The Metabolic Requirements for Transcellular Movement and Secretion of Collagen*

(Norman J. Kruse† and Paul Bornstein§)

From the Departments of Biochemistry and Medicine, University of Washington, Seattle, Washington 98195

SUMMARY

Cultures of chick tendon fibroblasts were capable of normal ATP production and protein synthetic activity even though the normally high rate of glycolysis was markedly reduced by substitution of pyruvate for glucose. Iodoacetate and 2-deoxyglucose reduced ATP levels and protein synthesis even in the presence of pyruvate. Under these conditions, both inhibitors were shown to have effects on the energy metabolism of cells which were apparently unrelated to an inhibition of glycolysis.

Selective inhibition of either glycolysis, by incubation in glucose-free medium, of oxidative phosphorylation, by incubation with an uncoupler, was shown to have little effect on cellular ATP levels or intracellular transport and secretion of collagen. However, inhibition of both glycolysis and oxidative phosphorylation resulted in decreased cellular ATP levels and an inhibition of collagen secretion. This effect was not due to a requirement for continued protein synthesis, since inhibition of protein synthesis with cycloheximide or puromycin had little effect on collagen secretion. The ATP requirement for intracellular transport and secretion is discussed in relation to the secretory pathway for collagen.

The collagen molecule exists intracellularly as a higher molecular weight biosynthetic precursor, procollagen (1, 2), and is synthesized primarily, if not exclusively, on membrane-bound ribosomes of the rough endoplasmic reticulum (3–5). Recent studies employing electron microscope autoradiography (6) and ferritin-labeled antibodies (7, 8) have implicated the Golgi complex in the pathway for secretion of procollagen, but the detailed mechanisms and the metabolic requirements for secretion have not been established.

There is evidence for the involvement of microtubules in the transcellular movement of procollagen since agents which disrupt microtubular function also inhibit conversion of procollagen to collagen (9) and secretion of procollagen (10–12). Conversion of procollagen to collagen is thought to occur extracellularly (1, 2, 13), hence lack of conversion would be expected to follow intracellular retention of the protein. In the study of Ehrlich and Bornstein (9), an energy requirement for collagen secretion was suggested since incubation of embryonic chick cranial bones with m-Cl-CCP, an inhibitor of oxidative phosphorylation, resulted in decreased conversion of procollagen to collagen. However, the possibility of nonspecific effects of the drug on the secretory process was not eliminated and metabolic consequences were not analyzed to establish that the production of ATP by oxidative metabolism was inhibited.

An ATP requirement has been established in pancreatic zymogen and insulin secretion (14–16). In both cases, ATP production by oxidative phosphorylation appeared to be essential for secretion, while inhibition of glycolysis had little effect (15–17). In the case of zymogen secretion, this apparently reflects the tissue's predominant aerobic metabolism and a limited glycolytic capacity (15). Little is known of the mechanisms by which ATP is utilized in secretory processes, and the question remains whether glycolytic and mitochondrial ATP are equivalent in meeting the cell's energy needs for this process. This is a pertinent consideration in light of reports suggesting such functional compartmentalization for a number of metabolic processes in animal cells (18–21).

The present work clearly shows an energy requirement for intracellular movement and secretion of collagen. The effect of selective inhibition of ATP-producing pathways on cellular energy metabolism and collagen secretion in cultured chick tendon fibroblasts was examined to determine whether specific metabolic pathways are required for the transcellular movement and secretion of collagen.

EXPERIMENTAL PROCEDURE

Materials—Powdered Dulbecco's modified Eagle's medium, 2.5% trypsin solution, fetal calf serum, and penicillin-streptomycin solution were obtained from Grand Island Biological Co., crude collagenase was from Worthington Biochemical. Firefly extract (FLE-250), crystalline disodium ATP, rabbit muscle lactic dehydrogenase, NAD+, 2-deoxyglucose, iodoacetate, and puromycin were all obtained from Sigma Chemical Co. L-Lactate, 1.0 N standard solution, was a gift from Boehringer Mannheim; m-Cl-CCP and cycloheximide were obtained from Calbiochem. Cables and center wells for CO2 determinations were from Kontes Glass Co. and plastic flasks were from Falcon Plastics. All radioactive compounds were obtained from New England Nuclear.

Cell Cultures—Seventeen-day-old chick embryos were obtained from a local hatchery. Chick tendon fibroblasts were isolated as

* This work was supported by National Institutes of Health Grants AM 11248 and DE 02600.
† Predoctoral Fellow of the National Institutes of Health.
§ Recipient of Research Career Development Award K4-AM-48882 from the United States Public Health Service.

1 The abbreviations used are: m-Cl-CCP, carbonyl cyanide m-chlorophenylhydrazone; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

4841
previously described (22) with the modification that digestion was performed with 0.05% crude collagenase in Ca2+- and Mg2+-free Puck's saline (0.9% NaCl solution) G digestion. Solution was continued for 60 to 90 min at 37° until nearly all of the tendon material was digested. After addition of growth medium and filtration through lens paper, the suspended cells were centrifuged at room temperature at 1000 g for 10 min and plated at a starting density of 2 to 4 x 10^4 cells/75 cm^2 Falcon flask. Dulbecco's medium with 27 mM NaHCO_3, 15 mM Heps, pH 7.4, 50 μg/ml of sodium ascorbate, 50 units/ml of penicillin, and 50 μg/ml of streptomycin, supplemented with 10% fetal calf serum, was used and cultures were grown under 5% CO_2-95% air at 37°.

Pulse-labeling experiments were done on confluent primary or secondary cultures. For these experiments, serum-free Dulbecco's medium was used with 25 mM Heps, pH 7.4, 4.2 mM NaHCO_3, and 50 μg/ml of sodium ascorbate, and incubations were carried out in an air atmosphere. In pulse and pulse-chase experiments, the growth medium was removed, the cell layer was washed once with serum-free medium, and the culture preincubated for 60 min. The preincubation medium was removed and medium with a radioactive amino acid was added. In chase experiments the labeling medium was removed, the cells were washed once with the chase medium containing 15 mM proline and then incubated in proline-containing medium. Incubations were terminated by removing the medium, washing the cell layer once with cold saline solution and then scraping the cell layer into cold 0.1 N acetic acid. The cells were sonicated with two 15-s bursts with the sonicator set at 10 on an intensity setting of 30. Aliquots of the cell sonicate were removed and saved for protein determinations (23). One milligram of bovine serum albumin was added as carrier protein per 10 ml of medium, and both cell and medium fractions were dialyzed extensively against 0.1 N acetic acid at 4°.

Collagen Assays—After dialysis, the volume of dialysates was determined and aliquots were counted in a toluene-based scintillation solution and then scraping the cell layer into cold 0.1 N acetic acid. The cells were sonicated with two 15-s bursts with the sonicator set at 10 on an intensity setting of 30. Aliquots of the cell sonicate were removed and saved for protein determinations (23). One milligram of bovine serum albumin was added as carrier protein per 10 ml of medium, and both cell and medium fractions were dialyzed extensively against 0.1 N acetic acid at 4°.

Radioactive collagen was also estimated by digestion with purified collagenase (25). Under a variety of experimental conditions, assays by collagenase digestion correlated well with values obtained by radioactive hydroxyproline determination assuming that approximately 45% of the prolyl residues in collagen are hydroxylated. Analysis of cold trichloroacetic acid-soluble peptides released by purified collagenase digestion revealed 40% radioactive hydroxyproline, indicating that prolyl hydroxylation was complete in these cultures.

Lactic and Glucose Determinations—Lactate determinations were performed using lactate dehydrogenase as described previously in this laboratory. Preliminary experiments indicated that under the incubation conditions used, approximately 95% of the lactate present in the cultures was released in the medium. Subsequently, only medium fractions were collected. After removal of the residual medium, bovine serum albumin carrier was added and protein precipitated with 0.4 N HClO_4. After centrifugation, aliquots of the supernatant were assayed in duplicate with identically treated fresh medium used as a blank.

Glucose was determined by the phenolsulfuric acid hexose assay (26). Assays were performed on replicate acts of five or six complete and values were subtracted from those obtained for fresh medium to calculate glucose utilized.

ATP Determinations—One bottle of firefly extract (FLE-230) was suspended in 25 ml of cold H_2O and stirred in the cold for 24 hours. The solution was centrifuged at 30,000 X g and aliquots were frozen for future use. A 1 ml stock solution of ATP was prepared in distilled water, and stored frozen in aliquots. Upon thawing, the luciferase was centrifuged at top speed in a clinical centrifuge to remove particulate matter formed by freeze-thawing, allowed to incubate for 60 min on ice to reduce endogenous luminescence, and diluted 1:50 into freshly prepared 0.01 N NaSO_4, 0.005 N MgSO_4, 0.025 N 2-mercaptoethanol, 0.5% bovine serum albumin, pH 7.4 (28). The ATP solution was diluted to 10^-4 M with the precise concentration determined spectrophotometrically.

Preparation of cell samples for ATP analysis was modified from the procedure of Stambrook and Siskin (29). After medium removal, 0.5 N HClO_4 was added and the cells were allowed to stand 30 min on ice. Cells were then scraped with a rubber policeman, homogenized in a ground glass homogenizer, and centrifuged at 15,000 X g. Protein determinations (23) were done on the pellet material. The supernatant was neutralized by addition of an equal volume of 0.4 N KOH, 0.1 N KHCO_3, and centrifuged to remove precipitate. The supernatant was then dialyzed extensively against 0.1 N acetic acid. Each reaction vial contained 0.9 ml of 50 mM glycylglycine, pH 7.45, 50 μl of 0.10 M MgSO_4, 20 to 100 μl of 10^-4 M ATP, or 10 to 20 μl of neutralized cell supernatant, and 20 μl of diluted luciferase were added to initiate the reaction. Determinations were carried out in a Beckman LS-230 liquid scintillation counter by a modification of an automated procedure (28). The luciferase was added manually with a repipette to the appropriate vial and mixed on a Vortex mixer precisely when the count cycle started on a previous vial so that the time from addition of the enzyme to the onset of count determinations was constant. Each standard point or sample was determined in triplicate.

Radioactive CO_2 Determinations—Radioactive CO_2 determinations were performed using bottles capped with a rubber plug equipped with an attached center well (30). For these experiments cells were grown to confluent monolayers in 50-ml glass prescription bottles. It was necessary to use glass bottles rather than the plastic Falcon flasks since the latter are permeable to CO_2. Following incubation with [2-14C]pyruvate, 200 μl of 70% HClO_4 were injected into the bottle. Hydroxide (500 μl) of Hyamine (Packard Instrument Co., Inc.) was then placed in the center well and bottles were incubated 90 min at room temperature. Control experiments indicated these conditions accomplished maximal absorpt of the 14CO_2. Three bottles containing confluent cell layers were used for each experiment. In these experiments it was found to be more accurate to express data on the basis of culture bottles rather than protein content. Protein determinations were unreliable due to the difficulty in quantitatively removing cell collagen from the glass surface.

RESULTS

Cell Culture—Isolated tendon cells adapted well to culture and exhibited a typical fibroblast-like morphology. Cell division was rapid with a doubling time of 18 hours estimated from the logarithmic growth phase of the curve shown in Fig. 1. Cultures reached a confluent density of 5 to 10 X 10^5 cells per flask at 4 to 5 days. Subsequently, cell division was slower as additional layers began to accumulate.

Pulse-Chase Experiments—It was found necessary to use relatively long incubation periods to obtain sufficient incorporation of proline into collagen for subsequent analyses. Thus, as seen in Fig. 2, radioactive peptideyl proline or hydroxyproline was not released into the culture medium in detectable quantity until 45 min after administration of the isotope. A similar lag in secretion was observed in cultured chick embryo fibroblasts (31), while freshly isolated chick tendon fibroblasts exhibited a shorter lag time (11, 22). By 60 min, 9% of the incorporated radioactivity but 20% of the radioactive hydroxyproline was present in the medium (Fig. 2A), indicating active secretion of radioactive collagen. A 60-min incorporation period was used to obtain maximal labeling of intracellular collagen in subsequent pulse-chase experiments. The data in Fig. 2A indicate that the conditions used during the chase period following a 60-min pulse were effective. Thus, total radioactivity incorporated in the cell layer at the start of the chase period closely approximates that present in the combined cell layer and medium at all times during the chase. The
FIG. 1. Growth of embryonic chick tendon fibroblasts in monolayer culture. Cells were plated at a starting density of approximately 0.5 x 10^6 cells/flask with an apparent plating efficiency of 75%. On successive days three or more plates of cells were trypsinized for 10 min at 37° with 0.05% trypsin in Puck’s saline A solution containing 0.02% EDTA. Post-confluent cultures were pretreated with 0.05% crude collagenase in Ca²⁺- and Mg²⁺-free saline G for 30 min at 37° in order to obtain a single cell suspension. After trypsinization, several volumes of growth medium were added and the cells were centrifuged at 350 x g for 10 min. Cells were resuspended in a known volume of growth medium, and three separate aliquots were counted in a hemocytometer. The cells were recenterfuged and suspended in 0.1 N acetic acid for protein determinations.

small increase in total radioactive hydroxyproline observed following institution of the chase period (Fig. 2B) presumably results from hydroxylation of proline present in nascent polypeptide chains at the start of the chase (32, 33).

Since the amount of radioactive collagen appearing in the culture medium was nearly maximal after a chase of 60 min (Fig. 2B), this period was selected for subsequent experiments. However, not all radioactive collagen is released from the cell layer during a chase period. Thus, even after 4 hours, 37% of the radioactive hydroxyproline for 30 min at 37° in order to obtain a single cell suspension. After trypsinization, several volumes of growth medium were added and the cells were centrifuged at 350 x g for 10 min. Cells were resuspended in a known volume of growth medium, and three separate aliquots were counted in a hemocytometer. The cells were recenterfuged and suspended in 0.1 N acetic acid for protein determinations.

Since the amount of radioactive collagen appearing in the culture medium was nearly maximal after a chase of 60 min (Fig. 2B), this period was selected for subsequent experiments. However, not all radioactive collagen is released from the cell layer during a chase period. Thus, even after 4 hours, 37% of the radioactive hydroxyproline for 30 min at 37° in order to obtain a single cell suspension. After trypsinization, several volumes of growth medium were added and the cells were centrifuged at 350 x g for 10 min. Cells were resuspended in a known volume of growth medium, and three separate aliquots were counted in a hemocytometer. The cells were recenterfuged and suspended in 0.1 N acetic acid for protein determinations.

small increase in total radioactive hydroxyproline observed following institution of the chase period (Fig. 2B) presumably results from hydroxylation of proline present in nascent polypeptide chains at the start of the chase (32, 33).

Effects of Metabolic Inhibitors on Protein Synthesis and Carbohydrate Metabolism—Preliminary experiments indicated the prominence of glycolysis in chick tendon fibroblast cultures, as evidenced by high rates of glucose utilization and lactate production. More than 90% of the glucose metabolized by such cultures was accounted for by lactate released into the medium (data not shown). This observation is consistent with previous studies of cultured diploid fibroblasts (36, 37). We hoped to utilize the glycolytic inhibitors iodoacetate and 2-deoxyglucose, in conjunction with an alternate energy source such as pyruvate, to examine the effect of selective inhibition of glycolytic ATP production on cellular energy levels and on the ability of the cell to transport and secrete collagen. Experiments with varying concentrations of iodoacetate in the presence of glucose indicated that a concentration of 0.05 mM iodoacetate produced a 85% inhibition of glucose metabolism, measured by lactate production. This concentration, and a concentration of 6.1 mM 2-deoxyglucose (1 mg/ml) were chosen in experiments to examine the selectivity of these agents.

Incorporation of [3H]tryptophan or [3H]proline into protein was unimpaired in cultures metabolizing pyruvate rather than glucose (Table I). Secretion of collagen was also normal as judged by appearance of radioactive hydroxyproline in the medium of cultures incubated with [3H]proline (Table II). When iodoacetate was added to pyruvate-containing medium, the reduction in labeled protein was predominantly in the medium fraction, reflecting an inhibition of both protein synthesis and secretion (Table II). Paradoxically, the addition of iodoacetate to cells cultured in the presence of both pyruvate and glucose resulted in a substantially greater reduction in protein synthesis (see below and “Discussion”). 2-Deoxyglucose produced a dramatic decrease in proline incorporation into protein, to a level less than 10% of that in control cultures (Table II).

To determine the contribution of a reduced synthesis of high energy compounds to these results, analyses of ATP content as well as lactate production were performed. As shown in Table
Ability of pyruvate to serve as energy source for chick tendon fibroblasts

| Carbohydrate          | Fraction     | Experiment 1 | Experiment 2 |
|-----------------------|--------------|--------------|--------------|
|                       | cpm/mg cell protein x 10^4 | cpm/mg cell protein x 10^4 |
| 1. 5.5 mM glucose     | Cell         | 17.0         | 12.5         |
|                       | Medium       | 1.7          | 2.92         |
| 2. 5.5 mM glucose     | Cell         | 18.0         | 15.4         |
|                       | Medium       | 1.96         | 3.91         |
| 3. 5 mM pyruvate      | Cell         | 18.9         | 15.7         |
|                       | Medium       | 1.56         | 3.76         |
| 4. 10 mM pyruvate     | Cell         | 21.3         | 13.0         |
|                       | Medium       | 1.43         | 3.18         |

- Cultures, consisting of two 25-cm² flasks, were preincubated for 60 min and then incubated in the presence of a labeled amino acid for 120 min.
- The medium contained 1 μCi/ml of [3H]tryptophan.
- The medium contained 3 μCi/ml of [3H]proline.

Effects of iodoacetate and 2-deoxyglucose on protein synthesis

| Condition | Fraction | Total incorporation | Hydroxyproline |
|-----------|---------|---------------------|----------------|
|           | cpm/mg cell protein x 10^3 |
| 1. Glucose| Cell    | 43.7                | 4.72           |
|           | Medium  | 16.5                | 6.77           |
| 2. Pyruvate| Cell  | 62.0                | 5.94           |
|           | Medium  | 20.1                | 8.45           |
| 3. Pyruvate| Cell  | 52.5                | 6.04           |
|           | Medium  | 19.8                | 7.93           |
| 4. No exogenous energy source | Cell | 46.7                | 5.05           |
|           | Medium  | 11.6                | 4.51           |
| 5. Pyruvate| Cell  | 37.7                | 2.45           |
|           | Medium  | 2.4                 | N.D.           |
| 6. Pyruvate| Cell  | 11.1                | 0.20           |
|           | Medium  | 0.97                | N.D.           |
| 7. Pyruvate| Cell  | 0.76                | N.D.           |

- Cultures were incubated for 120 min in the presence of the carbohydrate, or drug, or both. ATP and lactate determinations were performed on the cell layer and medium, respectively.
- Concentrations were: glucose, 5.5 mM; pyruvate, 5 mM; iodoacetate, 0.05 mM; 2-deoxyglucose, 6.1 mM.
- Lactate production was measured for the last 60 min of the incubation period.

III, glycolysis, measured by lactate production, was reduced to less than 10% of control values when pyruvate was used as a carbohydrate source, while the ATP content of the cells remained normal. Thus, under these conditions glycolysis should make only a minor contribution to ATP production. Yet, addition of either iodoacetate or 2-deoxyglucose to culture medium, whether supplemented with pyruvate or not, markedly decreased cellular ATP levels (Table III). This should not occur if these drugs act specifically as inhibitors of glycolysis, and suggests that other pathways of energy metabolism may be inhibited as well. Addition of glucose or pyruvate and glucose to pyruvate to cells cultured in the presence of iodoacetate increased lactate production but reduced ATP levels further to almost undetectable levels (see “Discussion”).

To obtain additional information on the specificity of these inhibitors, 14CO₂ production was monitored in cultures incubated with [2-14C]pyruvate. Production of radioactive CO₂ was found to be lowered in the presence of either drug (Table IV), indicating that aerobic utilization of pyruvate was impaired. Thus, decreased pyruvate metabolism via the Krebs cycle and electron transport system may play a role in decreasing cellular ATP in the presence of iodoacetate or 2-deoxyglucose (see “Discussion”).

To obtain specific inhibition of glycolysis in subsequent experiments, cultures were incubated in pyruvate-containing medium in the absence of glucose and inhibitors.

Effects of inhibition of energy metabolism on collagen secretion

- When 2 x 10⁻⁴ m m-Cl-CCP, an uncoupler of oxidative phosphorylation (38), was added to medium containing glucose, glycolysis increased by approximately 50%, and ATP levels were maintained at normal levels (Table V). However, when m-Cl-CCP was added in the absence of glucose and glycolysis was functioning at a low level, ATP levels dropped dramatically. Obviously, a very high rate of glycolysis is required to maintain cellular ATP levels in the presence of m-Cl-CCP. However, in the
absence of m-Cl-CCP, high ATP levels were maintained with a very low rate of glycolysis in pyruvate-supplemented medium or in the absence of carbohydrate for 1 hour. Apparently, respiratory ATP production maintained cellular ATP levels in the absence of glucose, and was inhibitable by m-Cl-CCP. To corroborate this, we examined CO₂ production from pyruvate in the presence of m-Cl-CCP. If m-Cl-CCP is, in fact, effective as an uncoupler of oxidative phosphorylation, an increased oxidation of pyruvate to CO₂ resulting from the release of respiratory control should be observed. In fact, a 4-fold stimulation of radioactive CO₂ production in the presence of m-Cl-CCP was observed (Table IV), indicating that the drug was effective under the experimental conditions used.

Collagen secretion, as monitored by the appearance of radioactively hydroxyproline in the medium, was maintained at control values as long as cellular ATP levels remained high (Table V). Thus, specific inhibition of glycolytic metabolism (by use of pyruvate as an energy source) or of oxidative metabolism (by use of glucose plus m-Cl-CCP) did not result in appreciably decreased collagen secretion. However, when both glycolysis and oxidative metabolism were interfered with (by use of m-Cl-CCP in the absence of an external energy source), ATP levels fell and collagen secretion was inhibited (Table V). The inhibition of collagen secretion cannot be ascribed to nonspecific effects of m-Cl-CCP since secretion was restored to near normal levels when glucose was present in addition to m-Cl-CCP. Thus, these results show that the secretion of collagen by cultured chick tendon fibroblasts is dependent on an energy source which can be provided by either glycolysis or respiration.

**Effects of Inhibition of Protein Synthesis on Collagen Secretion**—An inhibition of energy metabolism leading to a reduction in ATP levels is also likely to lead to an inhibition in protein synthesis. Previous studies had suggested that inhibition of protein synthesis did not interfere with conversion of procollagen to collagen (39) and therefore, presumably, did not interfere with secretion. Nevertheless, it was important to determine whether the inhibition of collagen secretion observed as a result of interference with energy metabolism could be attributed to reduced protein synthesis.

Experiments were therefore performed to determine the extent of inhibition of protein synthesis with varying concentrations of cycloheximide and puromycin. The incorporation of labeled proline into cell protein was inhibited by 98% in the presence of cycloheximide at a concentration of 25 μg/ml; 10⁻⁴ m puromycin produced an inhibition of 91% in comparison with control values. When this concentration of puromycin or cycloheximide

| Condition          | [CO₂] production | % pyruvate control |
|--------------------|-------------------|--------------------|
| Experiment 1       |                   |                    |
| Pyruvate           | 22.8              | 100                |
| Pyruvate Iodoacetate | 19.3              | 54                 |
| Pyruvate Iodoacetate | 7.15              | 31                 |
| Pyruvate 2-Deoxyglucose | 20.9              | 100                |
| Pyruvate m-Cl-CCP  | 98.7              | 390                |

**TABLE IV**

*Production of [CO₂] from [2,4C]pyruvate in presence of several metabolic inhibitors*

Cultures were preincubated for 60 mm in the presence of unlabeled pyruvate. At this time 1 μCi/ml of [2,4C]pyruvate was added and incubation continued for an additional 120 min. Concentrations used were: pyruvate, 5 mM; iodoacetate, 0.05 mM; 2-deoxyglucose, 0.1 mM; m-Cl-CCP, 2 × 10⁻⁷ M.

| Metabolic Inhibitor | [CO₂] production | % pyruvate control |
|---------------------|-------------------|--------------------|
| Experiment 1         |                   |                    |
| Glucose Glucose      | 73.3              | 100                |
| Glucose Glucose m-Cl-CCP | 79.3              | 100                |
| Glucose m-Cl-CCP     | 67.1              | 100                |
| Glucose m-Cl-CCP     | 66.3              | 100                |

**TABLE V**

*Dependence of collagen secretion on ATP production*

| Preincubation | Incubation conditions | Chase | Protein synthesis | Collagen secreted | ATP content | Lactate production |
|---------------|----------------------|-------|-------------------|-------------------|-------------|-------------------|
|               |                      |       |                   |                   |             |                   |
| Experiment 1  |                      |       |                   |                   |             |                   |
| 1. Glucose    | Glucose              | Glucose | 40.2              | 38.7              | 0.9         | 100               | 13/ 100           |
| 2. Pyruvate   | Pyruvate             | Pyruvate | 53.7              | 39.0              | 7.6         | 110               | 15/ 14            |
| 3. Pyruvate   | Pyruvate             | No carbohydrate | 56.8              | 39.5              | 8.0         | 116               | 8/ 6              |
| 4. Pyruvate   | Pyruvate             | m-Cl-CCP | 29.6              | 7.0               | 1.4         | 20                | 19/ 14            |
| 5. Glucose    | Glucose              | Glucose | 47.1              | 32.9              | 6.7         | 97                | 194/ 143          |
| Experiment 2  |                      |       |                   |                   |             |                   |
| 1. Glucose    | Glucose              | Glucose | 73.3              | 40.0              | 10.8        | 100               | 194/ 100          |
| 2. Pyruvate   | Pyruvate             | No carbohydrate | 79.3              | 45.8              | 14.8        | 137               | 20/ 10            |
| 3. Pyruvate   | Pyruvate             | m-Cl-CCP | 67.1              | 14.1              | 2.4         | 22                | 40/ 21            |
| 4. Glucose    | Glucose              | Glucose | 66.3              | 37.4              | 14.1        | 130               | 313/ 161          |

* All periods were of 60-min duration. Two 75-cm² flasks were used to determine protein synthesis and collagen secretion (incubation: 3 μCi/ml of [3H]proline; chase, 15 mm [3H]proline). Sister cultures consisting of three 25-cm² flasks per group were used for ATP and lactate determinations. Concentrations were: glucose, 5.5 mM; pyruvate, 5 mM; m-Cl-CCP, 2 × 10⁻⁷ M.

b Radioactivity in cell layer plus medium.

c Calculated from the ratio of counts in medium plus total counts per min in hydroxyproline.

d ATP levels in Experiment 1 were lower than in Experiment 2 due to a decay in activity which resulted from storage of samples in 0.5 N HClO₄ at -20°.

e m-Cl-CCP was also present during the last 15 min of the incubation period in Experiment 1 and during the last 5 min of the incubation period in Experiment 2. This accounts for the inhibition in incorporation of proline into protein in the absence of glucose in Experiment 1.
was included in the chase medium after a pulse with [3H]proline, only a small degree of inhibition of collagen secretion was observed (Table VI). Thus, collagen secretion remained largely unimpaired despite an inhibition of new protein synthesis exceeding 90%. The hydroxyproline present in the medium of inhibited cultures cannot be attributed to cell lysis since the ratio of radioactive proline to hydroxyproline in the medium of control and inhibited cultures was the same.

Both ribosome-bound polypeptides resulting from cycloheximide administration and prematurely terminated puromycin-containing nascent chains are likely to be retained intracellularly. These chains are at least partially hydroxylated (32, 33), and would therefore be detected by the hydroxyproline assay. Assuming a synthesis time of 6 min for a procollagen chain (40), a sufficient percentage of radioactive hydroxyproline could be present in nascent chains at the end of a 60-min pulse period to account for the small degree of inhibition of secretion observed in the presence of puromycin and cycloheximide.

**DISCUSSION**

A high rate of glucose utilization and lactate production is characteristic of cultured cells (41). Although we did not attempt to measure cellular respiration in the presence of glucose, the rate of glucose metabolism and respiration observed in other diploid fibroblast cultures indicates that both glycolysis and respiration are relatively active. The rate of glucose metabolism in cultured cells is limited by the availability of oxygen and by the rate of glycolysis (15, 17, 18) and has been reported to inhibit glycolysis specifically without appreciably affecting respiration in some systems (44). However, a survey of the effects of iodoacetate on respiration in the absence of an added energy source reveals a great deal of variability in specificity of action (44). In a number of cases, including mouse fibroblasts in culture, iodoacetate concentrations in the range of 0.05 mM produced significant inhibition of respiration (44). It is apparent from our results that it is necessary to determine the specificity of inhibition of energy metabolism by iodoacetate when the compound is used as an inhibitor of glycolysis. The decreased CO₂ production from [14C]-pyruvate may indicate direct inhibition of substrate utilization by the Kreb cycle and electron transport system. Alternatively, iodoacetate may inhibit transport of pyruvate into the cell either directly or as a result of selective use of ATP produced by glycolysis in active transport across the plasma membrane. In either case ATP production resulting from respiration would be reduced.

Similar considerations apply to the use of 2-deoxyglucose. 2-Deoxyglucose acts as a competitive inhibitor of glucose transport and is phosphorylated intracellularly, probably by hexokinase (45, 46). The accumulation of 2-deoxyglucose 6-phosphate apparently causes competitive inhibition of glucose-6-phosphate isomerase (47). The very low levels of ATP observed in the presence of 2-deoxyglucose (Table III) may result in part from the utilization of ATP in the phosphorylation of 2-deoxyglucose. However, as in the case of iodoacetate, decreased metabolism of pyruvate (Table IV) also appears to play a role.
2-Deoxyglucose has been used as an inhibitor of glycosylation in the synthesis of collagen (48), immunoglobulins (49), and yeast glycoproteins (50, 51). The mechanism of inhibition of glycosylation by 2-deoxyglucose may involve interference with uridine nucleotide metabolism with a resultant accumulation of UDP-2-deoxyhexose (52-54). Melchers (49) concluded, on the basis of the use of this compound, that glycosylation of immunoglobulins was necessary in order to permit the transcellular movement of these proteins in plasma cells. However, our results indicate that 2-deoxyglucose cannot be regarded as a specific inhibitor of glycosylation or glycolysis. These results are consistent with the observation that the inhibition of incorporation of lysine into these proteins in plasma cells. However, our results indicate that 2-deoxyglucose was necessary in order to permit the transcellular movement of 2-deoxyglucose, was not relieved by the addition of inosine as an energy source (48). The large decrease in ATP levels caused by 2-deoxyglucose must therefore be considered in experiments purporting to examine the role of glycosylation in intracellular transport and secretion of glycoproteins.

Acknowledgment The skillful technical assistance of Felicia Arguelles is gratefully acknowledged.

REFERENCES

1. Schofield, J. D. & Prockop, D. J. (1973) Clin. Ortho. Rel. Res. 97, 175-195
2. Bornstein, P. (1974) Annu. Rev. Biochem. 43, 567-603
3. Diegelmann, R. F., Bernstein, L. & Peterkofsky, B. (1973) J. Biol. Chem. 248, 5045-5052
4. Burns, T. M., Spears, C. L. & Kervar, S. S. (1973) Arch. Biochem. Biophys. 189, 680-684
5. Olsen, B. R., Berg, R. A., Kishida, Y. & Prockop, D. J. (1973) Science 182, 825-827
6. Weinstock, M. & Leblond, C. P. (1974) J. Cell Biol. 60, 92-127
7. Olsen, B. R. & Prockop, D. J. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2033-2037
8. Nist, C. von der Mark, K., Hay, F., Olsen, B. R., Bornstein, P., Ross, R. & Dehm, P. (1975) J. Cell Biol., 65, 75-87
9. Ehrlich, H. P. & Bornstein, P. (1972) Nature New Biol. 248, 269-276
10. Diegelmann, R. F. & Peterkofsky, B. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 892-896
11. Dehm, P. & Prockop, D. J. (1972) Biochim. Biophys. Acta 266, 385-388
12. Ehrlich, H. P., Ross, R. & Bornstein, P. (1974) J. Cell Biol. 62, 389-405
13. Layman, D. L. & Ross, R. (1973) Arch. Biochem. Biophys. 187, 451-456
14. Jameson, J. D. & Palade, G. E. (1968) J. Cell Biol. 39, 559-589
15. Jameson, J. D. & Palade, G. E. (1968) J. Cell Biol. 39, 569-603
16. Howell, S. L. (1972) Nature New Biol. 235, 85-86
17. Mateshinsky, F. M. & Ellerman, J. (1973) Biochem. Biophys. Res. Commun. 50, 193-199
18. Wu, R. & Racker, E. (1959) J. Biol. Chem. 244, 1036-1041
19. Randle, R. J., Garland, P. B., Hales, C. N., Newsholme, E. A., Dutton, R. M. & Pogson, C. I. (1966) Rec. Prog. Hormone Res. 22, 1-14
20. Young, D. A. (1969) J. Biol. Chem. 244, 2210-2217
21. Giddings, S. J. & Young, D. A. (1974) J. Cell. Physiol. 85, 409-418
22. Dehm, P. & Prockop, D. J. (1971) Biochim. Biophys. Acta 240, 338-339
23. Lowry, O. H., Rosebrough, N. J., Fare, A. L. & Randall, R. J. (1951) J. Biol. Chem. 186, 265-275
24. Monson, J. M. & Bornstein, P. (1973) Proc Natl Acad Sci. U. S. A. 70, 3521-3525
25. Peterkofsky, B. & Diegelmann, R. (1971) Biochemistry 10, 688-694
26. Hohorst, H. J. (1962) in Methods of Enzymic Analysis (Bergmeyer, H. U., ed) 1st Ed, pp. 296-297, Verlag Chemie, Weinheim
27. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. H. & Smith, F. (1956) Anal. Chem. 28, 350-356
28. Hamerstadt, K. H. (1973) Anal. Biochem. 52, 449-455
29. Stambrook, P. J. & Siskenn, J. E. (1972) Biochim. Biophys. Acta 361, 45-54
30. Warshaw, J. B. & Rosenthal, M. D. (1972) J. Cell Biol. 58, 293-291
31. Fessler, J. H. & Smith, L. A. (1970) in The Chemistry and Biology of the Extracellular Matrix (Balzacs, A. E., ed) pp. 457-464, Academic Press, London
32. Lazadens, E. L., Lukens, L. N. & Infante, A. A. (1971) J. Mol. Biol. 68, 831-846
33. Miller, R. L. & Udenfriend, S. (1970) Arch. Biochem. Biophys. 139, 104-113
34. Goldberg, B. & Sherr, C. J. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 361-365
35. Goldberg, B. & Green, H. (1964) J. Cell Biol. 23, 221-228
36. Bissell, M. J., Hattie, C. & Rubin, H. (1972) J. Natl. Cancer Inst. 49, 555-565
37. Morel, B. & Froesch, E. R. (1973) Eur. J. Clin. Invest. 3, 112-118
38. Heytler, P. G. & Preichard, W. W. (1962) Biochim. Biophys. Res. Commun. 7, 272-275
39. Bornstein, P., Ehrlich, H. P. & Wyke, A. W. (1972) Science 176, 544-546
40. Voutil, J. & Piez, K. A. (1972) J. Biol. Chem. 247, 855-862
41. Paul, J. (1965) in Cells and Tissues in Culture (Willmer, E. N., ed) pp. 239-276, Academic Press, New York
42. Cristafalo, V. J., Howard, B. V. & Kritchevsky, D. (1970) Prog. Org. Med. Biol. Chem. 2, 95-146
43. Schramm, M. (1965) Annu. Rev. Biochem. 36, 301-320
44. Wahren, J. L. (1965) in Metabolic Inhibitors, Vol. III, pp. 1-283, Academic Press, New York
45. Kipnis, D. M. & Cori, C. F. (1959) J. Biol. Chem. 234, 171-177
46. Smith, D. E. & Gorski, J. (1968) J. Biol. Chem. 243, 4169-4174
47. Hochstein, R. M. (1963) in Metabolic Inhibitors (Hochstein, R. M. & Quastel, J. H., eds) pp. 139-143, Academic Press, New York
48. Blumenkantz, N., Rosenbaum, J. & Prockop, D. J. (1969) Biochim. Biophys. Acta 192, 81-90
49. Melchers, F. (1973) Biochemistry 12, 1471-1476
50. Harkas, V., Svoboda, A. & Bauer, S. (1970) Biochem. J. 118, 755-758
51. Lorin, P. & Gascón, S. (1971) Eur. J. Biochem. 23, 160-165
52. Heredia, C. F., de la Fuente, G. & Solis, A. (1964) Biochim. Biophys. Acta 86, 216-223
53. Deuf, P. & Bauer, S. (1966) Biochim. Biophys. Acta 121, 219-214
54. Keppler, D. O. R. & Huber, G. (1973) Exp. Mol. Pathol. 19, 369-371
The metabolic requirements for transcellular movement and secretion of collagen.
N J Kruse and P Bornstein

J. Biol. Chem. 1975, 250:4841-4847.

Access the most updated version of this article at http://www.jbc.org/content/250/13/4841

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/13/4841.full.html#ref-list-1