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.

H. MOSER[24] and G. VECCHIO[25]

Station de Zoologie expérimentale de l'Université, 1200 Genève (Switzerland), October 1, 1966.

[23] Acknowledgments: The authors deeply appreciate the technical help of Dr. S. GRAFF and Mr. FRANK LUM in setting up apparatus.

[24] Recipient of Career Scientist Award of the Health Research Council of the City of New York under Contract No. I-428. Present address: Station de Zoologie expérimentale de l'Université de Genève, 154 route de Malagnou, Chêne-Bougeries, Geneva, Switzerland.

[25] Present address: Istituto di Patologia Generale, Università di Napoli, S. Andrea delle Dame, Naples, Italy.

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.

[1] D. E. WOLFE, L. T. POTTER, K. C. RICHARDSON and J. AXELROD, Science *138*, 440 (1962).

[2] J. H. BURN, Bull. Johns Hopkins Hosp. *112*, 167 (1963).

[3] J. H. BURN and M. J. RAND, Nature *184*, 163 (1959).

[4] T. MALMFORS, Acta physiol. scand. *65*, 259 (1965).

[5] D. D. SABATINI, K. BENSCH and R. J. BARRNETT, J. biophys. biochem. Cytol. *17*, 19 (1963).

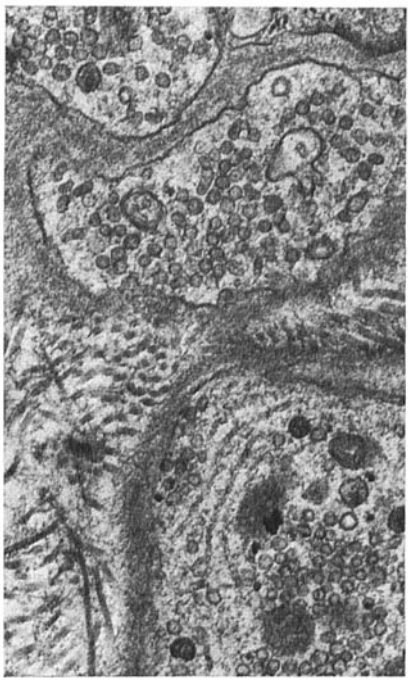

Fig. 1. Cat's iris, medium zone of the dilatator pupillae. OsO_4 fixation. Sections of autonomic nerves (preterminals) containing mostly 'empty vesicles'. ×32,000.

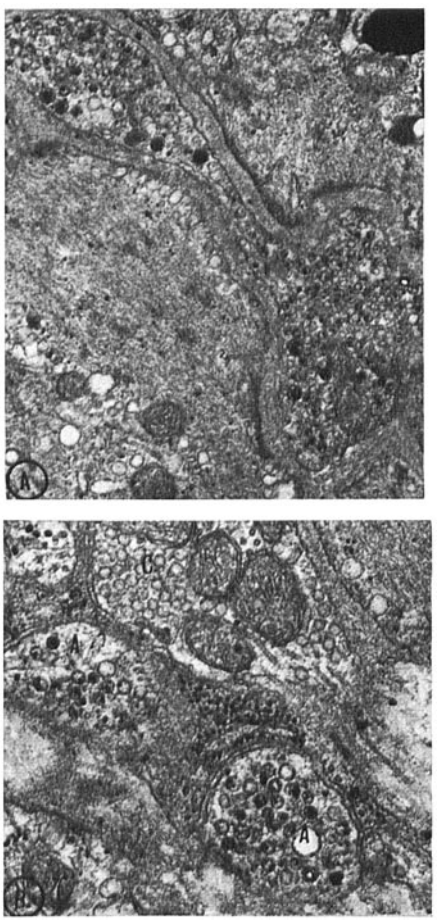

Fig. 2. Sections of cat's iris after in vitro incubation with norepinephrine and double fixation (glutaraldehyde and OsO_4). (a) Adrenergic nerve section containing mostly 'dense core vesicles'. × 20,000. (b) Closely related adrenergic (A) and cholinergic (C) nerve fibres. In the former virtually all the vesicles contain osmiophilic material, whereas in the latter all the vesicles remain empty. × 32,000.

Using this double fixation, many nerve terminals were now found to contain a large number of dense core vesicles. Thus it appears that the technique of fixation is of primary importance. This follows also from the fact that another biogenic amine, serotonin, can easily be demonstrated in blood platelets after glutaraldehyde fixation, but not after osmium fixation[6]. Although the proportion of vesicles with dense cores is much greater after combined fixation with glutaraldehyde and osmium tetroxide than after osmium fixation alone, we do not know whether all amines are completely retained by this technique. Even after this improved fixation the population of vesicles remained mixed. Many vesicles in sympathetic nerves did not contain any osmiophilic material, and with morphological criteria they could not be distinguished from the empty vesicles of parasympathetic nerve terminals.

To determine whether empty and dense core vesicles of sympathetic nerve terminals represent only momentarily differing states of function, i.e. differences in amine filling, or whether only a part of the vesicles is able to store amines, the following experiments were performed. Irides from cats pretreated i.p. with 50 mg/kg of pargyline 8 h earlier were incubated for 30 min at 37 °C in Krebs-Henseleit solution containing 20 μg/ml norepinephrine, bubbled with 95% O_2 and 5% CO_2. Double fixation (glutaraldehyde-osmium tetroxide) was performed as described before. After this procedure virtually all the vesicles in the sympathetic nerve terminals contained osmiophilic material, whereas the vesicles of parasympathetic nerve terminals remained empty as before (Figure 2a, b). That the nerve terminals with a homogeneous population of empty vesicles were parasympathetic and those with dense core vesicles sympathetic was verified by alternating chronic parasympathetic and sympathetic denervation. The results of these experiments will be published in detail elsewhere.

The present study has shown that (a) the amount of norepinephrine (osmiophilic material) present in the vesicles depends primarily on the method of fixation; (b) the storage capacity for norepinephrine is much higher than the amount of norepinephrine actually stored.

Empty and dense core vesicles in adrenergic nerves most probably represent only differing states of filling. However, it does not mean that these empty vesicles are not able to store norepinephrine. There is no justification, at least in the cat's iris, to consider them as a morphological correlate of a cholinergic link in postganglionic sympathetic transmission.

Résumé. Nous avons montré que dans les terminaisons nerveuses sympathiques de l'iris de chat, la proportion de vésicules à contenu osmiophile (noradrénaline) dépend essentiellement de la technique de fixation. Par ailleurs, après incubation des iris dans des solutions de noradrénaline et après fixation convenable, la quasi-totalité de ces vésicules renferment de la noradrénaline. Nous concluons que ces vésicules représentent une population potentiellement homogène, toutes étant capables de stoquer la noradrénaline.

J. P. TRANZER and H. THOENEN

Department of Experimental Medicine, F. Hoffmann-La Roche & Co., Basle (Switzerland),
10th November 1966.

[6] J. P. TRANZER, M. DA PRADA and A. PLETSCHER, Nature, in press.