Osmotic Concentration of the Contractile Vacuole of *Amoeba proteus* Following UV-Light Irradiation

The contractile vacuole of *Amoeba proteus* exhibits an atypical appearance, an enlargement in size, and altered contraction rate after micrurgical removal of the nucleus or after the *Amoeba* has been irradiated with UV-light[1,2,3]. Since these changes in the contractile vacuole are rapid with respect to other changes in the cell[1,4-6], it suggests that UV-irradiation may affect the osmoregulatory function of the contractile vacuole. In this paper we report on the osmotic concentration of the contractile vacuole following UV-irradiation.

Materials and methods. The methods for culturing and for UV-irradiation at 254 nm of *A. proteus* have been described[7]. The milliosmolal concentration of the contractile vacuole was determined by a modification[8] of the method described by SCHMIDT-NIELSEN and SCHRAUGER[9].

Results and discussion. About 1.0% of a population of unirradiated cells have enlarged contractile vacuoles, whereas after UV-irradiation about 10% have enlarged contractile vacuoles[1,8]. The milliosmols of the enlarged and unenlarged contractile vacuoles in the control cells were not statistically different (Table). The milliosmolal concentrations for the contractile vacuole contents of the control cells were larger than those reported by SCHMIDT-NIELSEN and SCHRAUGER[9]. This may be due to the different culturing conditions and strains of *A. proteus* used in the 2 investigations.

The UV-dosages used decreased the survival of the cells and altered the function of the nucleus[7]. Although UV-irradiation results in a larger % of the cells having enlarged contractile vacuoles, there is no statistical difference between the milliosmolal concentrations of the enlarged and unenlarged contractile vacuoles of the controls and the irradiated cells.

The similarity of the milliosmolal concentrations in the contractile vacuole between the controls and the irradiated cells suggests that the UV-light may not be affecting the mechanism(s) by which the contractile vacuole contents are regulated. However, there could be an altered ratio of ions, or non-electrolytes, which would not be detected by this method. In summary, UV-irradiation does not result in a detectable altered milliosmolal concentration of the contractile vacuole.

Zusammenfassung. Nach UV-Bestrahlung erweist sich die osmotische Konzentration der pulsatilen Vakuolen von *Amoeba proteus* als unverändert.

Milliosmolal concentration of the contents of enlarged and unenlarged contractile vacuoles of *Amoeba proteus*

	Unenlarged vacuoles	Enlarged vacuoles
Control	68 (30[a]) (30.33[b])	56 (12[a]) (18.61[b])
1000 ergs/mm²	76 (15) (28.56)	80 (15) (29.02)
2500 ergs/mm²	77 (40) (40.62)	49 (15) (16.90)

[a] = No. of samples. [b] = standard deviation of the mean.

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The Production of Stable Steady-States in Mouse Ascites Mast Cell Cultures Maintained in the Chemostat[1]

Sustained growth of randomly phased (asynchronous) fluid-suspension cultures of unicellular micro-organisms in a constant chemical environment, at a constant cell concentration and at a dictated exponential rate, has been achieved in the chemostat[2-9]. Similar attempts with animal cells have been only partially successful[10], limiting the usefulness of the chemostat for the biochemical, physiological and genetical analysis of animal cells in vitro. In this article we summarize chemostat experiments with neoplastic mouse mast cell cultures in which true stable steady-states were established.

The chemostat[3,10] is a device for growing cell cultures in steady-state[11] at preset doubling rates which are smaller than the maximum specific growth rate and which are controlled by one or more (limiting) nutrient factor(s). In essence the chemostat is an agitated culture vessel with an overflow setting the culture volume. Fresh nutrient liquid enters the culture vessel at a constant rate (dilution rate) and cell suspension, including supernatant medium, leaves the culture vessel, under ideal conditions of operation, at the same rate (specific washout rate). In the experiments reported here 2 types of automated mammalian-cell chemostats were used, models I and II, differing only in the design of the culture vessel and the stirring mechanism. Model I, which is an adaptation of the cytogenerator[12] to the principle of the chemostat, consists of a 2-arm culture vessel with an overflow on one arm and an inlet port for liquid nutrient on the other arm. Premixed gases (air, CO_2) under moderate pressures are alternately pulsed to the 2 arms of the growth chamber, accomplishing both agitation of the cell suspension and equilibration of the liquid phase with O_2 and CO_2 (pH control). Model II consists of a round reaction flask-type culture vessel with an overflow, a suspended inlet tube for the afferent nutrient stream which is broken by a dropping device, and a Vibro-Mixer,

Model E1 stirring device[13], and also inlet and outlet ports for the gas mixtures.

All the experiments reported in this article were carried out with the P815Y mouse ascites mast cell strain[14]. This cell strain grows, upon explantation from CBA/2 mice into adult horse serum supplemented FISCHER's growth medium, freely suspended and continues to express, in long-term culture, the characteristic properties of mast cells, namely serotonine, histamine and heparin synthesis.

In all our experiments the liquid medium introduced into the storage tank of the chemostat contained the 12 essential amino acids, L-glutamine, the 9 essential vitamins, D-glucose, and the essential electrolytes minus $CaCl_2$, and was supplemented with asparagine, L-serine, 10% adult horse serum, 100 U/cm³ of penicillin G potassium and 2.5 μg/cm³ of tetracycline. Except for the variable amount of D-glucose (50–300 mg%) the medium composition was that prescribed for murine leukemic cells by FISCHER[15]. During the entire course of the chemostat experiments the afferent medium was cooled and kept stored at a constant temperature (9–11 °C) in the dark and under a fixed partial pressure of CO_2.

Theoretically, under certain restricting conditions[16], stable steady-states of fluid-suspension cell cultures in the chemostat are possible at any value of the dilution rate which is smaller than the maximum doubling rate of the cell strain in the type of nutrient medium used. Mathematical proof of this theorem has been given for ideal bacterial culture systems[5–7,17]. The chemostat experiments of COHEN and EAGLE[10], performed with a variety of mammalian cell strains, failed to yield true thermodynamic steady-states at small dilution rates; the cell concentration could not be stabilized properly in these experiments and often varied in a cyclical pattern. In our own chemostat experiments, performed with P815Y cell cultures and conducted over periods of up to 5 months, we have now obtained evidence of true stable steady-states at dilution rates as small as $\omega = 0.0057$ h^{-1} (doubling time of culture, $\tau^* = 122$ h; Figure 1) and as large as $\omega = 0.022$ h^{-1} (doubling time of culture, $\tau^* = 31.5$ h; Figure 4). These steady-states are characterized by (1) a constant concentration of cells ($\bar{N}$ cells/cm³), as high as $18.2 \cdot 10^5$ cells/cm³,[18] (2) constant concentrations in the supernatant culture fluid of D-glucose ($\bar{c}_G$ mg%) and of L(+)-lactate ($\bar{c}_L$ mg%), and hence (3) a constant D-glucose to L(+)-lactate net conversion factor, (4) a constant doubling rate (specific growth rate, $\bar{k}^* = \omega = (\ln 2)/\bar{\tau}^*$ h^{-1} of the cell culture, equal to the dilution rate, ω, (5) constant rates per cell of D-glucose consumption ($\bar{Q}_G$ g cell^{-1} h^{-1}) and of net L(+)-lactate production ($\bar{Q}_L$ g cell^{-1} h^{-1}), (6) a constant cell population composition with respect to the fractions of cells in DNA synthesis, in observable mitosis and in RNA synthesis, according to the instantaneous thymidine-H³ labelling index ($\bar{J}_S$), the mitotic index ($\bar{J}_M$) and the instantaneous cytidine-H³ or uridine-H³ labelling index ($\bar{J}_{RNA}$). In a typical case (Figure 3) stabilization of culture growth was attained at a dilution rate of $\omega = 0.0123$ h^{-1}, a dilution rate which corresponds to a

[1] This investigation was supported by the Office of Naval Research under contract No. Nonr-266(76) and by the Health Research Council of the City of New York under contract No. I-428, and was carried out at Columbia University (Department of Biochemistry).

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[11] The steady-state is defined as a condition in an open system, in which the time derivatives of all concentrations of reactants (metabolites and cells, in our case) vanish while the flow of materials into and from the system continues at fixed rates. If such a condition maintains itself indefinitely, it is defined as a stable or true, thermodynamic, steady-state.
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[16] These conditions are: (1) Thermodynamic equilibrium of the molecular and macromolecular compounds in the incoming (i.e. stored or afferent) nutrient liquid (chemical stability of the afferent medium); (2) constant physical environment of the reaction mixture and culture vessel (constant temperature, illumination, etc.); (3) continuous transport at fixed rates of the incoming and the outgoing stream of liquid nutrient and gases into the culture and from the culture respectively; (4) complete mixing of the reactants in the culture vessel; (5) constant culture volume; (6) limitation of culture growth rate by one or more growth factors (substrates); (7) capacity of the cell strain for strictly asynchronous cell proliferation; (8) genetic stability of the cell strain, i.e. absence of population change-overs.
[17] C. C. SPICER, Biometrics *11*, 225 (1955).
[18] Ordinary batch cultures (stationary, sealed bottle cultures) of cell strain P815Y grow up to a maximum cell concentration of only $5 \cdot 10^5$ cells/cm³.

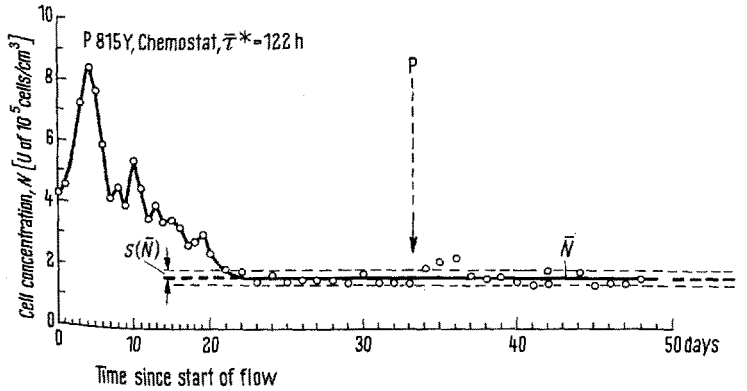

Fig. 1. Adjustment and maintenance of stable steady-state at a small dilution rate in a Model I chemostat. (In this case the afferent medium was stored at room temperature; 'P' indicates minor and temporary alteration of pH.)

doubling time of $\tau^* = 56.5$ h or 2.36 days. Under optimum conditions mouse ascites mast cell cultures double in the same medium every 12 h, that is about 4.7 times faster than during the prescribed steady-state. In this case the steady-state is demonstrated by a constant cell concentration maintained at $\bar{N} = 8.76 \cdot 10^5$ cells/cm³ (standard deviation of $\bar{N}$, $s(\bar{N}) = \pm 0.65 \cdot 10^5$)[19] for a period of 60 days[20], a constant D-glucose concentration in the supernatant medium of $\bar{c}_G = 2.36$ mg% (standard deviation of $\bar{c}_G$, $s(\bar{c}_G) = \pm 0.46$)[21], a constant L(+)-lactate concentration maintained in the supernatant culture fluid at $\bar{c}_L = 32.32$ mg% (standard deviation of $\bar{c}_L$, $s(\bar{c}_L) = \pm 3{,}23$)[22], and a constant population composition of about $100 \cdot \bar{J}_S = 26\%$ of the cells engaged in DNA synthesis. Since the concentrations, in the afferent nutrient

[19] Estimate of error of measurement of cell concentration, $\pm 0.56 \cdot 10^5$ cells/cm³.

[20] Steady-state interrupted on purpose.

[21] Estimate of error of enzymatic assay of D-glucose concentration, ± 0.27 mg%.

[22] Estimate of error of enzymatic assay of L(+)-lactate concentration, ± 1.58 mg%.

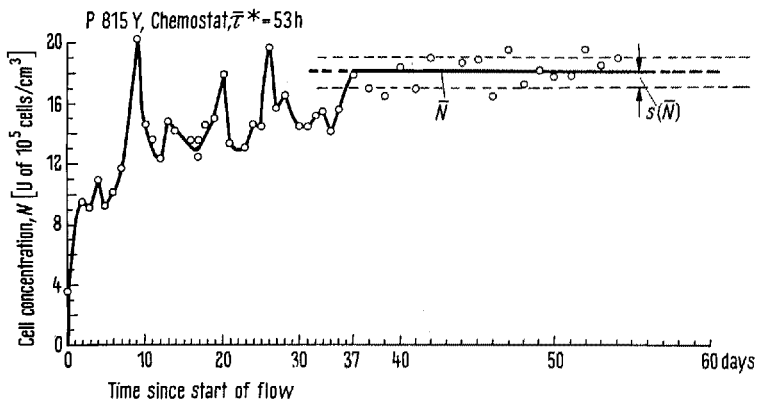

Fig. 2. Adjustment and maintenance of stable steady-state at a relatively large dilution rate in a Model I chemostat.

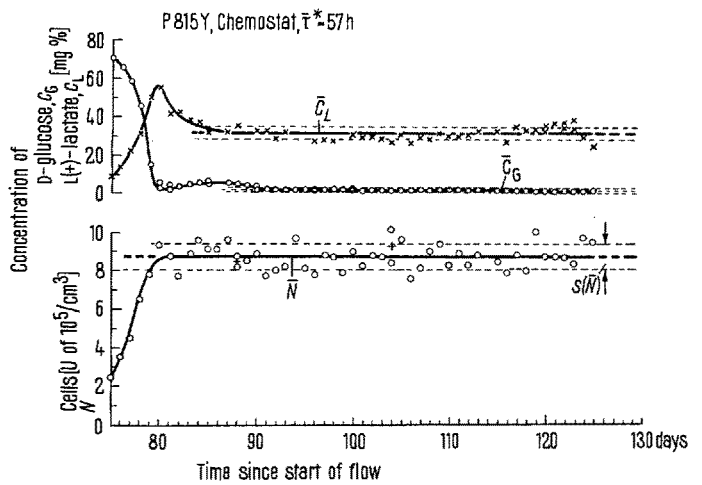

Fig. 3. Final readjustment and maintenance of stable steady-state in a 'perturbation of steady-state experiment' in a Model I chemostat. (The initial steady-state was interrupted in this case by addition to the culture fluid and to the stored medium of 20 mg% of D-glucose 1 month after the start of flow.)

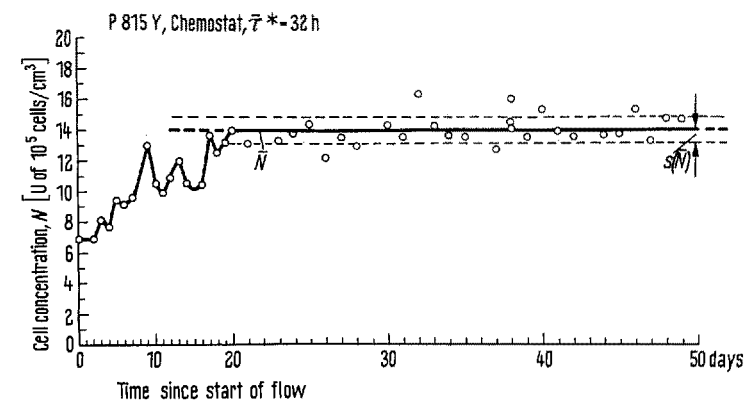

Fig. 4. Adjustment and maintenance of stable steady-state in a Model II chemostat.

stream, of D-glucose and of L(+)-lactate were kept in this case at $a_G = 83.32$ mg% and $a_L = 3.40$ mg% respectively, the specific rate of D-glucose consumption averaged during the steady-state $\overline{Q}_G = \omega(a_G - \overline{c}_G)/\overline{N} = 1.13 \cdot 10^{-11}$ g cell^{-1} h^{-1} and there was a net L(+)-lactate production in the culture averaging $\overline{Q}_L = \omega(\overline{c}_L - a_L)/\overline{N} = 0.33 \cdot 10^{-11}$ g cell^{-1} h^{-1}. Hence, we have, during this steady-state, a constant factor of net conversion of D-glucose into L(+)-lactate of $100 \cdot (\overline{Q}_L/\overline{Q}_G) = 35.5\%$ (pH = 6.9).

Stability of the steady-state condition in our experiments was indicated by minor oscillations of the culture parameters (N, c_G, c_L) around their mean steady-state positions ($\overline{N}$, $\overline{c}_G$, $\overline{c}_L$). Since the experimental error of the assays of cell concentration, D-glucose concentration and L(+)-lactate concentration were of the same magnitude as the standard deviations of the steady-state averages, $\overline{N}$, $\overline{c}_G$ and $\overline{c}_L$ respectively, the fundamental errors of the actual steady-state positions were insignificant and, therefore, probably the result of random oscillations. In the one case, where a larger than normal oscillation of cell concentration was observed (Figure 1), this event could definitely be related to an instrumental error (pH change due to change of CO_2 tanks).

In P815Y cultures inoculated into chemostats containing freshly prepared standard FISCHER's growth medium, stable steady-states are established only after very extensive adjustment periods (Figures 1, 2 and 4). Significant oscillations of the cultural parameters extend over periods ranging between 20 and 50 days of continuous operation of the chemostat. The duration of the adjustment period tends to decrease with increasing dilution rate. Undoubtedly the existence of such long adjustment periods in mammalian cell cultures has contributed to the failure of previous investigators[10] to observe stabilization of steady-states in the chemostat at small dilution rates.

The steady-states of mouse ascites mast cell cultures are extremely sensitive, and are readily displaced by alteration of practically any cultural parameter. Abrupt changes of metabolite concentration(s) in the culture fluid of the chemostat or in the incoming nutrient fluid (for example by addition of a few mg% of D-glucose to the culture fluid), alterations of the medium composition (for example by addition of minute amounts of thymidine to the culture fluid), or sudden change of pH, generate complex oscillations in the quantities measured, that is of N, c_G, c_L, Js, JM, JRNA, degree of culture asynchrony etc. During perturbation of steady-state the final adjustment to the original or to a new stable steady-state position is often preceded by short-lived semi-stable steady-states with a smaller yield of de-novo cell synthesis.

Because of their extreme sensitivity, steady-state P815Y cell cultures maintained in the chemostate are a far more efficient device for studying the effects of growth factors, hormones, antimetabolites and other drugs on mammalian cells in vitro than are ordinary log phase batch cultures[23].

Zusammenfassung. Neoplastische Mausmastzellen des Stammes P815Y wurden auf lange Dauer im Chemostaten gezüchtet. Solche Versuche, in denen es erstmals gelang, stabile stationäre Zustände des Zellkulturenwachstums auch bei sehr kleinen Durchfluss- bzw. Verdünnungskonstanten zu erzeugen, werden eingehend beschrieben.

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Significance of 'Empty Vesicles' in Postganglionic Sympathetic Nerve Terminals

Electronmicroscopic examination of postganglionic autonomic nerves reveals essentially 2 types of nerve endings: (a) parasympathetic nerve endings which contain a homogeneous population of 'empty vesicles', which are most probably the storage sites of acetylcholine, and (b) sympathetic nerve endings which possess a mixed population of 'empty vesicles' and of 'dense core vesicles'. The dense core vesicles most probably contain norepinephrine[1]. The functional significance of the empty vesicles in sympathetic nerve terminals (or preterminals) is not clear. It has been assumed that they contain acetylcholine, thus representing the morphological correlate[2] of a cholinergic link in postganglionic sympathetic transmission postulated by BURN and RAND[3].

The validity of this assumption was investigated in the present experiments on the cat's iris. Irides were removed from cats anaesthetized with pentobarbital and immediately fixed in 2% OsO_4 buffered at pH 7.4 with 0.1 M phosphate buffer for 1–2 h at 4 °C. After dehydration with alcohol and propylene oxide the irides were imbedded in epon 812. Ultra-thin sections were contrasted with uranium acetate and lead citrate.

Electronmicroscopic examination was concentrated on the medium zone of the dilatator pupillae, as it is known from fluorescence microscopic studies[4] that this region contains a large number of sympathetic nerves.

We were therefore surprised to find that virtually all vesicles of the autonomous nerves were empty (Figure 1). The preparations did not permit distinction between cholinergic and adrenergic nerves. To decide whether these findings were related to the mode of fixation, i.e. the classical technique of osmium fixation, irides were fixed in 3% glutaraldehyde[5] buffered with 0.1 M phosphate buffer (pH 7.4) for 1–2 h at 4 °C before treating them identically with osmium tetroxide.

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