (Fig. 3) at various albumin concentrations. The same trend appeared with [ $^{14}\text{C}$ ]leucine: After a period of

12 hours the incorporation rate in 1-AMS was 50%, in 0.5-AMS 65% of the controls. The kinetics of the uptake of [ $\alpha$ - $^{14}\text{C}$ ]aminoisobutyric acid into the cells grown in media supplemented with different concentrations of bovine serum albumin are similar to those of the controls, the incorporation of label from the amino acid being about 25% lower after 12 hours.

Exposure of the cells to a medium containing 1% human serum albumin results in an excessive adhesion to glass, while their viability was not severely affected. The number of dead cells never exceeded 3%. The incorporation activity of the cells with respect to [ $^{14}\text{C}$ ]thymidine was noted to be about 50% of the controls.

#### Discussion

For optimal growth EAT cells in suspension culture require the presence of horse serum in the culture medium. Nutrient medium with 10% calf serum has been found to be less favourable [14]. To investigate the growth and metabolism of EAT cells in a chemically defined medium we have cultured the cells in serum free medium containing various lots of albumin. The experiments of the present paper are limited to studies of the cells in the first passage in albumin medium.

Obviously, growth, viability and biosynthetic activities of the cells grown in serum free medium supplemented with albumin depend on the purity of the albumin used. In all experiments DNA- and protein synthesis were between 60–80% higher in medium containing bovine serum albumin of the purest grade than in medium supplemented with Cohn-fraction V. The differences between the growth promoting activity of the various albumin lots tested could be reduced but not completely equalized by dialyzing the albumin samples [14].

It is well known that serum plays an important role in the prevention of adhesion of the cells to glass. A strong adhesion of cells to glass was therefore observed in serum and albumin free medium. Albumin of different origin could not prevent this undesired effect, which renders it impossible to determine the number of cells of the cultures. The most reliable parameter of cell growth in our experiments seems therefore to be the incorporation of radioactivity from [ $^{14}\text{C}$ ]thymidine into the acid insoluble fraction. Taking as a basis the incorporation of [ $^{14}\text{C}$ ]thy-

Table III. Growth of EAT cells  $\left( \frac{dN}{dt} \cdot \frac{1}{N} \cdot 100 \right)$  in nutrient media with different albumin concentrations in the second passage *in vitro*.

Time [h]	Normal medium	0.5-AMS	1.0-AMS	2.0-AMS
6	15 ± 5	8 ± 3	7 ± 3	11 ± 8
8	22 ± 8	12 ± 1	13 ± 3	—
10	29 ± 6	20 ± 2	17 ± 3	—
12	33 ± 14	—	—	24 ± 8
24	79 ± 8	35 ± 16	38 ± 10	38 ± 9
	(n = 30)	(n = 4)	(n = 12)	(n = 10)

midine and [ $^{14}\text{C}$ ]leucine into the cells, it is not possible to attain growth rates in albumin media higher than 50% of the controls in complete, serum containing medium.

Since it was not the purpose of our experiments to identify unknown growth factors present in the horse serum, our interest was limited to the elucidation of optimal growth conditions of the cells in serum free medium. We therefore have directed our attention specifically to the modification of some further parameters of the culture conditions, which would possibly support an optimal growth and prevent the attachment of the cells to glass. One of these parameters was the pH of the medium, which was changed between 7.2 and 8.5 without reducing the adhesion of the cells to glass. The significantly reduced incorporation of [ $^{14}\text{C}$ ]thymidine into the cells at nonphysiological pH values is suggested to be a consequence of a prolongation of the G1-phase as was shown by Fodge and Rubin with fibroblasts and embryonal cells [21]. The pH dependence of glucose uptake and glycolysis of cultured EAT cells described by Kaminskas [22] could be confirmed by our experiments.

Continuous rotation of the culture flasks, which improves the constant supply of the cells with nutrients and oxygen, could also not prevent the attachment to glass but rather increased this trend and favoured coagulation. Perhaps this effect results from an inadequate protection and stabilisation of

the cell membranes by albumin. As was emphasized by Healy and Parker [23] glycoproteins of the serum, which are not present in the albumin media are of special importance in this respect. As was shown by Ryser [24] the adsorption of albumin by the cells is very high, but only a small amount of the adsorbed protein is taken up into the cells. Of course albumin has a stabilizing effect on the membrane of the cells, which is believed to be optimal already at 0.5% albumin in buffer [3]. Our experiments have shown that optimal conditions for cells grown in nutrient medium are achieved at 1% albumin.

The most striking observation concerning the amino acid metabolism of the cells in serum free medium supplemented with albumin is the enhanced uptake of leucine and isoleucine. Since radioactivity from [ $^{14}\text{C}$ ]leucine does not appear in the proteins and lipids [14] in a proportional quantity, we conclude an augmented oxidation of branched chain amino acids to take place in albumin medium. This assumption is supported by observations from Neff [25] who found a strong interdependence between the metabolism of ketogenic amino acids and the composition of the culture medium. As yet it is essentially unknown how serum or albumin influences the uptake of amino acids by cultured cells [26].

Growth and metabolism of the cells in long term cultures containing albumin will be described in a following publication.

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