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.

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

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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

removal of cells from the surface. The amount of cellular protein per dish was similar in pancreatin treated culture (40 min) and in culture not treated with pancreatin. Therefore, the longer incubation time did not cause any significant cell lysis under the present experimental condition. The protein was determined by the method of Lowry et al.[9].

The classical definition of cell viability based on a colony-forming assay cannot be applied to NB cells because many agents[10] transform dividing NB cells to differentiated non-dividing cells. The differentiated cells do not pick up trypan blue and contain several morphological and biochemical features of mature neurons. Therefore, the effect of various agents on the cell number is measured in the present study. The sodium butyrate-treated culture contained 10–15% large vacuolated cells, the majority of which did not pick up trypan blue, These vacuolated cells are clearly distinguishable from the rest of the cell population. We have presumed that these large cells would not renew cell division because of the presence of multiple large vacuoles in the cytoplasms. Therefore, the number of vacuolated cells has been subtracted from the total cell number counted in a hemacytometer in order to obtain the number of viable cells.

Results and discussion. Treatment of NB cells with sodium butyrate produced cell death, vacuolation and morphological changes as reported earlier[2]. One of the characteristic effects of sodium butyrate on mouse NB cells which was not reported earlier is the presence of a significant number (10–15% of total cell population) of vacuolated cells, the majority of which did not pick up trypan blue. Although 8–10% of sodium butyratetreated cells picked up trypan blue, some of these were not vacuolated. Unirradiated control cells were seldom vacuolated and rarely picked up trypan blue. The X-irradiated culture had 6–8% vacuolated cells, whereas the culture treated with X-rays and sodium butyrate had 40–50% large vacuolated cells.

Sodium butyrate potentiated the growth inhibitory effect of X-irradiation, 5-FU, CCNU, vincristine, adriamycin, R020-1724, papaverine and prostaglandin E_1 (tables 1 and 2). The combined effect of sodium butyrate with DTIC or methotrexate was less than additive. Sodium butyrate in combination with theophylline produced an effect which is similar to that observed with sodium butyrate alone. Sodium butyrate also enhanced the growth inhibitory effect of cyclic AMP stimulating agents on human melanoma cells in culture[11]. The exact mechanism of the combined effect of sodium butyrate and tumor therapeutic agents is unknown. A recent observation[12] shows that sodium butyrate inhibits anaerobic glycolysis by reducing the activity of lactate dehydrogenase (converts pyruvate to lactic acid) in NB cells in culture. We have previously reported that mouse NB cells are relatively more sensitive to an inhibitor of anaerobic glycolysis (DL-glyceraldehyde) for the criterion of growth inhibition[13], indicating that the survival of these cells may partly depend on anaerobic glycolysis. Since sodium butyrate inhibits anaerobic glycolysis, it is expected that the treatment of NB cells with sodium butyrate would produce growth inhibition. This mechanism may in part be responsible for the potentiating effect of sodium butyrate in combination with certain therapeutic agents. Based on these data, I propose a working hypothesis which suggests that the inhibition of anaerobic glycolysis after irradiation or chemotherapeutic treatment of cells (oxic and hypoxic) which use this pathway as a major source of energy would increase the lethal effect of certain tumor therapeutic agents. The dependence of oxic tumor on anaerobic glycolysis may be due to a mutational event. However, the dependence of hypoxic tumor cells on anaerobic glycolysis may be a transient adaptive phenomenon, since these cells can shift to aerobic glycolysis as soon as they enter the cell cycle following a proper stimulus.

Warburg's hypothesis that the high rate of anaerobic glycolysis in vitro is a unique requirement of neoplastic cells has been the subject of controversy since it was originally proposed. The fact that DL-glyceraldehyde, a potent glycolytic inhibitor[14], reduces the growth of many types of neoplasms[15-17] supports the hypothesis. However, some studies show that this metabolic anomaly is not due to a fundamental difference between normal and tumor cells but merely reflects their particular growth rate[18-21]. This general statement was disputed by another study[13] in which it was found that neuroblastoma cells have a much higher rate of glycolysis than chinese hamster ovary ($CHO\text{-}K_1$) cells, although the former cells have a longer doubling time than the latter. I suggest that some of these differences in results may be due to the fact that the number of cells relying primarily on anaerobic glycolysis for survival may vary from 1 type of tumor to another and may even vary from 1 individual to another for the same tumor type. Therefore, the effect of inhibitor of anaerobic glycolysis may quantitatively differ from 1 clone to another obtained from the same tumor.

Table 1. Effect of the combined effect of sodium butyrate and therapeutic agents on neuroblastoma cells in culture*

Treatment	No. of cells $\times 10^4$ ($\pm$SD)
Control	228 ±18
Sodium butyrate (0.5 mM)	53 ± 8
5-Fluorouracil (0.05 µg/ml)	163 ±12
Sodium butyrate + 5-fluorouracil	4.3± 1.5
X-irradiation (400 rad)	49 ± 7
X-irradiation + sodium butyrate	7.0± 2
DTIC (20 µg/ml)	77 ±13
Sodium butyrate + DTIC	25 ± 4
CCNU (20 µg/ml)	121 ±11
Sodium butyrate + CCNU	11 ± 4
Vincristine (0.002 µg/ml)	89 ± 8
Sodium butyrate + vincristine	15 ± 5
Adriamycin (0.008 µg/ml)	78 ±10
Sodium butyrate + adriamycin	14 ± 3
Methotrexate (0.1 µg/ml)	31 ± 4
Sodium butyrate + methotrexate	12 ± 2

*Neuroblastoma cells (50,000) were plated in plastic culture dishes. Drugs and X-irradiation were given 24 h after plating. The drugs and medium were changed 2 days after treatments. Each value represents an average of at least 6 samples.

Table 2. Effect of the combined effect of sodium butyrate and cyclic AMP stimulating agents on neuroblastoma cells in culture*

Treatment	No. of cells $\times 10^4$ ($\pm$SD)
Control	228 ±18
Sodium butyrate (0.5 mM)	53 ± 8
R020-1724 (200 µg/ml)	32 ± 9.0
R020-1724 + sodium butyrate	7.5± 2.9
Theophylline (0.5 mM)	117 ±13
Theophylline + sodium butyrate	50 ± 8
Papaverine (20 µg/ml)	77 ± 8
Papaverine + sodium butyrate	15 ± 4
Prostaglandin E_1 (10 µg/ml)	134 ±18
Prostaglandin E_1 + sodium butyrate	11 ± 3

*Neuroblastoma cells (50,000) were plated in plastic culture dishes (60 mm). Drugs were added 24 h after plating. The drugs and medium were changed 2 days after treatments and the cell number was counted 3 days after treatment. Each value represents an average of at least 6 samples.

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Action du *Corynebacterium parvum* sur les taux de lysozyme (muramidase) sérique

Effect of *Corynebacterium parvum* on serum lysozyme (muramidase) levels

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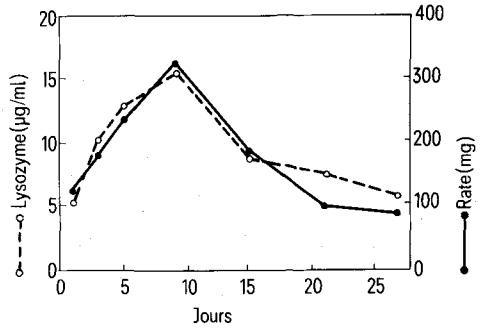
Summary. An i.v. injection of 548 µg of killed *Corynebacterium parvum* into C57B1 mice leads to significant changes in serum lysozyme (muramidase) levels. After an initial fall at 24 h, the activity of the enzyme increased progressively, reached a peak on the 9th day and returned to control range after the 15th day.

L'injection i.v. ou i.p. d'une suspension de *Corynebacterium parvum* tué, exerce chez la souris une action protectrice vis-à-vis d'un certain nombre d'infections expérimentales[2-4]. Il a été suggéré que cet effet protecteur était lié au pouvoir de la bactérie d'une part, de stimuler le système réticulo-endothélial[2,5] et d'autre part, d'augmenter la bactéricidie intracellulaire des macrophages[4]. Pourtant, des facteurs humoraux sont également impliqués dans le processus de défense de l'organisme. Dans le présent travail, on étudie l'action du *C. parvum* sur les taux d'une enzyme bactériolytique, du lysozyme (muramidase), dans le sérum.

Matériel et méthode. Des souris $C_{57}B1$ mâles, isogéniques, âgées de 10 à 12 semaines et d'un poids moyen de 20 g ont été utilisées dans toutes les expériences. Les animaux étaient soumis au temps zéro à une seule injection i.v. de 548 µg de *C. parvum* (Inst. Mérieux, Lyon). Le sang de divers groupes d'animaux ainsi traités, était prélevé par ponction retro-orbitaire dans les jours qui suivirent cette unique injection. Les taux de lysozyme sérique étaient évalués suivant la technique de Litwack[6] en utilisant comme substrat le *Micrococcus lysodeikticus* (Worthington Biochem. Corp., New Jersey).

Résultats. Les résultats sont présentés dans la figure. Après un declin initial au bout de la 24e heure, les taux de lysozyme sérique augmentent progressivement et donnent un pic 9 jours après l'injection de la bactérie. Ensuite, la courbe commence à décroître et des taux normaux s'observent à partir du 15e jour. Ces modifications d'enzyme dans le sérum suivent d'une manière hautement significative les modifications du poids de la rate ($p < 0.001$). Une faible liaison a également été trouvée entre les taux de lysozyme et le nombre de monocytes circulants ($p < 0.05$).

Discussion. Les granulocytes et les monocytes du sang, ainsi que les macrophages tissulaires sont considérés actuellement comme étant les cellules-sources de lysozyme sérique les plus importantes[7-10]. D'autre part, il a été observé que l'injection du *C. parvum* chez la souris entraînait une augmentation très importante du nombre de granulocytes et monocytes du sang[11]. Une hypertrophie du foie et de la rate a également été notée 3–15 jours après l'injection de la bactérie[5]. Des études histologiques ont montré que l'hépatomégalie était en grande partie constituée par l'augmentation numérique des cellules de Kupffer[12,13]. La splénomégalie était essentiellement due à l'accumulation dans la rate des cellules d'aspect macrophagiques et d'autres cellules mononucléaires issues de la moelle osseuse[14]. Il est donc bien évident que l'augmentation des taux de lysozyme sérique observée chez les animaux traités par *C. parvum*, reflète le renouvellement intense et accéléré des granulocytes et monocytes circulants et probablement des macrophages tissulaires. La forte liaison trouvée entre les taux



Modifications des taux de lysozyme sérique et du poids de la rate chez les animaux traités par *C. parvum* en fonction du temps.