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The pH Dependence of Disobutamide-Induced Clear Cytoplasmic Vacuoles in Cultured Cells[1] (42147)

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Abstract. Cellular uptake of disobutamide (D), and clear cytoplasmic vacuoles (CCV) induction by D in cultured rat urinary bladder carcinoma cells were dependent on the culture medium pH. At pH 6.0–6.7, drug uptake was slow and no CCV formed in 24 hr. At pH 7.0–8.0, the rate of D uptake and early appearance of CCV were directly proportional to increased basicity. This was explained by the increasing fraction of un-ionized D molecules at increasing basicity of the culture medium. It is only these electrically neutral D molecules which can penetrate the lipoidal cell membrane to induce formation of CCV. Intracellular presence of D was demonstrated by mass spectrometry methods. The results indicate that D is incorporated intracellularly, that D and not its metabolite(s) is in cells, and suggest that CCV are a result of drug sequesteration.

Disobutamide (D), α-(*o*-chlorophenyl)-α-[2-(diisopropylamine) ethyl]-γ-(1-piperidino)butyramide, (SC-31828, mol wt = 407) is an antiarrhythmic compound (1, 2). It induces clear cytoplasmic vacuoles (CCV) in many types of cells in the dog, and in the epithelium of the epididymis and choroid plexus of the rat (unpublished observations). Piperamide (3), chloroquine (4), tilorone, and other tertiary amines (5) induce histologically similar CCV in epithelial cells of the rat choroid plexus. The biological nature and chemical content of these CCV is unclear. Some investigators (3, 5) considered them as a sign of hydropic degeneration while others (4) believed they held primarily nonpolar lipid. We reported the induction of similar CCV by D, in 24–72 hr, in cultured mammalian cells originating from various species (6, 7), and found rat urinary bladder carcinoma cells (8) particularly useful for investigating the dynamic nature of CCV and the structural determinants of related amines that induce them. We also determined that the cultured rat cells incorporated ^{14}C-labeled D, and the rate of uptake paralleled the induction of CCV (unpublished observation); thus, we speculated that CCV were associated with drug storage. In one experiment we noticed that the CCV appeared in 1–2 hr rather than in 24 hr, so we investigated the cause of this temporal effect. We now report that the pH of the medium greatly affects the cellular uptake of the drug and the temporal appearance of CCV. Such a link between the culture medium pH, drug uptake, and CCV answers some basic questions on disobutamide–cell interaction and on the nature of CCV. The purpose of this study was to examine the effect of the pH of the culture medium on the cellular uptake of ^{14}C-labeled D and on the temporal appearance of D-induced CCV and to determine whether vacuolated cells contain D.

Material and Methods. *(A) Effect of medium pH on temporal appearance of CCV.* Rat bladder carcinoma (RBT CC-8) cells (8) (courtesy of Dr. Bendicht U. Pauli) were grown in Costar 25-cm^2 flasks with Medium-199 (M-199) supplemented with 30 m*M* Hepes (*N*-2-hydroxyethylpiperazine-*N*^{1}-2-ethane sulfuric acid), 30 m*M* sodium bicarbonate, and 10% fetal bovine serum. After 24 hr the medium was removed, the cells were washed in unbuffered saline, and fresh medium (without serum) containing 10^{-3} *M* D was added. The pH of the medium (determined by a Corning pH meter) for each flask (see Table I) was adjusted with HCl or NaOH.

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TABLE I. THE EFFECT OF MEDIUM pH ON THE TEMPORAL APPEARANCE OF CCV[a] INDUCED BY D[b] IN CULTURED RAT URINARY BLADDER CARCINOMA CELLS[c]

Hr: Parameter examined:	0 pH	1 CCV	2 CCV	2.5 pH	3 CCV	4 CCV	5 CCV	5.5 pH	24 CCV	24.5 pH
	6.0	–	–	6.50	–	–	–	6.60	–	6.60
	6.5	–	–	6.90	–	–	–	7.00	–	6.85
	6.7	–	–	7.19	–	–	–	7.15	–	7.02
	7.0	–	–	7.35	–	–	–	7.30	+	7.15
	7.2	–	–	7.51	–	+	+	7.40	+	7.26
	7.4	–	+	7.60	+	+	+	7.60	+	7.43
	7.6	–	+	7.66	+	+	+	7.57	+	7.50
	8.0	+	+	8.06	+	+	+	8.00	+	7.71

[a] CCV = clear cytoplasmic vacuoles.
[b] D = Disobutamide.
[c] Cultures in flasks were examined for presence of CCV at 1, 2, 3, 4, 5, and 24 hr of D exposure; + = CCV present; – = CCV not present. The pH of the culture was determined in homologous flasks at 0, 2.5, and 24.5 hr of D exposure.

Cultures in the same medium at the same pH, but without D, served as controls. Cells were observed *in situ* by a phase light microscope at 1, 2, 3, 4, 5 and 24 hr of drug exposure. The pH was recorded at 0, 2.5, 5.5, and 24.5 hr of drug exposure in both drug-exposed and control cultures. Throughout the experiments, cells were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

To exclude the effect of the acidic pH on the cells, cells were grown under similar conditions as described above, then incubated with 10^{-3} *M* D in medium at pH 6.0 for 4 hr. Then, they were washed with saline and further incubated with 10^{-3} *M* D in media at pH 6.0, 7.0, 7.2, 7.4, 7.6, and 8.0, and examined for induction of CCV at 1, 2, 3, 4, and 24 hr.

(B) Effect of medium pH on cellular drug incorporation. Rat cells were grown under similar conditions except that 5×10^{-4} cells were plated in each well of Costar 24-well plates. After 72 hr of incubation, the medium was removed, cells were washed, and M-199 (serum free, at the various pH levels) containing 10^{-3} *M* ^{14}C-labeled D (6.7 μCi/ml) was added. After 1, 2, and 24 hr of drug exposure, the medium was removed and cells were washed three times with saline. The cells in monolayer were digested with 0.5 *N* NaOH (0.5 ml per well) at 37°C for 24 hr, after which the NaOH was neutralized with an equal amount of HCl. The ^{14}C radioactivity accumulated in the cell layer was determined in a Mark III liquid scintillation counter using PCS (Amersham) scintillation fluid (each sample counted for 20 min). The experiments at each pH were done in triplicate. The chemical structure of D with its ^{14}C-radiolabeled carbons and protonated amines is presented in Fig. 1.

(C) Chemical analysis of cellular drug content. (a) Proton NMR spectrometry: Cells were exposed to 10^{-3} *M* D at pH 8.0 for 2 hr then fixed in *d*4-deuteromethanol. Spectra of fixed cells suspended in *d*4-deuteromethanol were determined on a Varian XL-200 using field frequency stabilization through an internal lock on the deuterium signal. To improve the dynamic range, the exchangeable proton signal near 5.7 ppm was continuously irradiated during the experiments and data

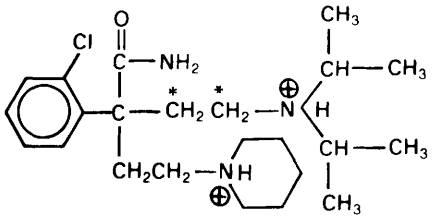

FIG. 1. The molecular structure of disobutamide including the two protonated amines. The two asterisked C's were equally radiolabeled as ^{14}C.

were collected in the double precision mode. The NMR spectra of the cells were referenced indirectly to the signal of tetramethylsilane. Cells not treated with D served as control; they were similarly grown, prepared, and analyzed. Theoretical NMR spectra of pure D in deuteromethanol were calculated using the spin simulation program resident in the Varian XL-200 instrument. (b) Mass spectrometry: Cells were grown and incubated with 10^{-3} *M* D at pH 8.0 for 2 hr under similar conditions as in studies for temporal appearance of CCV. Then, cells were removed, fixed in 100% methanol, and air dried. Several microliters of a methanol suspension of dried cells was transferred onto a glass probe tip and blown down with nitrogen. The glass probe was introduced directly into a Kratos MS-30 mass spectrometer and scanned continuously while heating until the low ion current indicated all volatile materials disappeared. Electron impact ionization was used with 300 μA ionizing current and 4 kV accelerating volts. Spectra were recorded with the Kratos DS-55 data system. Cells not exposed to D served as control; they were similarly grown, prepared, and analyzed. To ensure that the drug is in the cell and not merely on the cell surface, cells were exposed to 10^{-3} *M* D for less than 1 min, washed in saline, then similarly prepared, and analyzed. The electron impact spectrum of pure D was also determined by direct introduction probe. Chemical ionization mass spectra of D-treated vacuolated cells was determined by direct probe using methane gas at 0.35 Torr source pressure and a Finnigan 4000 mass spectrometer coupled to an Incos data system.

Results. The effect of medium pH of the temporal appearance of CCV is presented in Table I. Absence of CCV or delay in their appearance was associated with the lower pH levels. In cultures with an initial pH of 6.0, 6.5, and 6.7, there were no CCV even at 24 hr. In those with an initial pH of 7.0, CCV were present at 24 hr; of pH 7.2, at 4 hr; of pH 7.4 or 7.6, at 2 hr; and of pH 8.0, at 1 hr.

Typically, CCV appeared first in the perinuclear area (Fig. 2), and gradually filled the cytoplasm. As CCV occupied more of the cytoplasmic area, the cells became rounded, finally appearing almost spherical. This temporal morphologic progression was delayed at the lower pH levels.

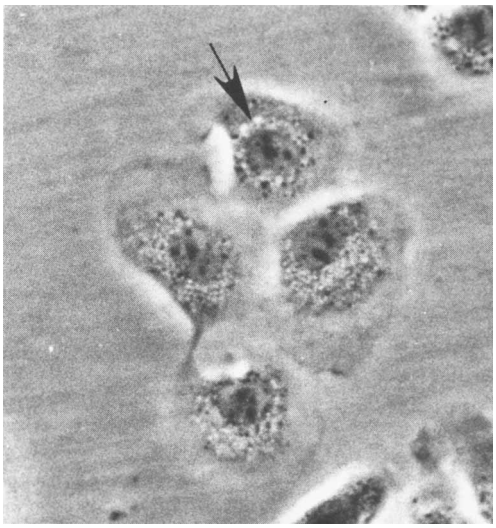

FIG. 2. CCV (clear cytoplasmic vacuoles) in the perinuclear area (arrow) in cultured rat urinary bladder carcinoma cells exposed to 10^{-3} *M* disobutamide at medium pH 7.2 for 4 hr (*in situ* photomicrograph).

The early temporal appearance of CCV in 24 hr following incubation of cells in culture medium at pH 6.0 was also dependent on the high pH levels of the medium. The correlation was similar to that presented in Table I, i.e., in cells incubated at pH 8.0, 7.6, and 7.4 CCV appeared in 1 hr, in those at pH 7.2 CCV appeared in 2 hr, at pH 7.0 in 24 hr, and in those incubated at pH 6.0 there were no CCV in 24 hr.

The effect of medium pH on the cellular incorporation of ^{14}C-labeled D is presented in Table II. Drug uptake increased with time at all pH levels. At 2 hr, the radioactivity was almost twice that at 1 hr, for the respective pH level. Cellular drug incorporation was dependent on pH. The lower the pH, the less drug was incorporated.

Tables I and II show a positive relationship between drug incorporation and temporal appearance of CCV. Drug incorporation into the cell monolayer of 2.67 μg (2 hr exposure, initial pH 7.4) or higher was associated with presence of CCV. CCV were absent when drug uptake was 2.58 μg (24 hr exposure, initial pH 6.7) or lower.

The aromatic proton NMR region of the

TABLE II. THE EFFECT OF MEDIUM pH ON UPTAKE OF ^{14}C-LABELED D[a] BY CULTURED RAT URINARY BLADDER CARCINOMA CELLS

Hr:	0	1	2	24
Parameter determined	pH	μg of ^{14}C-Labeled D[b]		
	6.0	0.07 ± 0.01	0.12 ± 0.03	0.32 ± 0.06
	6.5	0.21 ± 0.01	0.36 ± 0.03	1.76 ± 0.11
	6.7	0.28 ± 0.03	0.58 ± 0.02	2.58 ± 0.02
	7.0	0.44 ± 0.05	0.99 ± 0.05	4.33 ± 0.23
	7.2	0.68 ± 0.07	1.72 ± 0.04	6.72 ± 0.14
	7.4	1.10 ± 0.05	2.76 ± 0.18	9.69 ± 0.50
	7.6	2.03 ± 0.23	4.20 ± 0.24	11.52 ± 0.36
	8.0	3.14 ± 0.16	5.63 ± 0.91	9.60 ± 0.38

[a] D = Disobutamide.
[b] Calculated triplicate average of D ± SD taken up by cell monolayer (600 dpm = 1 μg D).

spectrum of D-treated vacuolated cells was essentially clear whereas the aliphatic proton region was highly overlapped with signals from cellular components. The spectrum of the aromatic region of D-treated vacuolated cells resembled but did not exactly match the aromatic spectrum of pure D in deuteromethanol. It was possible, though, to match the spectrum of vacuolated cells to that of pure D by making small changes in two chemical shifts and one coupling constant.

The mass spectrum of pure D is presented in Fig. 3 and of D-treated vacuolated cells in Fig. 4. The important diagnostic peaks, 307, 280, 114, 112 and 98, are interpreted on the spectrum of pure D. The spectra are essentially identical in all the major ions. Only minor differences due to volatile cell material are present in the spectrum of the cells. The chlorine isotope pattern is seen in the high mass ions labeled on Figs. 3 and 4. The spectrum of the cells not treated with D and of the cells treated with D for less than 1 min did not show the peaks of D or the chlorine isotope pattern.

The chemical ionization mass spectrum of D-treated vacuolated cells showed the protonated molecular ion at 408 atomic mass units as the most intense peak. A peak of approximately 35% the intensity of the 408 peak was present at mass 410 indicating the characteristic chlorine isotope pattern.

Discussion. The drug incorporation dependence on the pH can be deduced from the calculations presented in Table III. More molecules of D were un-ionized at the higher pH levels and therefore more were soluble in the lipid portion of the cell membrane, and passed into the cell. These calculations assume that the un-ionized form of the diamine is the major species diffusing through the lipid portion of membranes. This has been experimentally verified by Patel *et al.* (9), who state that the lipoidal "membrane permeability of these ammonium ions are about four or five orders of magnitude less than the membrane permeabilities of the corresponding amines." This pH dependence of CCV induced by D parallels the findings of CCV induced by methylamine or atropine, but differs from those induced by chloroquine whose uptake increased from pH 6.6 to 7.0 then decreased at pH 7.6 (10).

Although cultured cells incorporated ^{14}C-labeled D, it was not possible to determine definitely whether the radioactivity was due to D or its metabolite(s). Analysis of the NMR spectra was highly suggestive, but not definitive, of the presence of D in vacuolated cells. The presence of the same aromatic ring of D in cells indicated that this portion of D is unchanged. The slight chemical shifts in the spectra of cells suggest that intracellular magnetic environment of the aromatic ring is slightly different from that of pure D, possibly due to interaction of the drug with cellular components.

The near identity of the electron impact spectra of pure D and D-treated vacuolated cells (Figs. 3 and 4), and the appearance of

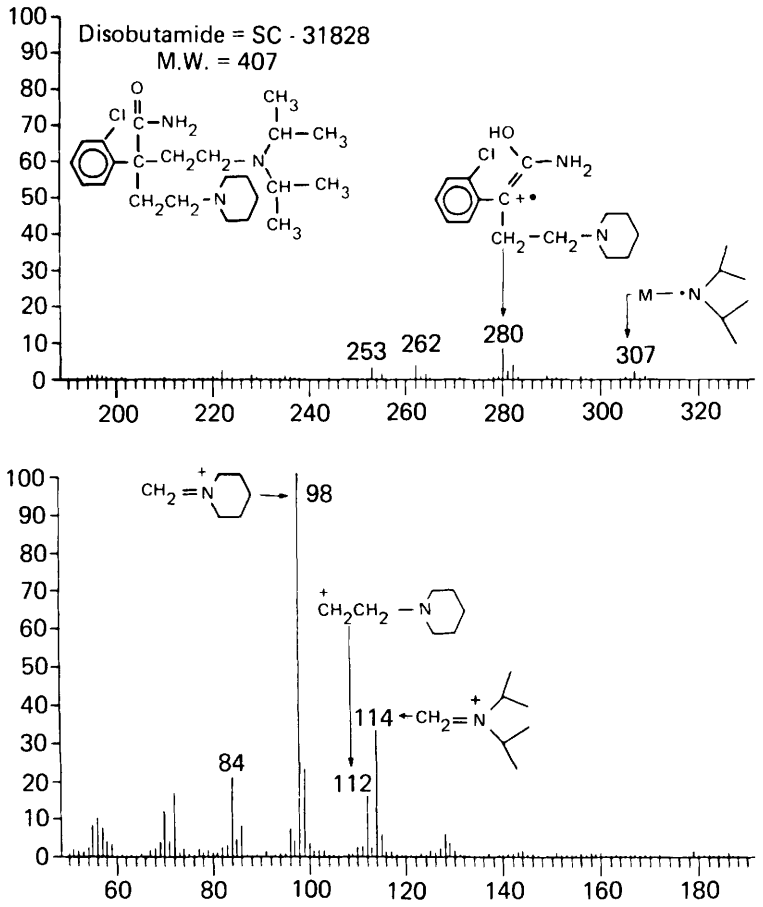

FIG. 3. The mass spectrum of pure disobutamide. The interpretation of the important peaks (307, 280, 114, 112, and 98) is provided. The chemical structure of the drug is in the upper left corner.

the protonated molecular ion at 408 atomic mass units in the chemical ionization spectrum of D-treated vacuolated cells, constituted conclusive evidence for the presence of D in the cells. In addition, the presence of the characteristic chlorine isotope peaks in the mass spectra was a convenient "tracer" for identifying drug-related compounds and excluding artifacts and other cell material. Even though our instrumental analyses were definitive for the presence of D in the cells, we could not entirely exclude the possibility that metabolite(s) were present in minor, nondetectable amounts.

The positive relationship between drug incorporation and temporal appearance of CCV supports the postulation that D-induced CCV sequester the drug and that CCV represent drug storage. CCV are distended lysosomes and components of the Golgi apparatus. In electron microscopic examination of D-induced CCV in cultured dog coronary artery muscle cells, most CCV were membrane-bound (lysosomes) and contained electron-lucent material (6). The electron microscopic features of D-induced CCV in coronary artery muscle cells, fibroblasts, renal tubular epithelial cells, and glomerular endothelium of intact dogs and in the epithelium of the choroid plexus of intact rats were the same (unpublished observations). In the coronary artery muscle cells of dogs *in vivo*, CCV

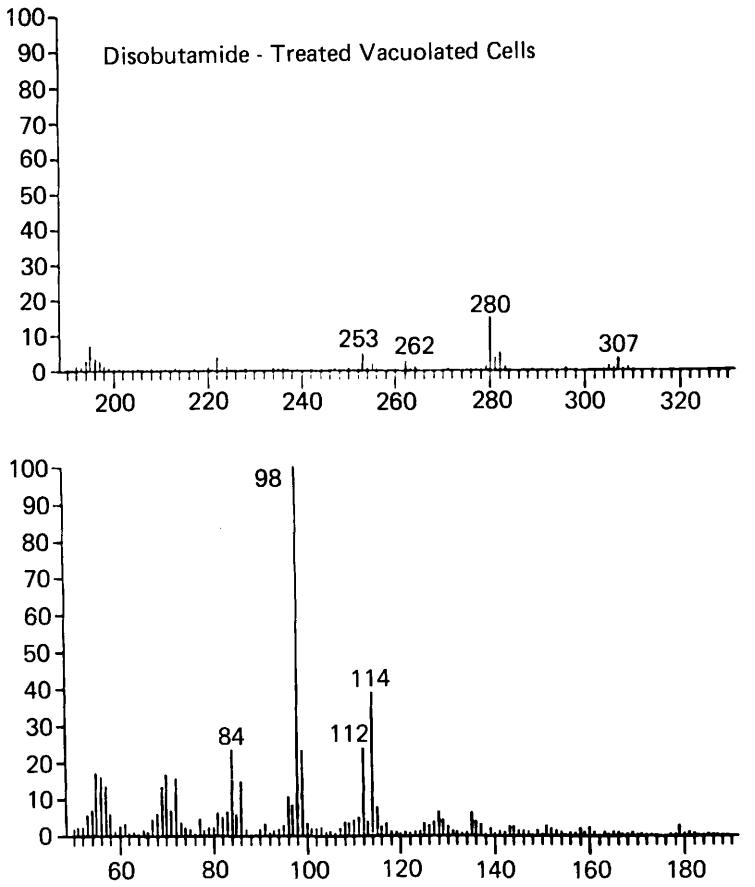

FIG. 4. The mass spectrum of disobutamide-treated vacuolated cells. The spectrum is essentially identical to that in Fig. 3 indicating the presence of the drug, and not of metabolite(s) in the cells.

appeared first in the Golgi apparatus area, and eventually occupied most of the cytoplasm. In this study, CCV appeared first in the perinuclear area, suggesting that CCV are formed first in the Golgi apparatus. Apparently, the drug enters the cell, first into the Golgi apparatus then into lysosomes.

The possible mechanism of induction of CCV by D may be similar to that proposed for induction of similar CCV by basic compounds (10, 11): (a) D enters cells and lysosomes by permeation of the unprotonated species. (b) The acidic lysosomes accumulate and entrap D in its diprotonated form. (c) Water enters lysosomes by osmosis resulting in their distention and clear vacuolar appearance. Diamines such as D are particularly potent CCV inducing agents since they are di-protonated within the acidic lysosome. The probability of both amines deprotonating simultaneously, so that the neutral diamine can exit through the lysosomal membrane is very small indeed. Consequently when a diamine enters the lysosome it is very securely "entrapped." Thus even quite dilute solutions of diamine outside the lysosome suffice to induce CCV.

In addition to D and water, CCV may also contain cellular material (e.g., soluble lipid) whose accumulation is induced by D. This biological aspect of D-induced CCV requires clarification. The assumption that soluble lipid accumulate in CCV has been proposed for vacuoles induced by chloroquine

TABLE III. CALCULATED[a] AVAILABLE MOLECULES OF DISOBUTAMIDE IN UN-IONIZED/IONIZED STATES AT VARIOUS pH LEVELS

pH	$\frac{[Base]}{[Acid]}\ pK_{a1}$[b]	$\frac{[Base]}{[Acid]}\ pK_{a2}$[b]	$\frac{[Base]}{[Acid]}\ pK_{a1}$	$\frac{[Base]}{[Acid]}\ pK_{a2}$[c]
6.0	1/15,850[d]	1/398		1/6,306,300
6.5	1/5,012	1/126		1,631,512
6.7	1/3,162	1/80		1/252,960
7.0	1/1,585	1/40		1/63,400
7.2	1/1,000	1/25		1/25,000
7.4	1/631	1/16		1/10,096
7.6	1/393	1/10		1/3,930
8.0	1/158	1/4		1/632

[a] Calculations are based on the Henderson–Hasselbalch equation: pH = pK_a + log[Base]/[Acid]; or, log[Base]/[Acid] = pH − pK_a. Base = unprotonated amine = un-ionized amine; acid = protonated amine = ionized amine.

[b] Disobutamide pK_{a1} = 10.2 and pK_{a2} = 8.6

[c] The probability of both nitrogens being un-ionized.

[d] Example of a calculation: pH = pK_a + log[Base]/[Acid]; then, log[Base]/[Acid] = pH − pK_a. When pH = 6.0 and pK_a = 10.2, log[Base]/[Acid] = 6.0 − 10.2 = −4.2; then, [Base]/[Acid] = $10^{-4.2}$ = 1/15,850 or, un-ionized amine/ionized amine = 1/15,850.

in the choroid plexus epithelium of intact rats (4).

The pH of the medium may affect not only D but also the cells, rendering them susceptible or resistant to D incorporation and CCV formation. The fact that early CCV formation in the cells preincubated at pH 6.0 was dependent on the higher pH (as was the case in the experiment of Table I) supports our interpretation that the main effect of pH is on ionization of D and not on cells. Further support to this argument is absence of CCV or any morphologic changes in the control cultures, exposed to the same pH's but without D. Additionally, gradual increases from 0 to 10% of bovine fetal serum supplement to the culture medium did not cause a temporal change in appearance of CCV (unpublished observations). This excludes the effect of serum on the cells or on the drug as a cause of early CCV appearance.

There were minor fluctuations in the medium pH in both control and drug-treated cultures throughout the experiments. The fluctuations were caused by exposure to atmospheric CO_2 and products of cell metabolism.

We conclude that at the lower pH of culture medium, cellular incorporation of D is hindered, and that the appearance of the induced CCV in cultured cells is prevented or delayed. At the lower pH levels, the number of doubly un-ionized D molecules is greatly decreased, fewer molecules are soluble in the lipid portion of the cell membrane, and fewer can enter the cell. The data indicate that cells take up D, that D and not its metabolite(s) is in cells, and suggest that CCV sequester the drug.

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Note added in proof. Fully vacuolated cells were incubated in culture medium without D. CCV disappeared by Day 6 of drug withdrawal. This disappearance paralleled a decrease in cell radioactivity from 1832 to 84 dpm (from 3.05 to 0.14 μg D). These data indicate that the formation of CCV in cultured cells is a reversible process associated with the release of D from the vacuolated cells.

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