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FACTORS INFLUENCING THE UPTAKE AND DISPOSITION OF INDOMETHACIN-[^{14}C] IN CELL CULTURES

MICHAEL A. BEAVEN and BARBARA M. BAYER

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20014, U.S.A.

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Abstract—Previous studies have shown that indomethacin arrests growth of rat hepatoma (HTC) cell cultures in the G_1 phase, and that removal of the drug leads to resumption of synchronous growth. The arrest was dependent on the concentration of the drug and the serum content of the medium. To determine how these and other factors affect the uptake of distribution of the drug in cell cultures, further studies were undertaken with indomethacin[^{14}C]. The passage of indomethacin into the cells was rapid and reversible. The labeled drug was taken up by two processes; one was highly concentrative and possessed a high affinity for the drug but was saturated by therapeutic concentrations of the drug; the other was nonsaturable over the range of concentrations studied (5–800 μM). In the absence of serum, the drug reached concentrations six to twelve times that in the medium. With increasing concentrations of serum in the medium, the ability of the cells to concentrate the drug was diminished, and with a high concentration of serum (> 30 per cent), the intracellular level of drug in the cells was one to two times that in the medium. Small decreases in pH (± 0.4 units) induced by changes in CO_2 levels or buffer resulted in a 2- to 3-fold increase in intracellular drug levels. The findings indicate that small changes in protein content, pH and CO_2 levels can have a marked influence on the cellular levels of indomethacin, and point to the possible usefulness of cell cultures to study the dynamics of drug distribution at the cellular level.

Our earlier studies showed that indomethacin and other acidic nonsteroidal anti-inflammatory drugs arrest growth of transformed and nontransformed cells [1–3] in the G_1 phase of the cell cycle [1, 3]. The inhibition was dependent on drug concentration and serum content of the medium, and paralleled the anti-inflammatory activity of the drugs. The effect was reversible and, upon removal of drug, all cells resumed cycling in synchrony to produce a surge and decline in DNA synthesis and, after 24 hr, cell division [1, 3]. Although the 24-hr period was thought to reflect the time required for the cells to pass from the G_1 state through the remainder of the cycle, it was not determined whether drug or metabolite was retained by the cells for a significant period of time and contributed to the delayed resumption of growth. The present studies were designed to answer this question and to obtain additional information on the effects of pH, serum concentration and other factors that are likely to influence the distribution and potency of the drug both in cell cultures and *in vivo*.

METHODS

Materials. The study was made possible by a gift of indomethacin[(2C)-^{14}C],* 12.5 mCi/mmole (Lot No. L590, 226-02E86), from Merck, Sharpe & Dohme, Rahway, NJ. A stock solution, 50 μCi in 200 μl dimethylsulfoxide, was prepared and stored at −20°. This solution was diluted with Eagle's medium containing 10 per cent fetal calf serum as needed.

Unlabeled indomethacin (Sigma Chemical Co., St. Louis, MO), 14.3 mg, was dissolved in 200 μl of dimethylsulfoxide, neutralized with 1 N NaOH (final pH 7.4) and brought to a final volume of 10 ml with Eagle's medium containing 10 per cent fetal calf serum to make a 4 mM solution.

Media were prepared by the Media Unit, Division of Research Services at the National Institutes of Health. Other reagents were obtained from sources described in earlier papers [2, 3]. All reagents and media were sterilized by filtration before use.

Preparation of cultures. Rat hepatoma mother cultures were grown in flat-bottom, plastic, screw-capped flasks (Falcon Division, Becton-Dickinson & Co., Rutherford, NJ, Cat. No. 3024, 75 cm^2 growing surface) and were replated weekly. The cells were grown in Eagle's medium, supplemented with Earle's salts, 2 mM glutamine and 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2 at 36°.

For individual experiments, 5-day old confluent cultures were shaken vigorously, and the resultant suspensions were diluted with medium to give 20,000–30,000 cells/ml. Aliquots (1 ml) of the suspension were dispensed into parallel rows of tissue culture cluster plates (Costar, Cambridge, MA, Cat. No. 3524). One row of wells was used to determine indomethacin uptake, the other for measurement of cell count. Unless stated otherwise, cultures were grown to 50–70 per cent confluency (5–7 × 10^5

* An attempt to prepare tritium-labeled indomethacin by catalytic tritium exchange in glacial acetic acid was unsuccessful. Analysis by chromatography and crystallization with unlabeled indomethacin indicated that less than 0.1 per cent of the tritiated product was indomethacin.

cells/well) before each experiment. Each value quoted is the mean ± S.E. of six wells.

Indomethacin[^{14}C], 10 nmoles (125 nCi), was added to each well, alone or as a mixture with unlabeled indomethacin, in a volume of 10–100 μl. Sufficient medium was removed from the culture beforehand to maintain a final volume of 1 ml. Where the composition of the medium was varied, the cultures were washed twice with the new medium before addition of 1 ml of the new medium.

Measurement of uptake and efflux of indomethacin[^{14}C]. Incubations were carried out in a Forma Scientific model 3171 incubator (Marietta, OH) with an automatic air/CO_2 control adjusted to give an atmosphere of 95 per cent air, 5 per cent CO_2 and 100 per cent humidity. All other experimental manipulations were carried out in an adjacent sterile laminar-flow hood heated to 37°.

The cellular levels of indomethacin[^{14}C] were determined after removing the culture medium, washing the cultures twice with 0.1 ml of ice-cold Ca^{2+}- and Mg^{2+}-free Dulbecco's medium (pH 7.4), and adding 0.2 ml of 0.4 mM perchloric acid. The perchloric acid was removed and the precipitated cells, which adhered to the bottom of the well, were extracted twice with 0.1 ml of an ethanolic solution (0.4 mM) of unlabeled indomethacin. The ethanolic extracts were assayed for radioactivity by liquid scintillation spectrometry (efficiency of counting, 85 per cent). Preliminary studies indicated that the two washes with the Ca^{2+} - and Mg^{2+} - free Dulbecco's medium removed all of the extracellular label and 6–8 per cent of the drug in the cells. The drug remained in the cell residue after precipitation with perchloric acid but was extracted quantitatively from the residue into ethanol or an ethanolic solution of unlabeled indomethacin.

To study the time course of uptake, labeled indomethacin (20 nmoles or 250 nCi/well) was added to cultures under an atmosphere of air and 5 per cent CO_2. These were then returned to the incubator. Plates were removed from the incubator at intervals, and the amount of label taken up by the cells was determined as described above. To study the time course of release, single cultures were incubated with 60 nmoles (750 nCi) indomethacin[^{14}C] for 60 min. The cultures were washed quickly, twice with 0.1 ml Ca^{2+} - and Mg^{2+} -free Dulbecco's medium, and 1 ml of Eagle's medium with 10 per cent fetal calf serum was added back to the cultures. At the indicated times thereafter, cultures were gently shaken and 10-μl samples of media were removed for assay of radioactivity. The amount of radioactivity remaining in the cells at the end of the experiment was also determined.

Determination of cell number and size. Cultures were washed with Ca^{2+} - and Mg^{2+} -free Dulbecco's medium and incubated with 0.025 per cent trypsin solution at room temperature (24°). Detachment of cells was assessed by microscopic examination and, when complete, the reaction was stopped by addition of soybean trypsin inhibitor (0.5 per cent) solution. Dispersion of the cells was aided by repeated aspiration of the cultures through the tip of a 1-ml Eppendorf pipette. Dispersion was complete within 3–5 min, and the cells suffered no visible damage by this procedure. For the majority of experiments, cells were counted in a Neubauer counting chamber.

Cell size was determined with a Particle Data Counter (Particle Data, Inc., Elmhurst, IL) that was coupled to a logarithmic amplifier and a computer with an X–Y printout. The instrument was equipped with a 1000-μl flow meter and a 120-μl orifice, and was calibrated with latex particles of known diameter. The trypsinized cell suspensions were diluted with Isoton (Fisher Scientific, Washington, DC) to give 3–6 × 10^3 cells/ml. Size distribution analysis of the trypsinized cell suspension indicated a single peak (mean cell volume, 1.7–1.9 μl/10^6 cells) and an absence of cell fragments or clumps. A value of 1.8 μl/10^6 cells was selected to estimate cellular concentration of drug.*

Metabolism studies: chromatography of medium and cell extracts. Six HTC cultures were incubated for 4 days with 125 nCi (10 nmoles) indomethacin[^{14}C]. The culture medium was pooled, and unlabeled indomethacin (in 100 μl dimethylsulfoxide) was added to make a 0.4 mM solution. Ethanolic extracts of the cells were prepared, as described above, and pooled. Ten μl of the pooled samples was spotted onto thin-layer 5 × 20 cm precoated silica gel plates (LQD plates from Quantum Industries, Fairfield, NJ) and the plates were placed in a solvent system consisting of chloroform–ethanol–heptane–water–ammonia (40:40:40:2.8:1.6 parts by volume). Indomethacin was located by exposing the plates to iodine vapor, and the radioactivity was assayed in 2-mm wide segments of gel scraped from the plate. The recovery of indomethacin[^{14}C], added to Eagle's medium (with 10 per cent serum) and treated in the same manner was 75 per cent.

Measurement of binding of indomethacin[^{14}C] *to serum protein.* A solution of indomethacin[^{14}C] (final concentration 10 μM) in Eagle's medium with 10 per cent fetal calf serum was incubated for 15 min at 37°. The mixture (volume 100 μl) was centrifuged for 4 hr at 160,000 *g* (max) in a Beckman Airfuge Ultracentrifuge Centrifuge (Beckman Corp., Los Angeles, CA) equipped with an A-100 rotor. Samples (5 μl) were removed carefully from the surface and assayed for radioactivity. Under these conditions, the clearing time of albumin, based on a sedimentation coefficient of 4.6 *S*, was calculated to be 2.4 hr. Completeness of sedimentation was tested by recentrifugation of the sample for 2 hr. Incubation of indomethacin[^{14}C] with 100 per cent fetal calf serum indicated 97–98 per cent binding of drug to serum. Samples containing indomethacin[^{14}C] and Eagle's medium without fetal calf serum were also run as controls and showed no measurable binding of drug.

RESULTS

Uptake and metabolism of indomethacin by HTC

* Cell volumes (μl/10^6 cells), measured on different days of culture growth, were: 1.86 ± 0.12 on day 1 (64,000 cells/well); 1.89 ± 0.05 on day 2 (94,700 cells/well); 1.80 ± 0.10 on day 3 (200,500 cells/well); and 1.74 ± 0.04 on day 4 (715,000 cells/well). Measurement of the tritiated water and [^{14}C]inulin space in untrypsinized cultures gave variable results for cell volume (1–2 μl/10^6 cell).

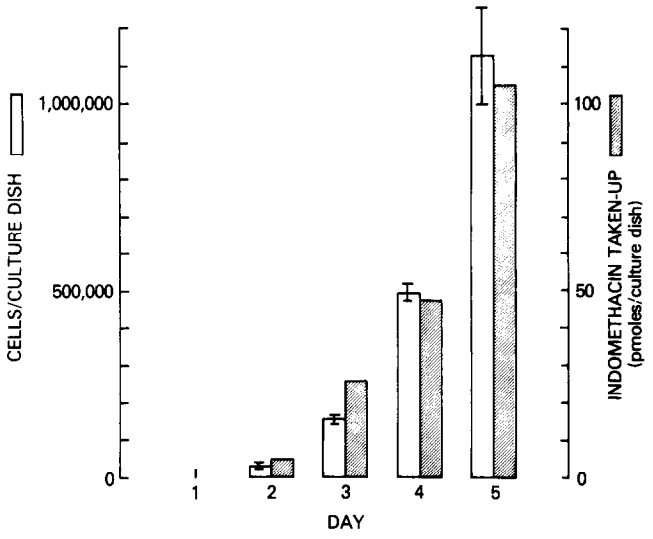

Fig. 1. Uptake of indomethacin [^{14}C] on different days of HTC culture growth. Two- to five-day cultures were incubated with indomethacin [^{14}C] (10 nmoles/ml culture) for 60 min, and the uptake was determined as described in Methods. Each is the mean ± S.E. of six cultures. Total volume of the cells, as measured in the Particle Data Counter, was 0.06, 0.27, 0.85 and 2.07 μl/well on days 2, 3, 4 and 5 of growth, respectively. Calculation of cellular concentration of drug, based on these measurements, indicated that the cellular concentration of drug was 3.0–4.7 times that in the medium, and showed no significant change from day to day.

cultures. At 10 μM concentrations, the uptake of indomethacin[^{14}C] was similar at all stages of culture growth and was proportional to the number of cells in the culture (Fig. 1). Measurement of cell volume and drug content indicated that the cellular concentration of drug was four to five times that in the medium.

The uptake of labeled drug was rapid (Fig. 2A), as was its rate of release (Fig. 2B), and equilibrium of drug between cell and medium was reached within a few minutes. Uptake was rapid both for low (20 μM) and high (400 μM) concentrations of drug (Fig. 2A). In previous studies [3], drug was removed by washing the cell cultures twice with 1 ml of Eagle's medium (with 10 per cent fetal calf serum). This procedure was found to remove > 98 per cent of the label from the cells.

Little or none of the drug was metabolized. In cultures incubated for 5 days with 10 μM indomethacin[^{14}C], most of the label in the medium and cells

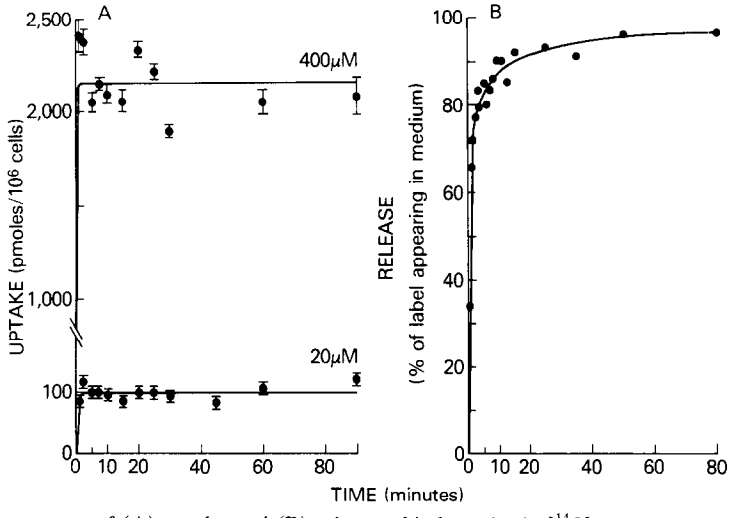

Fig. 2. Time course of (A) uptake and (B) release of indomethacin [^{14}C] in and out of cells of 4-day-old HTC cell cultures. Panel A: the amount of indomethacin[^{14}C] taken up by cells after addition of indomethacin[^{14}C] (20 or 400 nmoles/ml). Panel B: the percentage of label appearing in the medium from cells previously labeled by incubation with indomethacin[^{14}C]. Each value in panel A is the mean ± S.E. for six cultures. The data in panel B are representative of two experiments (see Methods for details). Average cell counts were 604,000 cells/well for experiment A and 640,000 cells/well for experiment B.

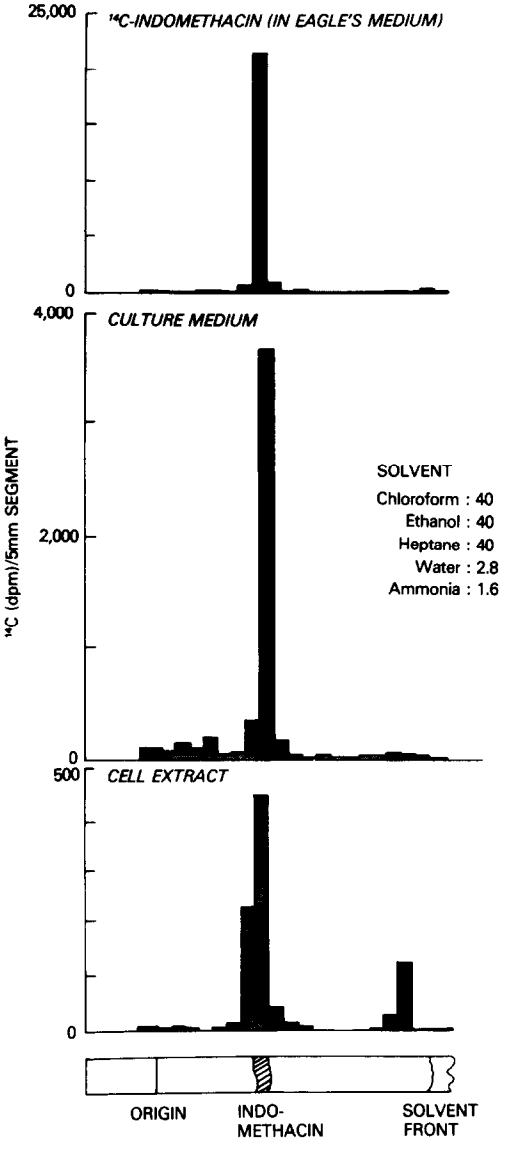

Fig. 3. Thin-layer chromatograms (silica gel) of radioactive material in medium and cells of cultures incubated with indomethacin[^{14}C] (10 nmoles/ml culture) for 5 days. The profile of radioactivity for incubations containing indomethacin[^{14}C] and Eagle's medium only is shown in the upper panel.

migrated as indomethacin upon chromatography on thin-layer plates (Fig. 3). A small amount (> 0.2 per cent of the label added) of the radioactivity in the cells migrated with the solvent front (Fig. 3). This material was not identified.

Influence of drug concentration on indomethacin uptake. The uptake of indomethacin was not saturable (solid lines, Fig. 4A and B) over the range of concentrations studied (5–800 μM), although a plot of the ratios of cell to medium drug levels (dashed lines, Fig. 4A and B) showed that uptake was biphasic. There was a sharp decline in the ratio when the concentration of drug was increased from 5 to 50 μM, and a much shallower decline thereafter. The pattern was similar in the absence (Fig. 4A) or pres-

Table 1. Effect of pH on cellular levels of indomethacin[^{14}C]*

	Indomethacin[^{14}C] levels				Indomethacin[^{14}C] cells
	Cells		Medium		
Incubation condition†	c.p.m./total	c.p.m./μl‡	c.p.m./μl		Indomethacin[^{14}C] medium
(1) MEM/air (pH 7.8)	1086 ± 44	603 ± 24	256		2.4 ± 0.1
(2) MEM/95% air + 5% CO_2 (pH 7.4)	2213 ± 30	1224 ± 17	257		4.8 ± 0.1
(3) MEM + Hepes (50 mM)/95% air + 5% CO_2 (pH 7.0)	3545 ± 52	1969 ± 29	259		7.6 ± 0.1

* Each value is the mean ± S.E.M. for six cultures.

† Four-day HTC cell cultures (990,000 cell/well) were washed with modified Eagle's medium (MEM), and 1 ml of MEM, with or without 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) buffer, was added. The plates were incubated for 10 min at 37° in air or in air with 5% CO_2. Indomethacin[^{14}C] (130 nCi) was added (to give a final concentration of 10 μM) and the plates were incubated for a further 90 min. The pH (Radiometer pH meter) was determined in cultures containing unlabeled indomethacin.

‡ A total cell volume of 1.8 μl was assumed for the calculation (see text).

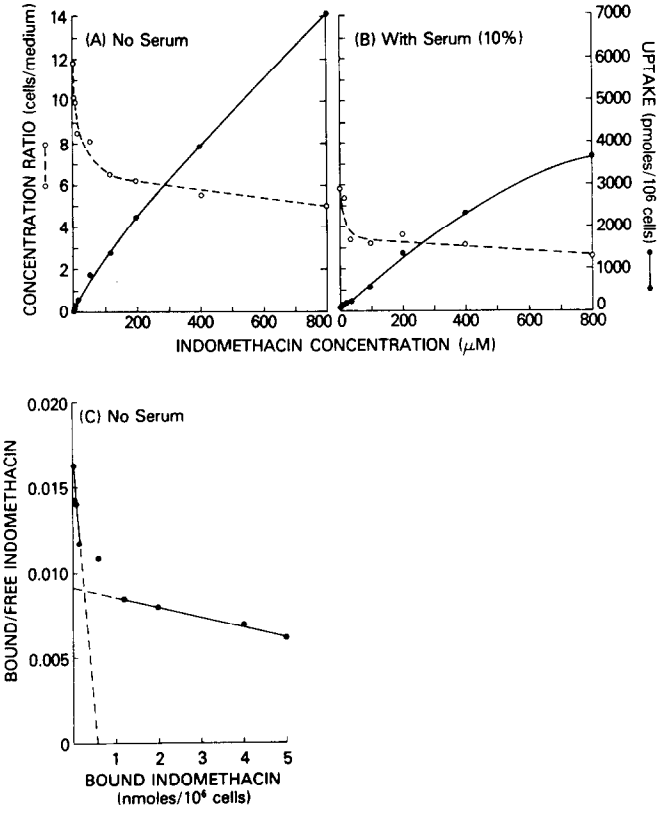

Fig. 4. Relationship of indomethacin uptake and drug concentration. Four-day HTC cultures were incubated for 30 min with indomethacin[^{14}C], and various amounts of unlabeled indomethacin in the absence (A), and in the presence (B) of 10 per cent fetal calf serum. A Scatchard plot of the data in panel A is shown in panel C as described in text. The range of indomethacin concentrations studied was 5–800 μM. Mean cell count was 740,000 cells/well and a cell volume of 1.8 μl/10^6 cells was assumed (see Methods). Each value is the mean of six cultures.

ence (Fig. 4B) of fetal calf serum, but uptake was reduced in the latter case.

Assuming that the levels of free drug in the cells and medium were the same, and that the additional drug held in the cell was bound or sequestered, a Scatchard plot (Fig. 4C) indicates two components, one having an apparent dissociation constant (K_b) of 35 μM and 0.5 nmole binding sites/10^6 cells (10^7 molecules/cell), and the other a K_b value of 600 nM and 20 nmoles sites/10^6 cells. Thus, two processes may be present, one involving high affinity and a limited number of binding sites and another involving low affinity and high capacity for sequestering indomethacin.

Effect of changes in serum concentration on drug uptake. The cells accumulated high levels of drug in the absence of fetal calf serum, and drug concentrations in the cell ranged from 5.4 to 11.8 (mean 9.5) times that in the medium in four experiments. In the presence of serum, the ability of the cells to concentrate drug diminished sharply. In one experiment, the ratio of cell to medium drug levels declined from 5.4 to 2.5 (Fig. 5), and in another from 11.5 to 5.8, when the concentration of serum was increased from 0 to 10 per cent. With high concentrations of serum

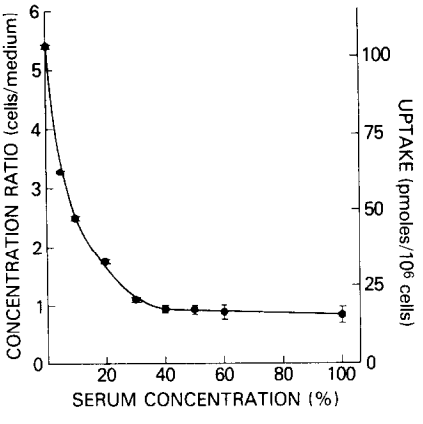

Fig. 5. Influence of fetal calf serum concentration on the uptake of indomethacin[^{14}C] by HTC cultures. The drug (10 nmoles/ml culture) was added to 4-day-old HTC cultures in which medium had been replaced with Eagle's medium containing the indicated concentration of fetal calf serum. To calculate the ratio of concentration of drug in cells to that in the medium, cell volume was assumed to be 1.8 μl/10^6 cells (see Methods). Mean number of cells was 754,000 cells/well. Each value is the mean ± S.E. of six wells.

(30 per cent and beyond), there was little further change (Fig. 5), and intracellular levels of drug were one to two times that in the medium. Protein binding measurements indicated that with 10 per cent fetal calf serum in the medium and a drug concentration of 10 μM (the concentration used in these studies) 56–80 per cent of the drug was bound to the serum protein.

pH and drug uptake. Small changes in pH, produced by changes in CO_2 concentration or by the addition of buffer, had a profound effect on the distribution of drug. The ratio of cell to medium drug levels varied from 2.4 in the absence of CO_2 (pH 7.8) to 7.6 in the presence of 5 per cent atmospheric CO_2 and a neutral pH (7.0) (Table 1).

DISCUSSION

The above studies indicate that (1) indomethacin distributes rapidly between medium and HTC cells and, in the absence of serum, becomes concentrated within the cells, (2) the intracellular levels of drug are dependent on serum concentration and pH of the medium, and (3) small changes in either of these two parameters will result in large changes in drug levels in the cells. The ability of indomethacin to bind to albumin in plasma [4–7] or, as shown in these studies, to serum protein(s) in the culture medium is the most likely reason for the increased uptake of drug in the absence of serum and for the fact that the cytostatic activity of indomethacin is increased when the serum content of the culture medium is reduced (unpublished data).

If indomethacin (pK_a 4.5) crosses the lipid cell membrane in the nonionized state and equilibrates with its lipid-insoluble ionized form on both sides of the membrane, the distribution of drug will be governed by the pH of the intra- and extracellular spaces, the higher concentrations of drug being present in the space with the higher pH. The partition of drug between the lipid and aqueous phases of the cell will also be influenced by pH. The dissociation constants of most nonsteroidal anti-inflammatory drugs are such that small changes in pH markedly alter drug distribution in tissues. For example, Brune *et al.* calculated that a shift from 7.4 to 6.8 in the extracellular pH and from 7.0 to 7.2 in the intracellular pH will result in a 4-fold increase in the intracellular concentration of phenylbutazone (pK_a 4.5) and showed that such a shift is accompanied by an increased inhibition of the phagocytic activity of blood granulocytes by this drug [9]. The 3- to 4-fold increase in indomethacin concentration in the HTC cells (noted in Table 1) also illustrates the importance of pH in regulating the distribution of the drug. The relation between uptake and drug concentration (Fig. 4), however, indicates that additional factors come into play, and that at least two processes are involved in the uptake of indomethacin. One process appears to be saturated at low and therapeutic [2] concentrations of drug. The other is not saturated even at relatively high concentrations of indomethacin. Two phenomena that would be consistent with the data are binding of drug to protein or other components in the cell and solubilization of nonionized drug in lipid structures. Whatever the mechanism, it is apparent that at therapeutic concentrations (10–20 μM) the drug is highly concentrated within the cell. Fluorescence microscopic studies with human fibroblast cultures suggest that the drug does become localized in intracellular lipid bodies (B. M. Bayer and H. Kruth, unpublished data).

The studies confirm that changes in both pH [8, 9] and protein composition of the extracellular fluid produce large changes in the cellular concentration of the acidic anti-inflammatory drugs. These effects are important in relation to the therapeutic and toxic actions of the drug, as inflammatory exudates tend to be acidic [8, 10], and these drugs concentrate in inflamed tissues [8, 11]. Extravasation of plasma proteins [12] and changes in the CO_2/carbonate equilibrium might also contribute to the higher levels of drug in inflamed tissue.

Although indomethacin is extensively metabolized in man, rats and other species [4, 12–14], and the liver is a major site of metabolism [4, 10], the rat HTC cells do not metabolize indomethacin. Unlike the isolated rat hepatocyte preparations that have been used as a model to study drug metabolism *in vitro* [15–18], the transformed cells appear to have lost the ability to perform this specialized function. The absence of metabolism also suggests that the effects of indomethacin on cell proliferation are due to the drug itself rather than to a metabolite.

The ability to remove indomethacin completely from the cultures by simple washing techniques enhances the possible usefulness of this drug in inducing synchronous growth in cell cultures. The time sequence of changes in DNA synthesis, mitosis and cell division after removal of the drug indicates that the cultures resume growth with a high degree of synchrony [1, 3]. Moreover, the cells can be held in the G_1 state for prolonged periods of time without impairment of viability or the ability of the cells to resume growth once indomethacin is removed [1, 3].

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