Journal of Supramolecular Structure 5:591 (443)–600 (452) (1976)

Pyruvate-Induced Changes in Hepatoma Cell Morphology and Macromolecular Synthesis

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Cells maintained in basal growth medium with 0.2–1.0% serum often require citric acid cycle intermediates for optimal viability. We have found that pyruvate added to minimal growth medium causes cellular flattening and formation of external processes accompanied by increased DNA synthesis in cultured hepatoma cells (HTC cells).

Cells were cultured in plastic T-flasks (0.5, 1.0, or 2.0×10^6 cells/flask) containing 5 ml medium (90% Eagle's Basal Medium (BME) and 10% Swim's S-77) with various concentrations of fetal calf serum (0.2, 0.25, 0.5, 1.0, 2.0, 10%) and either pyruvate (50, 100, 250, 500, 1,000 μg/ml), or one of: dibutyryl cAMP (DBcAMP) or dibutyryl cGMP (DBcGMP) at 10^{-3}, 10^{-4}, or 10^{-5} M. At 44–48 hr cultures were pulsed with tritiated thymidine, uridine, or leucine. Cells became attached to the plastic surface within 24 hr. Cells in medium with 0.25 to 2.0% serum had a rounded appearance. With added pyruvate, cellular flattening, process formation, and an increased adherence to the substratum was observed. By 48 hr, cultures without pyruvate grew in rounded clusters; with pyruvate, cells formed extensive interconnecting processes that appeared loosely attached to the monolayer surface. At the cell densities tested, process formation was maximal with 250 to 500 μg/ml pyruvate. Cytochalasin B blocked flattening and process formation; EDTA (1 mg/ml) caused retraction of processes within 3 min, and a slow dissolution of these structures within cells was observed. DBcAMP or DBcGMP did not induce process formation. Flattening and process formation in pyruvate-enriched cultures were accompanied by marked stimulation of DNA synthesis and smaller increases in RNA and protein synthesis. Cell number was not affected.

These pyruvate-induced changes suggest that alterations in energy metabolism, or precursors that enhance viability and macromolecular synthesis in mammalian cell cultures, may exert marked effects on cellular morphology without corresponding changes in growth of neoplastic liver cells.

Key words: pyruvate, hepatoma cells, cell shape, macromolecular synthesis

INTRODUCTION

Support of cell growth in culture appears dependent upon multiple, as yet undefined, factors in mammalian serum. Studies aimed at characterization of these factors and their effects on cells (e.g., glycyl-histidyl-lysine, fibroblast growth factor, epidermal growth factor, and somatomedin B) are usually performed with culture medium containing low

concentrations of serum (0.2 to 1.0%) sufficient to maintain cell viability, but permitting only minimal growth rates (1–5). Cell viability under these conditions is often enhanced by supplementation of the basic medium with metabolic substrates such as pyruvate, acetate, or citric acid cycle intermediates (1). For this reason, many of the commonly used culture media contain high and unphysiological concentrations of these compounds. In the case of pyruvate, physiological concentrations in serum usually range between 4 and 20 μg/ml (6), while typical pyruvate concentrations are usually in the 50–500 μg/ml range (Table I).

We have found that the addition of pyruvate to cultures of hepatoma cells (HTC) in minimal serum medium causes cellular flattening, formation of external cellular processes, and increased adhesiveness to the monolayer substratum. These morphological effects are accompanied by a marked increase in DNA synthesis without corresponding enhancement of cell growth. Since growth stimulation by growth factors is often associated with alterations in cell morphology (2–4), the role of other medium constituents in producing these changes must be considered in any assessment of the effects of growth factors on cells.

METHODS

The general methods of cell culture were as previously described (7). The HTC line, a chemically induced rat hepatoma that is maintained in spinner culture, has a generation time of about 24 hr when 10% serum is in the medium. At serum concentrations of 0.5–1%, the cells remain viable but do not grow. At lower serum concentrations, the cells detach from the surface, begin to swell and extrude cytoplasm, and subsequently die. In our experiments, the cells were cultured in closed plastic T-flasks (Falcon Plastics, Oxnard, Ca.) with a 24 cm^2 monolayer surface. Cells were added in concentrations of 0.5, 1.0, or 2.0 $\times 10^6$ cells per flask to 5 ml of a pyruvate-free culture medium (90% Eagle's BME with glutamine and 10% Swim's S-77) with fetal calf serum (Grand Island Biochemical Co., San Jose, Ca.) at concentrations varying from 0 to 10%. Prior to the addition of cells the medium was treated with either pyruvate (0, 50, 100, 250, 500, 1,000 μg/ml) or one of two cyclic nucleotides (10^{-3}, 10^{-4}, 10^{-5} M) dibutyryl adenosine 3′:5′-cyclic monophosphate (DBcAMP) and dibutyryl guanosine 3′:5′-cyclic monophosphate (DBcGMP) (Sigma Chemicals, St. Louis, Mo.). Cytochalasin B (Aldrich Chemicals, Milwaukee, Wis.), in concentrations of 0.5, 1.0, or 2.0 μg/ml, was added to medium in other experiments.

At 24 hr from the beginning of incubation another 5 ml of starting medium was added to each flask. At 44 hr, the cell culture was pulsed with 1 μCi/flask of ^{3}H-thymidine

TABLE I. Pyruvate Concentrations in Typical Cell Media (23, 24)

	μg/ml
Brinster's (BMOC-3)	56
Holmes (A2 + APG)	100
Leibowitz (L-15)	550
Ham (F-10, F-12)	110
Scherer's Maintenance	500
Neuman and Tytell	110
Dulbecco's Modified Eagles (MEM)	110
IMEM-ZO	110

(17.6 mCi/mM), ^{3}H-uridine (2,000 mCi/mM), or ^{3}H-leucine (55 mCi/mM) (New England Nuclear, Cambridge, Mass.). Incubation was terminated at 48 hr, the cultures were photographed, and incorporation of labeled precursors into DNA, RNA, and protein was measured as previously described (7).

RESULTS

Pyruvate-Induced Changes in Cell Morphology

By 24 hr, the hepatoma cells had attached to the support surface. Cells maintained in pyruvate-free medium with 0.5–2.0% serum (low serum) adhered to the surface but had a generally rounded appearance. In medium with added pyruvate, cells were beginning to flatten and to extend cellular processes.

At 48 hr, cells in low serum (0.5–2.0%) without pyruvate were growing in rounded clusters (Fig. 1). In the presence of pyruvate, the cells tended to flatten, and to form long cellular processes (Fig. 2). The processes often appeared to fuse into smooth interconnecting links, so that cells and processes tended to become organized into extended matrices (Figs. 3 and 4). The unattached ends of processes had a flattened and splayed appearance (Fig. 5). Process formation was maximal at pyruvate concentrations between 250 and 500 μg/ml, and increased only slightly at 1,000 μg/ml. Concentrations of pyruvate as low as 50 μg/ml caused pronounced cellular flattening but little process formation (Fig. 6). Neither flattening nor formation of processes were influenced by cell density, being similar at all tested cell concentrations ($0.5–2.0 \times 10^{6}$ cells/flask).

In serum-free medium, cells adhered poorly and were not affected by pyruvate. The morphological effects of pyruvate were maximal when the serum concentration in medium was 0.5%. In 10% serum most cells were moderately flattened and did not form processes, and pyruvate had no observable effect.

DBcAMP at 10^{-3} M induced cellular flattening but not to the extent observed with pyruvate. Cellular processes were not formed. DBcAMP at lower concentrations, and

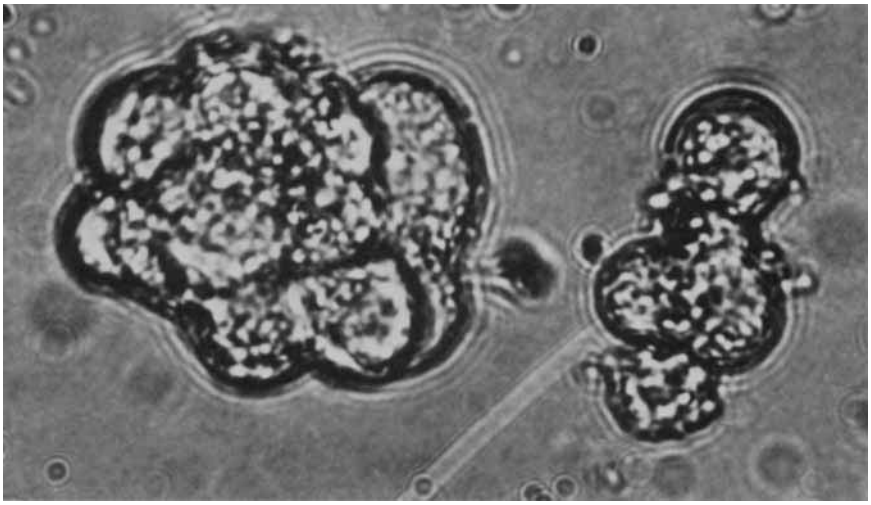

Fig. 1. Hepatoma cells maintained for 48 hr in medium with 0.5% serum without pyruvate. Cells form rounded clusters without flattening or process formation (phase contrast, × 500).

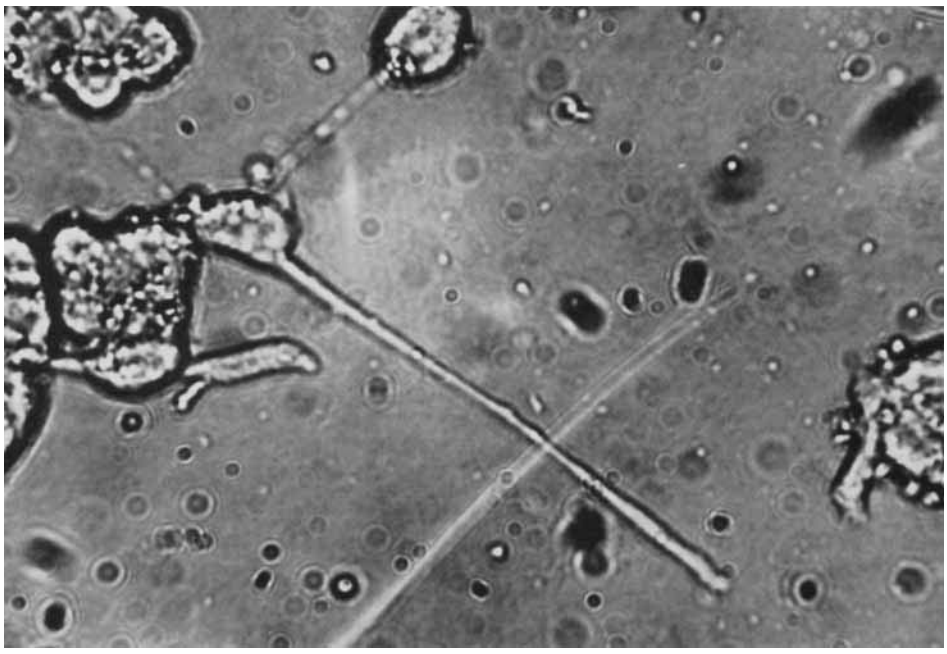

Fig. 2. Hepatoma cells maintained for 48 hr in medium with 0.5% serum and 500 μg/ml pyruvate. Cells extrude long processes with a hollow appearance (phase contrast, × 375).

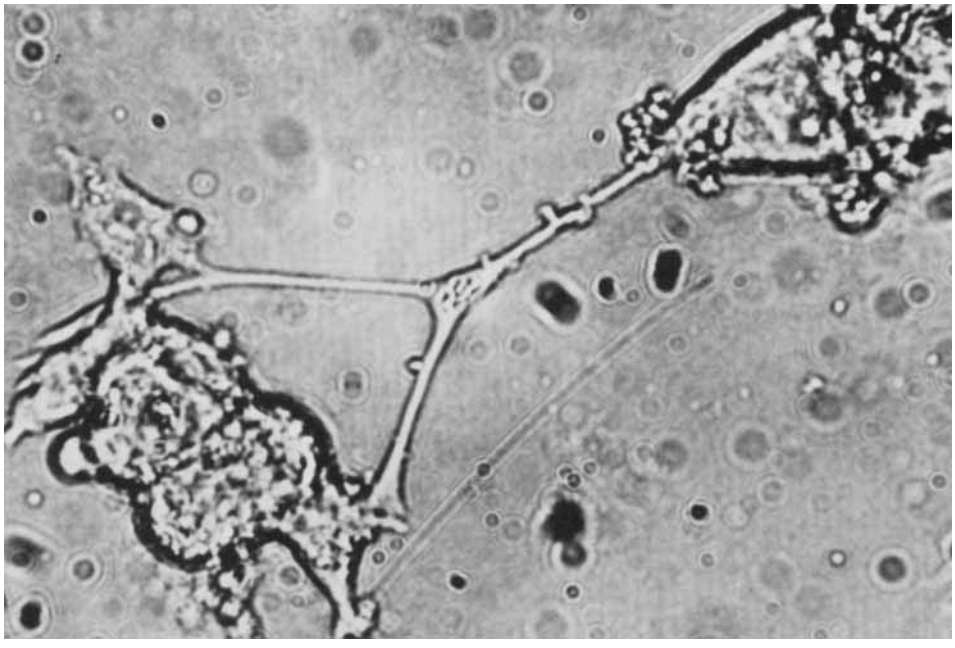

Fig. 3. Hepatoma cells maintained as in Fig. 2. Fusion of processes connecting three cells (phase contrast, × 375).

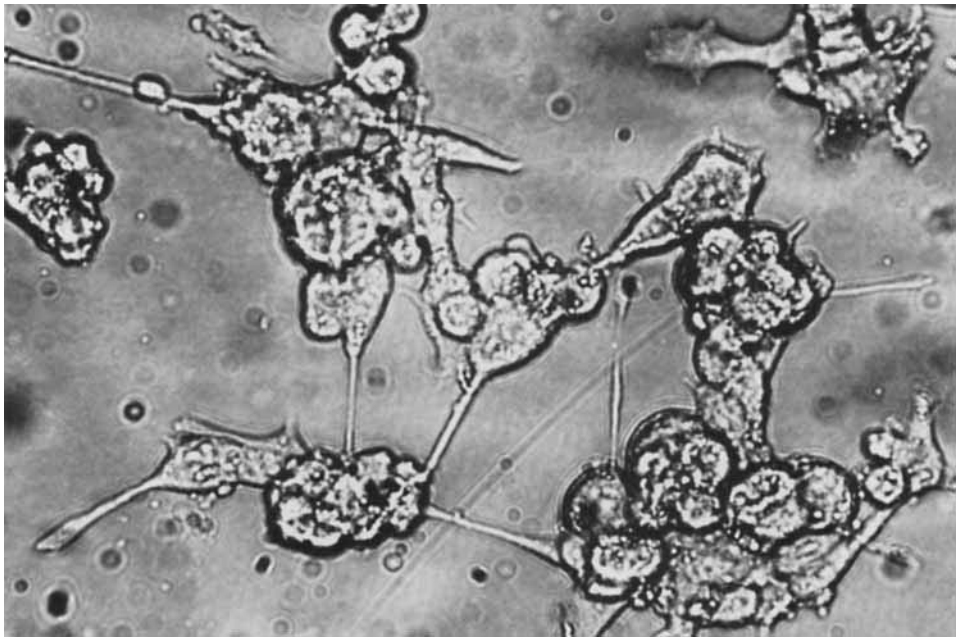

Fig. 4. Hepatoma cells maintained as in Fig. 2. Cells are flattened and interconnected by a matrix of cellular processes (phase contrast, × 188).

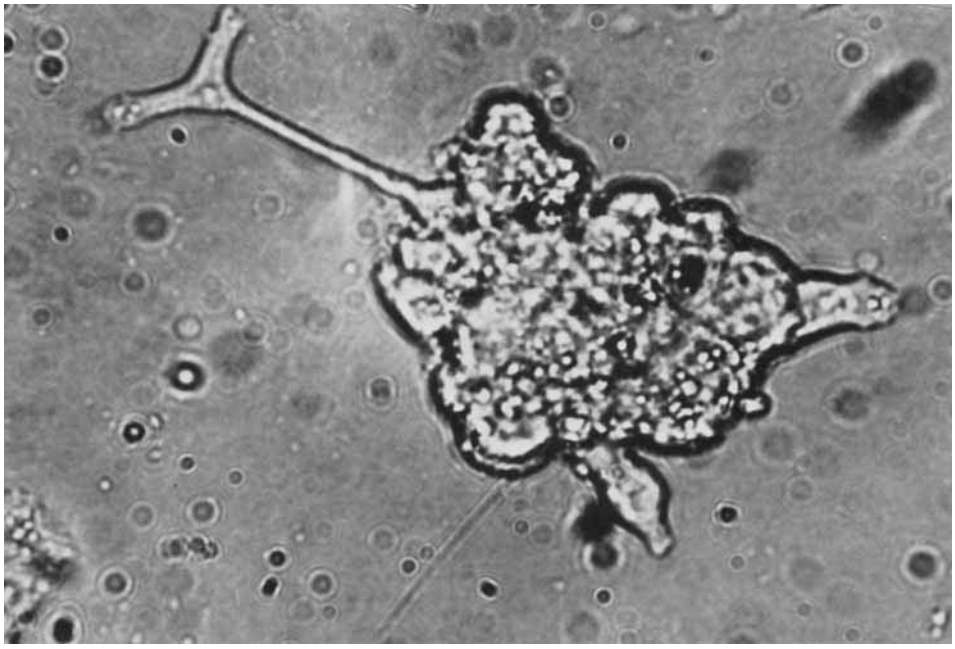

Fig. 5. Hepatoma cells maintained as in Fig. 2. A cluster of cells extruding a single process. Unattached end of process is flattened and splayed (phase contrast, × 375).

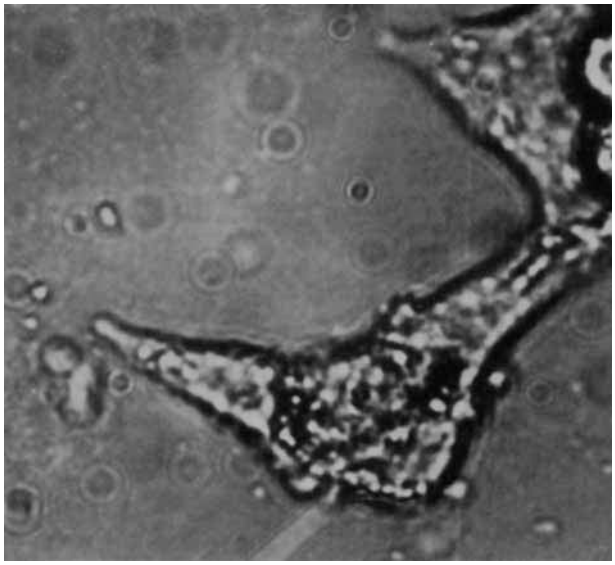

Fig. 6. Hepatoma cells maintained for 48 hr in medium with 0.5% serum and 50 μg/ml pyruvate. Cells are flattened, but processes are poorly formed (phase contrast, × 350).

DBcGMP at all concentrations tested, had no effect on morphology. Cytochalasin B at 1 μg/ml completely blocked the development of cellular flattening and process formation in pyruvate-enriched cultures grown in 1% serum but had no effect on cells in 10% serum.

When the calcium chelator EDTA, at 1 mg/ml, was added to cells with developed processes, the processes retracted into the cells within 1–3 min, suggesting that the pseudopodia were loosely attached to the surface. Segments of processes approximately equal in length to the cellular diameter were often absorbed intact by the cells and appeared to dissolve slowly in the cytoplasm.

Effects of Pyruvate on Cell Adhesion

Cells supplemented with sodium pyruvate were much more resistant to removal from the plastic surface of culture flasks than were the controls. Gentle shaking released less than 3% of the cells into medium in pyruvate-supplemented cultures, compared with 20% to 30% in control cultures (Table II). Complete detachment of cells from plastic surfaces required repeated incubation with trypsin (0.01%) and EDTA (0.005%). Cells that were incubated in the presence of cytochalasin B (1 μg/ml) and pyruvate-supplemented medium adhered poorly to the surface and a majority of cells (75%) could be removed with gentle shaking.

Effects of Pyruvate on Macromolecular Synthesis

The concentrations of pyruvate that provided for maximal changes in cellular morphology also enhanced macromolecular synthesis. Flattening and process formation were associated with a 3- to 15-fold increase in DNA synthesis, and smaller increases in RNA and protein synthesis (Fig. 7). The specific activities of labeled thymidine in the acid soluble fractions of cytosol from cells grown with and without pyruvate were similar, indicating that intracellular thymidine pools were not affected by pyruvate. Despite increased

TABLE II. Effect of Pyruvate and Cytochalasin B on Hepatoma Cell Adhesiveness

Treatment	Percentage of total cells recovered		
	Basal medium	Basal medium + pyruvate	Basal medium + pyruvate + CB
1. Wash-off procedure	26	3	73
2. 1st trypsinization + EDTA	78	32	85
3. 2nd trypsinization + EDTA	96	60	98
4. 3rd trypsinization + EDTA	99	93	100
5. 4th trypsinization + EDTA	100	98	–

Cultures (1×10^6 cells) were plated in closed T-flasks of 24 cm^2 area (Falcon Plastics, Oxnard, Ca.) in the basal medium containing 1% serum under conditions described in Methods. Sodium pyruvate (500 μg/ml) and cytochalasin B (CB) (1 μg/ml) were added as indicated. After 48 hr, the culture medium was gently washed back and forth over the cells, then removed. The remaining cells were detached by a series of 10 min incubations with a 0.9% saline solution, pH 7.4 containing 0. 01% trypsin and 0.005% EDTA. Cells were counted in a hemocytometer.

DNA synthesis, cell number did not rise above control values in the pyruvate-treated cultures.

DISCUSSION

The stimulatory actions of serum on cell growth, survival, viability, and macromolecular synthesis in vitro are usually attributed to protein factors in serum (8). The present study suggests that simpler and smaller components of serum, such as intermediates of energy metabolism, may also exert multiple effects on the structure and synthetic activities of cultured cells. The biological properties of these nonprotein constituents of serum appear to vary with their concentrations (e.g., cellular flattening at low pyruvate concentrations, formation of processes at higher concentrations) and with the concentration of other serum components in culture medium. Thus, the relative lability, multiplicity, and broad concentration range of energy intermediates in serum may contribute to variability of results obtained with cells cultured in media reinforced with different batches of serum (8).

Effects on cellular morphology similar to those produced by pyruvate in hepatoma cells have been observed with high concentrations of cAMP, DBcAMP, butyrate, theophylline, and hormones in a variety of other culture systems. Myoblasts form intercellular processes upon addition of cAMP, DBcAMP, or theophylline (9). Treatment of fish melanophores with melanocyte stimulatory hormone causes extrusion of processes concomitant with release of cAMP, an effect that may be reversed with norepinephrine, an antagonist to cAMP in this system (10). Cellular flattening, process formation, increased adherence to the substratum, restoration of contact inhibition, loss of cancer cell surface antigens that bind to lectins, and inhibition of growth can be induced in many neoplastic cell cultures by cAMP, DBcAMP, or theophylline (11–13), while butyrate exerts analogous effects on cell lines that are morphologically unresponsive to cAMP or its analogs (14–15). The factors that induce these changes in cell morphology may all act through common stimulation of cellular energy metabolism. The effect of pyruvate may be similar, since the addition of exogenous pyruvate is known to increase citric acid cycle activity in neoplastic tissue (16).

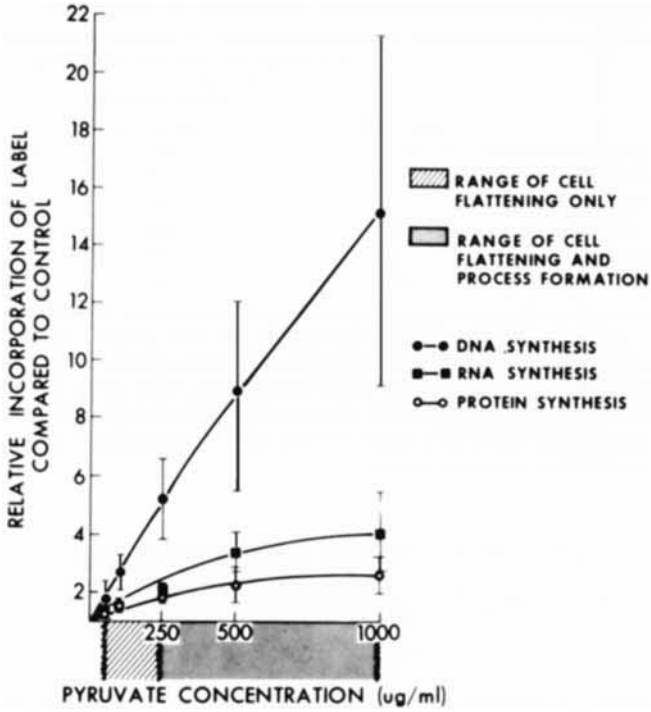

Fig. 7. The effect of pyruvate in medium on DNA, RNA, and protein synthesis. Hepatoma cells were cultured in medium containing 0.5% fetal calf serum as described in the Methods section. Bars represent standard deviation from the mean. Incorporation of label in control cultures was normalized to unity. Each point represents the average of at least six separate experiments.

Holley (17) has proposed that the sequence of events in cell replication is tightly coupled to the intracellular concentrations of certain "critical" substrates. One of the first events in the sequence leading to cell replication is greatly increased energy metabolism necessary for cell division. The addition of pyruvate to cells in minimal growth medium may mimic the early stages of cell replication by increasing cellular energy production. The pyruvate-induced stimulation of DNA synthesis accompanied by the lack of increase in the cell number suggests that under these conditions the cells proceed through the "S" phase (DNA replication) of the cell cycle, then stop. The idea that the exogenous pyruvate may allow the cells to proceed only through part of the cell cycle is in accord with the concept that concerted action of several serum factors is necessary for cell replication. No single factor has yet been isolated that allows serum-dependent cells to replicate in defined medium (1–5, 17).

It is unlikely that the increase in DNA synthesis observed in pyruvate-treated hepatoma cell cultures is directly linked with the morphological changes induced by pyruvate. While cellular flattening and spreading occur in many cell types during the stage of active DNA replication (S phase), flattening and extrusion of long filamentous processes is also accompanied by a modest decrease in DNA synthesis in cultured rat and human hepatoma cells exposed to lysozyme (18).

The pyruvate-induced effects on hepatoma cell morphology were blocked by the action of cytochalasin B in minimal growth medium. While this drug induces cellular contraction, inhibition of locomotion and membrane movements, and pseudopod extension in many cell types (19–21), its mechanism of action at concentrations effective on cells (10^{-6} M) is still obscure. Inhibition by the drug of cytoplasmic gelation reactions has been proposed (19, 20). The decreased adhesiveness of hepatoma cells in the presence of cytochalasin B may be due to inhibition of cellular attachment, as reported by Miranda et al. (19). These workers also observed that once cells become attached, cytochalasin B may enhance adhesion to the substratum. The difference between this observation and our results may be due to the low serum concentration (1%) used in the minimal growth medium. Under these conditions, the hepatoma cells attach poorly to the substratum and may be more sensitive to inhibitory effects of cytochalasin B, whereas when grown in 10% serum in the presence of a similar concentration of the drug (1 μg/ml), the cells attach in a normal manner.

Many highly purified growth factors influence the morphology as well as the replication rate of cultured cells. It is of interest that these effects are usually induced with medium containing pyruvate in high concentrations (3–5, 11, 12, 14, 22), which may contribute to changes in normal and neoplastic growth patterns. For example, BHK 21/13, a line of thin, elongated fibroblasts, assume a rounded shape, exhibit loss of contact inhibition, and grow in patterns associated with transformed or neoplastic cells when insulin in concentrations (8 μg/ml) three orders of magnitude above the physiological is added to the medium (22). In view of the effects of pyruvate on hepatoma cells, it is possible that high concentrations of insulin may be required because of interference by pyruvate with insulin-induced changes in morphology and growth patterns of BHK cells.

Our observations demonstrate that energy intermediates in medium may exert striking effects on cellular morphology, intercellular associations, and macromolecular synthesis in cultured cells. These findings suggest that cellular responses to biological and synthetic growth-promoting factors may be altered or modified by nonproteinaceous, small molecular weight constituents of most defined growth media. The influence of these components must be carefully evaluated in in vitro studies of cell growth and morphology.

ACKNOWLEDGMENTS

This work was supported by USPHS research grant HD 03148, the Liver Center Grant P17 AM 18520, and grant 717 from the American Cancer Society (California Division).

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