vated in vitro and the changes induced by the dosis of 100 µg/ml were more severe and more widespread than those induced by 50 µg/ml. Experiments were performed with the dosis of 25 µg of chloroquine per ml, but the results were inconstant and therefore the results of these experiments have not been reported. Chloroquine at the dosis of 150 µg/ml caused a complete destruction of the lens.

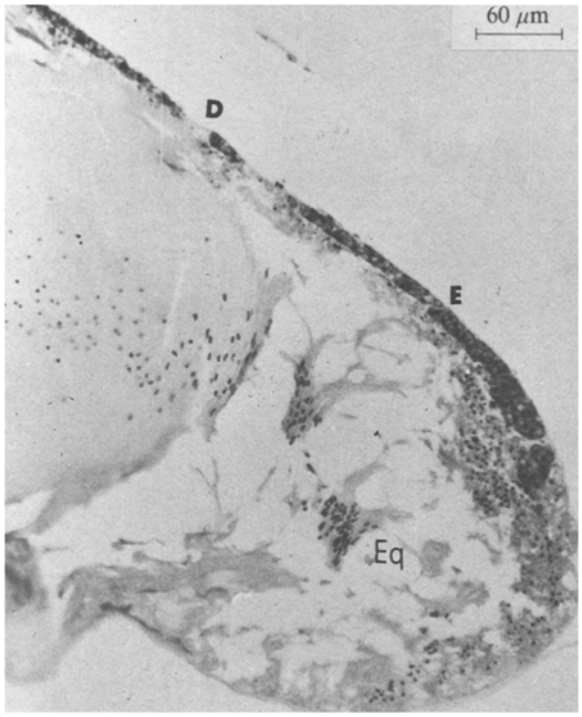


Fig. 3. Lens treated with 100 µg/ml of chloroquine. Severe destruction of equatorial fibres. × 160.

Ciak and Hahn[9] showed that chloroquine blocks protein synthesis by inhibiting DNA and RNA polymerases. This mechanism might explain the lack of elongation of lens fibres. As to the destruction of lens fibres, this might be explained by the formation of toxic vacuoles as described by Fedorko et al.[10]. The results reported above suggest that chloroquine treatment of women during the period of fertility should be undertaken with caution, in spite of the fact that susceptibility of the developing lens of the chick embryo to chloroquine could not be compared to that of human embryo.

1 This work was supported by a grant from the office of the Chief Scientist, Ministry of Health, Israel.
2 H.E. Hobbs, S.P. Eadie and F. Sommerville, Br. J. Ophthal. *45*, 284 (1961).
3 P. Henkind and N.F. Rothfield, N. Engl. J. Med. *269*, 433 (1963).
4 N.G. Lindquist and S.E. Sjostrand, Nature *227*, 1257 (1970).
5 N.G. Lindquist, Acta radiol. *325*, 1 (1973).
6 J. Piatigorsky and S.S. Rotschild, Dev. Biol. *28*, 382 (1972).
7 M.O. Tso, D. Albert and L.E. Zimmerman, Invest. Ophthal. *12*, 554 (1973).
8 Y.R. Barishak, L. Barr-Nea and Y. Messer, Ophthal. Res. *8*, 17 (1976).
9 J. Ciak and F.E. Hahn, Science *151*, 347 (1966).
10 M.E. Fedorko, J.G. Hirsch and Z.A. Cohn, J. Cell Biol. *38*, 392 (1968).

^{125}I-Insulin is preferentially internalized in regions of the hepatocytes rich in lysosomal and Golgi structures

J.-L. Carpentier, P. Gorden[1], P. Freychet[2], A. Le Cam[2] and L. Orci[3]

Institute of Histology and Embryology, Geneva University School of Medicine, CH-1211 Geneva 4 (Switzerland), 28 February 1979

Summary. ^{125}I-Insulin initially localizes to the plasma membrane of isolated rat hepatocytes but is subsequently internalized and preferentially associates with lysosomal structures. In the present study, we show that this preferential association to lysosomes occurs in regions of the cell rich in lysosomal and Golgi structures.

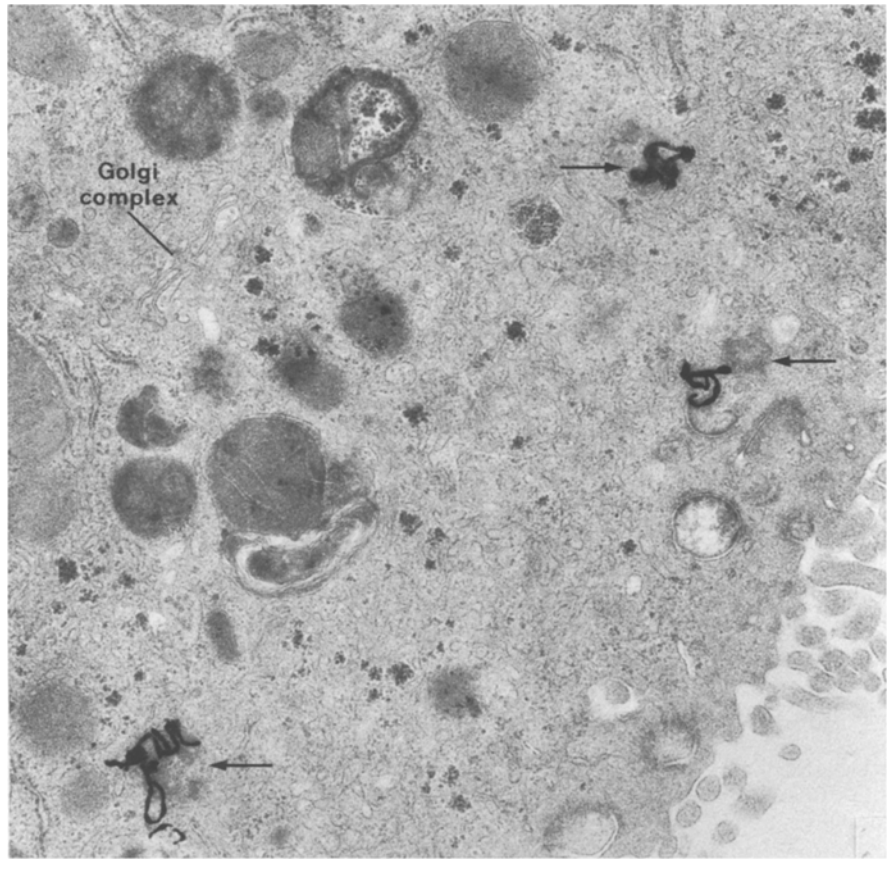
We have demonstrated that ^{125}I-insulin initially localizes to the plasma membrane of isolated rat hepatocytes[4-6]. A portion of the labelled material is subsequently internalized by the cell as a constant function of binding and preferentially associates with lysosomal structures intracellularly[7,8]. Qualitatively it appeared that not only were intracellular autoradiographic grains preferentially localized to lysosomal structures but, in addition, grains appeared to be localized in regions of the cell rich in lysosomal and Golgi structures. In the present study we have quantitatively verified this observation.

Materials and methods. Hepatocytes were isolated from normal 6-8-week-old Wistar rats fed ad libitum, using a modification of the method described by Seglen[9]. The percentage of parenchymal cells in the purified cell suspension exceeds 95%; the viability of the cell suspension estimated by the trypan blue exclusion and by morphological criteria exceeds 85-95%. ^{125}I-insulin was prepared at a sp. act. of 250 µCi/µg by a modification of the chloramine-T-method[10]. The labelled insulin was purified by filtration on G-50 Sephadex at 4 °C prior to each experiment. Hepatocytes (1×10^6 cells/ml) were incubated in 0.5 ml of modified KRB (pH 7.7) containing 25 mg/ml bovine serum albumin (fraction V) and 0.8 mg/ml of bacitracin with 5×10^{-10} M (3 ng/ml) ^{125}I-insulin at 37 °C for a maximum of 60 min. Incubation, fixation and processing for autoradiography were carried out as previously[5,7,11]. Samples were examined in a Philips EM 300 electron microscope (Philips Instruments Eindhoven, The Netherlands) and photographs taken at a magnification (× 9000) calibrated with a reference grid 2160 lines/mm (Fullam Inc., Schenectady, N.Y.). For each experimental condition tested (unincubated cells, cells incubated for 5 min at 20 °C and cells incubated for 30/60 min at 37 °C) 2 types of pictures were taken: pictures with autoradiographic grains and random pictures. Pictures with grains were taken in cells that were

Relationship of lysosomal structures and Golgi elements to region of the cells selected for the presence of autoradiographic grains

	Volume density of lysosomal structures		Volume density of Golgi elements	
	Random	Regions with grains	Random	Regions with grains
Control*	1.7±0.3 n=50	–	0.2±0.1 n=50	–
5 min/20 °C**	NS		NS	
	2.0±0.3 n=50	2.3±0.4 n=58	0.3±0.1 n=50	0.2±0.1 n=58
30–60 min/37 °C (related to membrane)***	NS		NS	
	1.9±0.4 n=67	1.9±0.3 n=49	0.2±0.1 n=67	0.2±0.2 n=49
30–60 min/37 °C (internalized)****	$p<0.001$		$p<0.05$	
	1.9±0.4 n=67	4.9±0.6 n=60	0.2±0.1 n=67	1.1±0.4 n=60

* Isolated hepatocytes incubated for 60 min in the absence of insulin. ** Cells were incubated for 5 min at 20 °C with ^{125}I-insulin. Under these conditions the analysis was performed for grains at 0±250 nm from the plasma membrane. *** Cells were incubated for 30–60 min at 37 °C with ^{125}I-insulin. This analysis was done for grains at 0±250 nm from the plasma membrane. **** Cells were incubated for 30–60 min at 37 °C with ^{125}I-insulin. This analysis was done for grains beyond 250 nm from the plasma membrane.



View of the periphery of isolated hepatocytes incubated for 60 min at 37 °C with 5×10^{-10} M ^{125}I-insulin. Autoradiographic grains are seen overlying or in the vicinity of lysosomal structures (arrows). This region of peripheral cytoplasm contains numerous lysosomal structures and a Golgi complex. ×21,500.

judged to be well preserved. For pictures without grains (to be used as control), random sampling was insured by photographing the closest hepatocyte cytoplasm to each corner of 3 consecutive squares of the supporting grid (12 pictures per grid). For each condition, 4 different Epon blocks were cut and a total of 48 pictures analyzed (12×4). Morphometric determinations were made on prints enlarged 3 times (final magnification: ×27,000). Since in similar experimental conditions ^{125}I-insulin was found internalized within a limited rim of cytoplasm extending 1.6–2.0 µm beyond the plasma membrane[5], all analysis were restricted to this cytoplasmic region. Morphometric determinations were made using a test screen (160×205 mm) as previously described[7]. The cytoplasmic surface scored in each picture represented about 15 µm^2. On this material the volume density Vv of lysosomal structures and Golgi apparatus was determined by point counting[12] as previously described[7].

Results and discussion. As shown in the figure, within the range of magnification chosen for our study, both lysosomal structures and Golgi elements were easily detected.

The volume density of these structures was analyzed in the peripheral rim of cytoplasm defined in 'Methods'. The volume density of lysosomal and Golgi structures was first evaluated in random pictures from the 4 experimental conditions shown in the table and found to be similar. Next, the volume densities of these structures were evaluated in two series of pictures selected for the presence of grains related to the plasma membrane (5 min of incubation at 20 °C and 30/60 min of incubation at 37 °C.) In both of these conditions, the volume densities were again unchanged and similar to previously evaluated random pictures. By contrast, when pictures were selected for the presence of internalized autoradiographic grains at 30/60 min of incubation at 37 °C, there was a 2.6-fold increase in the volume density of lysosomal structures and a 5.5-fold increase in the volume density of Golgi elements (table). These results indicate that ^{125}I-insulin not only localizes to lysosomal structures[7], but in addition localizes to regions of the cell in which the volume density of lysosomal structures and Golgi elements are increased. Although in the isolated hepatocytes, the polarity of the cell is undetectable, it is likely that the specific regions described above correspond to the biliary pole of the cell, regions known to be rich in lysosomal and Golgi structures[13]. Further studies in intact liver will be necessary to verify this point.

1 This work was performed while Dr Gorden was visiting professor of the Institute of Histology and Embryology, University of Geneva, Geneva, Switzerland.
2 INSERM U145, Department of Experimental Medicine, Nice, France.
3 This work was supported by grant No. 3.120.77 from Swiss National Science Foundation. We thank M.M. Sidler-Ansermet, O. Jerotic and N. Maalaoui for their valuable help.
4 J.-L. Carpentier, P. Gorden, A. Le Cam, P. Freychet and L. Orci, Diabetologia *13*, 386 (1977).
5 P. Gorden, J.-L. Carpentier, P. Freychet, A. Le Cam and L. Orci, Science *200*, 782 (1978).
6 J.-L. Carpentier, P. Gorden, P. Freychet, A. Le Cam and L. Orci, Diabetologia *14*, 224 (1978).
7 J.-L. Carpentier, P. Gorden, P. Freychet, A. Le Cam and L. Orci, J. clin. Invest., in press (1979).
8 J.-L. Carpentier, P. Gorden, P. Freychet, A. Le Cam and L. Orci, submitted for publication.
9 P.O. Seglen, Meth. Cell Biol. *13*, 29 (1976).
10 P. Freychet, in: Methods in Receptor Research, part II, p. 385. Ed. M. Blecher, Marcel Dekker Inc., New York 1976.
11 J.-L. Carpentier, P. Gorden, M. Amherdt, E. Van Obberghen, R. Kahn and L. Orci, J. clin. Invest. *61*, 1057 (1978).
12 E.R. Weibel, Int. Rev. Cytol. *26*, 235 (1969).
13 Ch. Rouiller and A.M. Jézéquel, in: The Liver. Morphology Biochemistry, Physiology, vol. I, p. 195. Ed. Ch. Rouiller. Academic Press, New York, London, 1963.

Effect of sodium butyrate in combination with X-irradiation, chemotherapeutic and cyclic AMP stimulating agents on neuroblastoma cells in culture

K.N. Prasad

Department of Radiology, University of Colorado Medical Center, 4200 East 9th Avenue, Denver (Colorado 80262, USA), 26 September 1978

Summary. Sodium butyrate, X-irradiation, chemotherapeutic agents and cyclic AMP-stimulating agents caused reduction in the cell number (due to cell death and reduction in cell division) when added individually to mouse neuroblastoma cells in culture. However, the combination of sodium butyrate with X-irradiation, chemotherapeutic and cyclic AMP-stimulating agents produced a greater reduction in the cell number than that produced by the individual agents.

Butyric acid, a 4-carbon fatty acid, occurs naturally in the body where it is formed by the hydrolysis of ethylbutyrate. Sodium butyrate (0.5–1.0 mM) appears to be either innocuous or produces reversible growth inhibition, and morphological and biochemical alterations in several mammalian cells in culture[2-5]. Sodium butyrate causes cell death and increases the expression of several differentiated functions in neuroblastoma (NB) cells[2]. Sodium butyrate has been used clinically first by Dr Tom Voute (Spinozastraat 51, Postgiro 2388, Amsterdam), and then by Dr L. Furman Odum, Children's Hospital in Denver (personal communication). Although the clinical value of sodium butyrate cannot be evaluated at this time, high doses (7–10 g per day) of sodium butyrate produce no clinically detectable toxic effect in patients with neuroblastomas. Because of its potential usefulness as a tumor therapeutic agent, I wondered if it, in combination with X-irradiation, chemotherapeutic agents and cyclic AMP stimulating agents, would enhance the growth inhibitory effect of these agents. I now report that the combination of sodium butyrate with X-irradiation and therapeutic agents produces a greater degree of growth inhibition (due to cell death and reduction in cell division) on NB cells in culture than that produced by the individual agents.

Materials and methods. The procedures of culturing and maintaining mouse NB cells were previously described[6]. Clone NBP_2, which has both tyrosine hydroxylase and choline acetyltransferase, was used in this study[7]. The procedure for X-irradiation was previously described[8]. Sodium-n-butyrate (K & K Co.) was dissolved in water (50 mM) and the pH was adjusted to 7. 5-fluorouracil (5-FU), 5(3,-3-dimethyl-1-triazeno) imidazole-4-carboxamide (DTIC), (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea(CCNU), adriamycin sulfate, vincristine, theophylline, and methotrexate were dissolved in water. (4-(-3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), papaverine and prostaglandin E_1 (PGE_1) were dissolved in 50% ethyl alcohol. All solutions were stored at 4 °C. Cells (50,000) were plated in Falcon plastic dished (60 mm), and drug or X-irradiation was given 24 h later. Control cultures received an equivalent volume of solvent. The drug and medium were changed 2 days after treatment and the cell number was counted 3 days after treatment by a hemacytometer. To count the cell number the cells were removed from the dish surface by incubating them in 0.25% pancreatin solution. Since sodium butyrate-treated cells appear to attach rather firmly not only with the surface of the dish but also with each other, longer incubation time (40 min) in the presence of pancreatin solution was needed in order to prepare a single cell suspension. The unirradiated control cells and X-irradiated cells require only 15 min of incubation in the presence of pancreatin solution for a complete