Differences in the Lipid Distribution in Subcellular Fractions of Mouse Fibroblasts Derived from Logarithmic and Stationary Growth

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(Z. Naturforsch. **31 c**, 179–185 [1976]; received October 27/November 20, 1975)

Lipid Distribution, Mouse Fibroblasts, Growth Curve, Subcellular Fractions

The lipid class compositions of subcellular fractions of SV40-transformed mouse fibroblasts derived from the logarithmic and stationary growth phase were compared. Cell populations of the stationary growth phase showed a relative decrease of the protein content and an increase of triglycerides and alkoxydiglycerides which could be located in the non-sedimenting fraction and in the nuclei and mitochondria containing fraction, respectively. Furtheron, distinct shifts in the subcellular distribution of those lipid classes could be observed which exhibited no relative overall increase or decrease when the cells of both growth phases were compared. In the crude plasma membrane fraction the ratio "lipid class/protein" remained about constant with the exception of the phospholipids and alkoxydiglycerides.

Introduction

The search for chemical differences characterizing distinct states of animal cells has become a field of central interest in biochemistry. The results of numerous studies provide substantial information on the biochemical and molecular changes which have become established in neoplastically transformed cells. However, much less is known about biochemical differences which characterize cells in the state of logarithmic growth and cells in the state of stationary growth.

Stimulated by the observation that various strains of mouse cells exhibited an increased content of triglycerides when they have reached the stationary growth phase [1], studies were now undertaken to correlate the accumulation of triglycerides with distinct subcellular fractions. The present paper describes investigations performed with a line of SV 40-transformed mouse fibroblasts which can be grown in suspension culture. Subcellular fractions of cells of the stationary growth phase were compared with those of logarithmically growing cells on the basis of lipid distribution. Special emphasis has been put on the adaption of a simple and rapid technique of cell fractionation to avoid secondary biochemical alterations.

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Material and Methods

Chemicals

L-[1-^{3}H]fucose, 1.5 Ci/mmol, and [6-^{3}H]thymidine, 21 Ci/mmol, were obtained from the Radiochemical Centre Amersham, England.

All Chemicals used were of analytical grade. Bis(4-nitrophenyl)-phosphate, 4-nitrophenylphosphate, 4-nitrophenol, stearic acid, glycerol monostearate and glycerol tripalmitate were purchased from E. Merck, Darmstadt; glucose-6-phosphate and NADH from Boehringer, Mannheim; cholesterol and cholesterol palmitate from Sigma Chem. Comp., St. Louis, USA. Solvents (E. Merck) were freshly distilled before use.

Deoxyribonucleic acid from calf thymus was purchased from Fluka, Basel, and bovine serum albumin was a gift from Behringwerke, Marburg.

Cells

STU-51A/232B mouse fibroblasts, a subline derived from SV 40-transformed embryonic cells of STU mice [1], were grown at 37 °C in suspension in 500 ml of growth medium containing 10% of heat inactivated fetal calf serum under an atmosphere of about 5% CO_2 in air with rapid stirring (670–700 rpm) in a 1 l flask. The starting cell population density was 1×10^6 cells/ml. Logarithmic growth of cells was maintained by appropriate dilution with fresh medium. The stationary growth phase was attained when cells were grown without further dilution.

The growth of cells was monitored by determination of the mitotic index using the chromosome spreading technique [2] as well as staining with cristal violet [3]. The determination of the S-phase, *i.e.* of

that part of the cell population which was actively synthesizing DNA, was carried out in a separate experiment: 10 ml portions of the cell suspension were subjected to a 15-min pulse of [^{3}H]thymidine (2 μCi/ml) and treated further as described by Prescott [4] and Stanners and Till [5].

For radioactive labeling the cells were grown in the presence of 1 mCi[^{3}H]fucose per 500 ml of cell suspension. The radiochemicals were added shortly before the cells started with logarithmic growth.

Cell disruption and fractionation

The zinc ion method as described by Warren and Glick [6] had to be slightly modified to achieve optimal cell fractionation with STU-51A/232B cells. The cells were harvested by centrifugation and washed three times with phosphate buffered saline, containing 10 mM non-labeled fucose, followed by one wash with 1 mM $ZnCl_2$ solution. The washed cells were resuspended in 1 mM $ZnCl_2$ solution to give a suspension of 5×10^7 cells/ml, which was incubated at room temperature for 15 min. The cell suspension was then cooled in an ice-bath and homogenized in a Dounce homogenizer. Homogenization was monitored by phase contrast microscopy.

Shortly before the homogenate was layered on to a discontinous gradient of 5, 40 and 50% sucrose solutions (w/w), composed of 7,7 and 1 ml, respectively, EDTA was added to the homogenate to give a final concentration of 0.5 mM. The gradients were centrifuged with $1000 \times g$ at 4 °C for 30 min. The resulting sucrose containing fractions were diluted with 5 volumes of water and the material of each fraction was sedimented to pellets by centrifugation at $100\,000 \times g$ for 60 min. Aliquots of each fraction were subjected to electron microscopy. The overlay fraction, which contained no sucrose, was lyophilized.

Enzyme assays

In order to characterize the fractions of the cell homogenate obtained after sucrose gradient centrifugation, the following enzyme marker assays were performed:

Alkaline phosphatase (EC 3.1.3.1) and phosphodiesterase (EC 3.1.4.1) were assayed as described by Wolff and Jones [7] and by Bosmann, Hagopian and Eylar [8].

NADH diaphorase activity (NADH: (acceptor) oxidoreductase, EC 1.6.99.3) was measured using the procedure described by Wallach and Kamat [9].

Glucose-6-phosphatase (EC 3.1.3.9) was assayed according to the method of Hübscher and West [10] and cytochrome c oxidase (EC 1.9.3.1) according to the method of Hageboom and Schneider [11].

Electronmicroscopy

Cell fractions were sedimented to pellets, fixed in 2.5% glutaraldehyde for 30 min, washed three times in phosphate buffered saline and embedded in 1% agar. Small pieces of the agar were post-fixed with 1% osmic acid solution and then dehydrated with 70% ethanol containing 2% uranyl acetate and with ethanol. The further procedure was analogous to that used by Atkinson and Summers [12]. The thin-sections were examined in a Siemens Elmiscope 1A electron microscope.

Isolation and fractionation of lipids

After ultrasonication at 10 kilocycles for 3 min the total lipids were isolated from the pellets and lyophilisates of the sucrose gradient fractions according to the partition procedure described by Folch, Lees, and Sloane Stanley [13]. The neutral and polar lipids were either preseparated by silicic acid chromatography followed by thin-layer chromatography according to the method of Snyder, Blau, and Morris [14] or directly separated by thinlayer chromatography according to the method used by Freeman and West [15]. The individual lipid spots were visualized by short exposure to iodine vapor, scraped off the thin-layer plates and eluted with chloroform/methanol = 4 : 1.

Colorimetric determinations

The glycerides and alkoxydiglycerides were determined by the method of Van Handel, Zilversmit, and Bowman [16], cholesterol and cholesterolesters by the method of Zlatkis and Zak [17], free fatty acids by the method of Lauwerys [18] and phospholipids by the method of Bartlett [19]. For the estimation of DNA the Dische-reaction as modified by Burton [20] was used and for protein measurements the Folin-reaction [21] was applied.

Determination of radioactivity

Portions of the radioactive samples were either dissolved in 1 ml water and then mixed with 9 ml of Bray's scintillation mixture [22] or directly dissolved in 10 ml of the scintillation mixture. The radioactivities were counted in a Packard-Tri-Carb scintillation counter using [^{3}H]fucose as external standards.

Results

Biological and chemical parameters of the logarithmic and stationary growth phase

In the logarithmic growth phase the cell population is in a steady state of growth in which the

fraction of cells in each stage of the life cycle as well as the duration of the stages are constant. The population doubling time (generation time) of STU-51A/232B mouse fibroblasts, grown in suspension culture, can be calculated from the linear portion of the growth curve given in Fig. 1. This growth curve

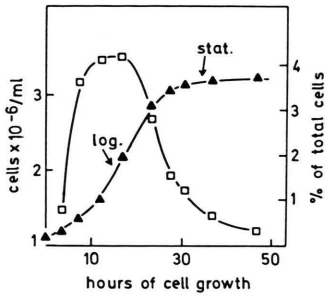

Fig. 1. Growth curve of a population of STU-51A/232B mouse fibroblasts grown in suspension culture. Population density (▲), mitotic index (□). The arows indicate the time of harvesting the cell population in the logarithmic and stationary growth phase, respectively.

refers to a cell culture grown without further dilution with fresh media. The generation time was found to be 15 h ± 5%. This value is consistent with the generation time which was estimated for suspension cultures maintained in the logarithmic growth phase by adequate dilutions with fresh medium.

The mitotic index of a cell population maintained in logarithmic growth was found to vary with the method used. The spreading technique [2] yielded a value of 4.0 ± 0.4% and a value of 3.3 ± 0.2% was obtained when the cells were stained with cristal violet [3]. In order to characterize the cell population exhibiting the growth curve given in Fig. 1, the mitotic index was determined at various times of cell growth by means of the spreading technique. The mitotic index obtained between 11 and 18 h of cell growth is compatible with the value found in logarithmically growing cell populations. In the stationary growth phase the mitotic index drops to about one tenth of the maximum value. The states of cell growth, marked by arrows in Fig. 1, represent the cell populations of the logarithmic growth phase and stationary growth phase, respectively, which were used for all comparative investigations of the lipid distribution in subcellular fractions.

The cell populations of both growth phases were further characterized by estimations of the fraction of cells which was actively synthesizing DNA. In the logarithmic growth phase, the percentage of cells which became labeled after a 15 min pulse of [^{3}H]-thymidine was found to be 69 ± 5%. This value was calculated from a set of five slides of the same experiment scoring a total of 5000 nuclei. Only those cells were taken as positive which showed more than 5 grains above their nuclei after a 14-day exposure of the Ilford K-5 emulsion. When the cell population had entered the stationary growth phase, the fraction of cells still showing active DNA synthesis at the given time (marked by the upper arrow in Fig. 1) was negligible. Less than 1% of the cells was then found to exhibit active DNA synthesis. During the whole growth period the fraction of damaged cells, *i. e.* cells stainable with trypan blue, was in the range of 2 – 4%.

From the point of logarithmic growth to the point of stationary growth phase, as indicated in Fig. 1, the cell population increased from 2.0×10^6 to 3.1×10^6 cells/ml cell suspension. In order to characterize the chemical differences which became established when the cell population had entered the stationary growth phase, aliquots of the cell population of each growth phase were analyzed. The overall increases of the individual lipid classes of the cell population present in 400 ml of the suspension culture of each growth phase are listed in Table I. In the period between the marked points of the growth curve (Fig. 1) the cell population had increased by a factor 1.55. Essentially the same factor was obtained when the contents of total phospholipids were compared. The ratios of the contents of cholesterol, mono- and diglycerides and free fatty acids were only slightly lower, but the ratios of the contents of protein and of the chole-

Table I. Cell population, protein content (mg) and lipid content (nmol) present in 400 ml of cell suspension of the logarithmic (log.) and stationary (stat.) growth phase.

		log. Phase	stat. Phase	Ratio: log. ph./ stat. ph.
cells		8.0×10^8	12.4×10^8	1.55
protein [mg]		21.0	27.2	1.29
phospholipids	[nmol]	5204	8158	1.57
cholesterol	"	1764	2546	1.44
cholesterolesters	"	356	356	1.00
monoglycerides	"	648	964	1.49
diglycerides	"	582	848	1.46
triglycerides	"	604	1256	2.08
alkoxydiglycerides	"	226	388	1.72
free fatty acids	"	1102	1654	1.50

sterolesters were significantly lower than the ratio of the cell populations. This would mean that either the synthesis of proteins and of cholesterolesters was diminished or their catabolic rates increased when the cell population had entered the stationary growth phase. On the other hand, the ratio of the contents of the alkoxydiglycerides appeared to be slightly, and that of the triglycerides drastically increased, when compared with the ratio of the cell populations.

In order to correlate these chemical differences with distinct subcellular fractions, the cell population of the logarithmic as well as of the stationary growth phase were subjected to homogenization and fractionation under the same conditions.

Fractionation of the cell homogenates

The procedure used for homogenization and fractionation of STU 51A/232B cells was technically simple and could be performed in a very short time thus minimizing secondary biochemical alterations. The procedure yielded fractions which still allowed to correlate differences in lipid distribution with distinct subcellular fractions. Among the methods generally used for isolation of plasma membranes, the zinc ion method [6] as compared to the Tris-method [6] appeared to have the advantage of a better stabilization of the plasma membranes of STU 51A/232B cells, most likely due to the inhibition of degradative enzymes as had been also suggested for the stabilizing effect of iodoacetate [12]. The discontinous sucrose gradient centrifugation of the cell homogenates yielded four fractions: the 50% sucrose cushion with the sediment, the 40% and the 5% sucrose fraction, and the fraction of non-sedimented material still present in the overlay. The fractions were characterized by phase contrast and electron microscopy and by enzyme marker assays using phosphodiesterase and alkaline phosphatase activity as markers for plasma membranes [23], NADH diaphorase and glucose-6-phosphatase activity as markers for the endoplasmic reticulum [24] and cytochrome c oxidase activity as a marker for mitochondria [24]. For chemical markers the DNA content, the incorporation of [^{3}H]fucose which had been reported to occur preferentially into plasma membranes [12, 25], and the molar ratio of cholesterol/phospholipids of each fraction were determined. The data are listed in Table II. The sediment contained only nuclei and mitochondria and, possibly, a few non-disrupted cells. Essentially all the DNA as well as the cytochrome c oxidase activity were found in this fraction. Membrane material was present in the fractions containing 5% and 40% sucrose but only in the 40% sucrose fraction predominantly membranes exhibiting a ribosome-containing "rind" on only one side were observed in the electron microscope (see Plate I A)*. This membrane fraction also showed the highest molar ratio of cholesterol/phospholipids, a very high specific incorporation of [^{3}H]fucose and a characteristic enrichment of the phosphodiesterase and alkaline phosphatase activities. Since, simultaneously, the activities of the enzyme markers specific for the endoplasmic reti-

* Plate I see on page 182 a.

Table II. Characterization of the fractions obtained after discontinous sucrose gradient centrifugation of the homogenate of logarithmically growing cells by enzyme markers and chemical markers. Specific enzyme activities are given in μmol of reaction product/h per mg protein at 37 °C if not stated otherwise. All values are means of the data of four experiments except for [^{3}H]fucose incorporation.

Markers	Homogenate	Fractions 0% Sucrose	5% Sucrose	40% Sucrose	Sediment
phosphodiesterase	0.29	0.55	0.24	0.65	0.13
alkaline phosphatase	0.20	0.59	0.37	0.66	0.09
NADH diaphorase	8.4	6.6	13.8	3.0	9.0
glucose-6-phosphatase	0.35	0.60	0.85	0.25	0.35
cytochrome c oxidase (min × mg)$^{-1}$	16.2	<0.2	<0.4	<0.3	28.2
% protein distribution	100 (100)[a]	23.8 (27.9)[a]	10.0 (10.2)[a]	13.2 (14.6)[a]	53.0 (47.3)[a]
μg DNA/mg protein	130	<2	<4	<5	250
^{3}H-Fucose (cpm × 10^{-3}/mg)	14	16	11	35	11
cholesterol/phospholipids (molar ratio)	0.32	0.30	0.25	0.56	0.29

[a] % Distribution in cellular fractions derived from the stationary growth phase.

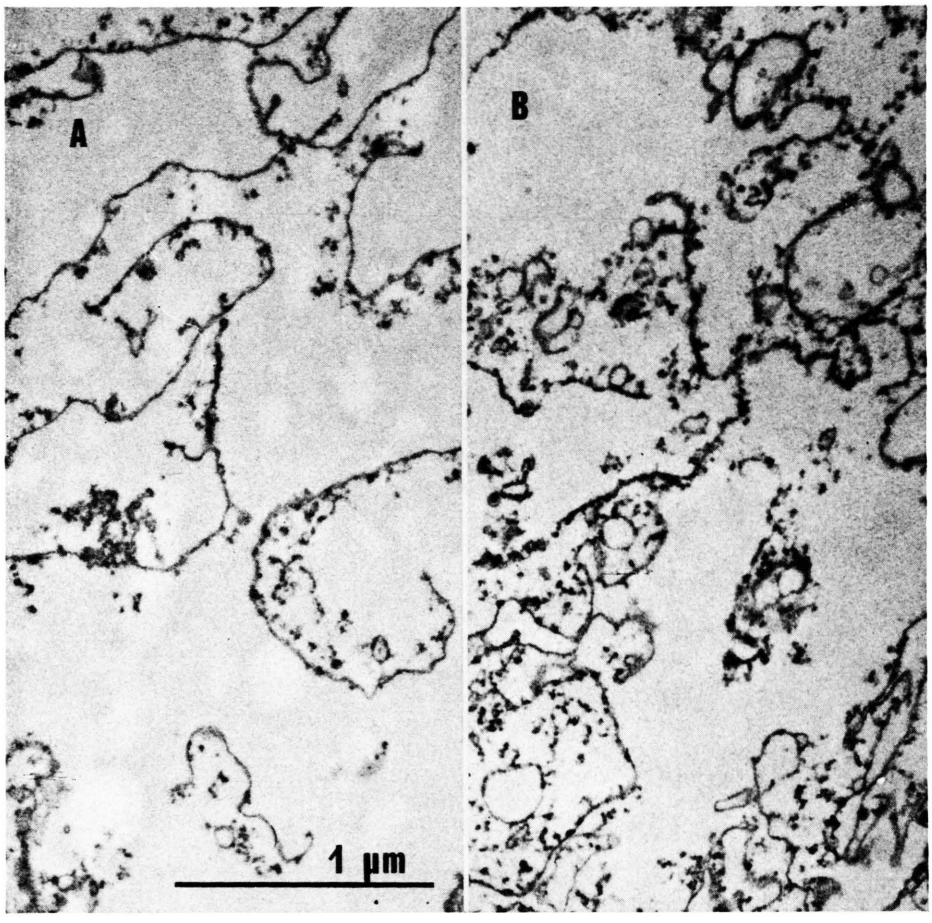

Plate I. Membrane containing fractions obtained after sucrose density gradient centrifugation of the cell homogenate: A. Membrane fraction isolated from the 40% sucrose layer (× 35 000), B. Membrane fraction isolated from the 5% sucrose layer (× 35 000). Bar: 1 µm.

culum were found to be very low, this fraction represented a "crude plasma membrane fraction". The maxima of the specific activities of NADH diaphorase and of glucose-6-phosphatase were measured in the 5% sucrose fraction. Electron microscopy of this fraction showed that most of the membranes had a layer of ribosome like structures on both sides (Plate I B), which indicated that the membranes preferentially derived from the endoplasmic reticulum. The amount of plasma membranes still present in this fraction must be low, since the ratio of cholesterol/phospholipid and the [^{3}H]fucose incorporation as well as the corresponding enzyme marker activities were drastically diminished. In the non-sedimenting material, *i. e.* the overlay fraction without sucrose, no typical membrane structures could be demonstrated.

The data of enzyme marker activities and of the ratio of cholesterol/phospholipid, as given in Table II for the subcellular fractions of logarithmically growing cells, were compatible within ± 12% with the data obtained with cells derived from the stationary growth phase.

Distribution of lipids

The content of lipids of distinct classes were determined for each of the four fractions which have been characterized in the preceding chapter. All the analytical data given in Table III are expressed in nmoles per mg protein. The numerical comparison of the data which refer to the cell populations of the logarithmic and the stationary growth phase, respectively, is complicated by the fact that the increase of the amount of protein in the period between both points of the growth curve (Fig. 1) did neither parallel the increase of the cell number nor the increase of most of the lipid classes (see Table I).

This difficulty can be overcome by comparing the ratios R of the specific contents of the lipid classes in corresponding fractions of cell homogenates derived from the logarithmic and stationary growth phase. The ratios R given in the Table III for the homogenates correspond essentially to those which can be calculated from the factors of increase of lipid classes and protein listed in Table I.

The major chemical differences which could be demonstrated in the cell populations of the logarithmic and stationary phase (see Table I) can now be located in subcellular fractions (Table III). In the stationary phase the triglycerides were accumulated mainly in the non-sedimenting material and partly in the sediment and the alkoxydiglycerides predominantly in the sediment. Changes in the distribution were observed in the following lipid classes: monoglycerides showed a relative increase in the non-sedimenting material and in the fraction containing the endoplasmic reticulum but a decrease in the sediment, diglycerides a relative increase in the sediment, and free fatty acids a drastic relative increase in the non-sedimenting material which appears to be compensated by a decrease in the sediment. An increase of the relative content of the phospholipids was found in the fraction of the non-sedimenting material as well as in the fractions containing the endoplasmic reticulum and the plasma membranes but a slight decrease in the sediment fraction. The cholesterolesters, which showed no overall increase in the cell populations (see Table I), revealed differences in their relative content in

Table III. Distribution of lipids in the fractions obtained after sucrose gradient centrifugation of homogenates derived from cell populations of the logarithmic (log.) and of the stationary (stat.) growth phase. The lipid contents are given in nmol per mg protein. R represents the ratio "stat./log." of the specific contents of a lipid class in corresponding fractions.

Fractions (% sucrose)	Homogenate			0			5			40			Sediment		
Growth phase and ratio R	log.	stat.	R	log.	stat.	R	log.	stat.	R	log.	stat.	R	log.	stat.	R
phospholipids	249	300	1.21	159	234	1.47	342	488	1.43	360	512	1.42	243	233	0.96
cholesterol	84	94	1.12	55	60	1.09	94	106	1.13	201	229	1.14	67	69	1.03
cholesterolesters	17	13	0.77	27	21	0.78	26	15	0.58	12	11	0.92	12	9	0.75
monoglycerides	31	36	1.15	26	40	1.54	30	69	2.30	31	30	0.97	33	27	0.82
diglycerides	28	32	1.13	40	41	1.03	62	56	0.90	40	39	0.98	13	18	1.38
triglycerides	28	46	1.61	30	67	2.23	57	61	1.07	32	29	0.91	22	36	1.63
alkoxy-diglycerides	11	14	1.33	12	11	0.92	25	22	0.88	13	16	1.23	7	13	1.86
free fatty acids	52	61	1.16	42	97	2.31	64	87	1.36	44	46	1.05	58	39	0.67

single subcellular fractions: *i. e.* maximal decrease in the fraction containing the endoplasmic reticulum and scarcely any change in the crude plasma membrane fraction. It is noteworthy that in the crude plasma membrane fractions of both growth phases the ratio of "lipid/protein" of most lipid classes maintained constant with $R \sim 1$ except for the phospholipids and the alkoxydiglycerides.

Discussion

A valuable consideration of comparative studies of chemical parameters of biological significance is dependent on well defined states of the biological system. We choose a strain of mouse fibroblasts which would be grown in suspension culture thus offering some technical advantages. Cell populations in suspension cultures are better to handle than those kept in culture dishes, cell growth is easier to monitor and all manipulations performed with the cells can be made without trypsination. In the logarithmic growth phase the cell population exhibited a mitotic index and a percentage of DNA synthesizing cells comparable to other animal cells whereas in the stationary phase the cells ceased to synthesize DNA and the mitotic index decreased to less than one tenth of that found in the logarithmic phase.

The major chemical differences, which became established in the stationary phase with respect to the lipid content, have been found to reside in the accumulation of triglycerides and alkoxydiglycerides. The correlation of these differences with distinct subcellular fractions is mainly dependent on the quality of cell fractionation. Although the technique of fractionation used in this work yielded only crude subcellular fractions, it allows to distinguish between the fraction containing nuclei and mitochondria, that containing preferentially plasma membranes, another one containing predominantly the endoplasmic reticulum and a fraction with non-sedimenting material.

The accumulation of the triglycerides and alkoxydiglycerides found in the stationary phase can be located in subcellular fraction when the specific lipid contents are compared (see Table III). Accumulation of triglycerides occurred in the non-sedimenting material, most likely due to the formation of triglyceride liposomes in the cell, and accumulation of alkoxyriglycerides was found in the nuclei and mitochondria containing fraction. Besides the accumulation of the triglycerides and the alkoxydiglycerides in the stationary phase, distinct shifts could be observed in the subcellular distribution of those lipid classes which exhibited essentially no relative overall increase or decrease when the intact cells were analysed. After fractionation of the cell homogenates free fatty acids and monoglycerides were found to increase in the non-sedimenting material and in the endoplasmic reticulum fraction but to decrease in the nuclei and mitochondria containing fraction, whereas diglycerides showed only a slight increase in the nuclei and mitochondria fraction.

All these differences are most likely a result of complex regulation mechanisms which can be interpreted as criteria of the stationary growth phase. One criterion of primary importance may be seen in the fact that the relative protein content of the stationary cell population was distinctly lower than that of the logarithmically growing cells population (see Table I). This may imply a diminished synthesis of enzymes as well as of structural proteins. Cell populations in the stationary phase, as given in Fig. 1, are still capable of starting logarithmic growth when the suspension culture is diluted with fresh medium. Cell populations, however, which were maintained in the stationary phase about 15 – 20 h longer than indicated in Fig. 1 for the harvest of the stationary cell population, show then a rapid increase of the number of damaged or dead cells. This phenomenon must be due to further cellular alterations, most likely due to a regulatory break down.

The authors wish to thank Mrs. R. McWilliams and Mr. G. Berger for the preparation of the electron microscopical probes.

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