CARBON DIOXIDE FIXATION

IN CULTURED ANIMAL CELLS

by

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CHAPTER I

INTRODUCTION

Carbon dioxide is essential for the growth and maintenance of nearly all living cells because it is involved in one or more critical physiological functions. Among these functions are: 1) carbon dioxide, by way of carbonic acid, bicarbonate and carbonate, provides an important buffering system for hydrogen ions in solution; 2) carbon dioxide can change protein structure by transforming uncharged amino groups into negatively-charged carbonate groups (99); and 3) carbon dioxide is utilized for many carboxylation reactions.

The present study is concerned with only the last function, that of the importance of carbon dioxide fixation in animal cells. Carbon dioxide fixation can be carried out by many enzymatic reactions in almost every form of life. The metabolic significance of carbon dioxide fixation is many-fold in that it is involved in: a) fatty acid synthesis; b) gluconeogenesis; c) de novo synthesis of purines; d) synthesis of urea; e) synthesis of pyrimidines; and f) supplying four carbon dicarboxylic acids for replenishing key intermediates of the Krebs citric acid cycle that were utilized for forming amino acids, porphyrins, and other compounds.

The writer decided that the simplified environment provided by the methods of cell and tissue culture offers an excellent system for studying carbon dioxide fixation in animal cells and for determining the ramifications of these processes in the metabolism of carbohydrates,

amino acids, and other important cell substances. Extensive carbon dioxide fixation studies have been carried out using slices or perfused organs but there is a scarcity of information on these processes and their related functions in mammalian cells grown in vitro. Furthermore, tissue culture offers a convenient and readily reproducible experimental system compared to intact animals which are subject to many uncontrollable variables.

Artificially cultured mammalian cells are useful biochemical subjects because they exist as a relatively uniform population of free-living cells, of similar physiological state, age and presumably biochemical composition (184). An established line of mouse cells NCTC clone 929 (strain L, Earle) which was used in these investigations offered the advantage of competence to grow in a defined synthetic medium. Large amounts of bicarbonate were normally included for buffering purposes in the medium for growing these cells. Although several published reports (89, 198, 240, 242) imply that carbon dioxide deficiency adversely affects L-cells, no studies have focused on the incorporation or, more specifically, enzymatic fixation of carbon dioxide by these cells.

CHAPTER II

HISTORICAL REVIEW

The Cultivation of Animal Cells in the Presence and Absence of Carbon Dioxide

Carbon dioxide is considered to influence the metabolic activities of animal cells because: 1) when bicarbonate is left out of the medium, many cellular processes are adversely affected; 2) when C-14 labeled bicarbonate is used, the label is found in many products; and 3) many isolated enzymes have been shown to fix carbon dioxide. One of the problems that is encountered in trying to grow animal cells under non-bicarbonate conditions is that, even when bicarbonate is left out of the medium it can be made available to the cells from three other sources: carbon dioxide dissolved in the medium; that present in the atmosphere; and that produced by the cells' own metabolism. In a closed system most of this carbon dioxide can be removed by the use of an alkali trap. Another problem that must be considered in using non-bicarbonate buffers involves distinguishing whether an observed cellular response is due to the lack of carbon dioxide or to the possible toxicity of the new buffer.

Inhibition of multiplication of human cells (HeLa and conjunctival Chang cells) grown in cell culture was one of the first observed effects of carbon dioxide depletion (79). Similarly, L-cells were observed to multiply erratically in non-bicarbonate medium (Tris or phosphate buffered media) and to exhibit abnormal cell morphology (the cells appeared granular and rounded) (89, 242, 198, 240). The outgrowth of

fibroblasts from chick hearts was irreversibly inhibited in the absence of bicarbonate (96). Carbon dioxide is known to stimulate the growth of many bacteria and is a recognized growth requirement for several anaerobic spirochetes (95). Sodium bicarbonate (0.05 to 0.1 per cent) shortened the lag phase and increased both the growth rate and total yield of the anaerobic spirochete Treponema microdeutium (95).

The optimum carbon dioxide tension for respiration of L-cells (53) and three other lines of cultured mammalian cells was found to be one per cent (52). In rat liver slices, increasing the carbon dioxide or bicarbonate concentration from 10 to 45 mM at constant pH caused six to seven fold increases in glycogen synthesis from glucose (99). Hastings and Longmore found no effect of carbon dioxide on the enzymes: hexokinase, glucokinase, glycerol kinase, glycogen synthetase, phosphorylase, or pyrophosphatase. However, at its optimum pH the activity of adenosine triphosphatase was increased by carbon dioxide. Also, the same investigators found that increasing the carbon dioxide concentration from 10 to 40 mM at constant pH stimulated the conversion of acetate into fatty acids four to six fold. By increasing the carbon dioxide concentration from nine to 44 mM at constant pH, glycerol uptake was increased five fold and the percentage of glycerol converted to glycogen increased three fold in rat liver slices (151).

In isolated perfused livers from fasted rats, glucose synthesis from glycerol was not affected by altering the carbon dioxide concentration, but both glycerol conversion to glycogen and the glycogen level increased nearly 64 per cent when the bicarbonate level was increased from 17 to 30 mM (150). Glucose uptake and its conversion to glycogen by rab-

bit-kidney cortex grown in vitro increased in a higher bicarbonate medium (100). In Trichophyton mentagrophytes, the enzymes glucose-6-phosphate dehydrogenase, glucose-6-phosphate isomerase, and UDPG pyrophosphorylase were induced by carbon dioxide and glucose metabolism was stimulated (40).

The time-dependent profile of the activities of lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and glutamate-oxaloacetate transaminase of L-cells grown in bicarbonate buffered media were different from those of cells grown in phosphate buffered media (55). Loomis (152) found that sexual differentiation was reversibly induced in Hydra by specified changes in the pCO_2 of the aqueous environment. He stated " . . . the level of pCO_2 in the environment of a living cell is one of the most labile and neglected of all biological variables, yet one that is capable of regulating both the rate of cell division and the process of differentiation."

Substitutions for Carbon Dioxide

In the absence of bicarbonate, human (HeLa and conjunctival Chang) cells failed to grow rapidly and underwent slow death. However, by adding bicarbonate (80) or normal cell extracts (79) to the medium at any time up to fourteen days, growth was restored. With the same human cells, most of the carbon dioxide was fixed into acid soluble and nucleic acid fractions. It was found that ribonucleosides substitute partially for carbon dioxide in supporting cell multiplication (34). Oxaloacetate (OAA) alone was ineffective, but when combined with nucleosides it gave better growth than ribonucleosides alone (34). In HeLa and L-cells, ribonucleosides, deoxyribonucleosides, or extracts of normal cells could substitute partially for carbon dioxide (198). Since normal cell extracts

did not replace carbon dioxide completely, the critical compounds are either present in limited amounts or are in bound or labile forms.

In non-bicarbonate medium HeLa and L-cells did not plate out satisfactorily. When OAA was added to this medium, plating efficiency for these two cell types was substantially improved but not for monkey-kidney cells (89). Even in cases where it can partially substitute for carbon dioxide, OAA may not be the essential product but may rapidly break down to form carbon dioxide. OAA is easily decarboxylated enzymatically or non-enzymatically and, in fact, undergoes spontaneous decarboxylation in the medium (198). The failure of a compound to substitute for carbon dioxide does not mean that it is not an essential product of carbon dioxide fixation. The added compound may not be able to penetrate the cell membrane easily, not be in an active form, or not be present in appropriate concentration. It was pointed out by Runyan and Geyer (198) that efforts to substitute for carbon dioxide may never be completely successful because cells may have a requirement for carbon dioxide or bicarbonate per se. For example, carbon dioxide deficiency may produce damaging physical alterations in the cell such as swelling due to ionic changes across the membrane.

It can be concluded that bicarbonate is an essential nutrient for mammalian cells (160, 240) in that it is required for their growth and maintenance (241, 162). Carbon dioxide was considered essential for survival of human cells and their rapid multiplication (34, 80). Runyan and Geyer (198) and Chang et al. (34) considered that the major function of carbon dioxide in HeLa and L-cells was to provide precursors for the synthesis of purines and pyrimidines.

Even though Eagle (56) stated that bicarbonate is not essential for HeLa and other cells, his results were equivocal as pointed out by Swim and Parker (241). Eagle used stoppered flasks with no carbon dioxide trap, with the result that the cells probably produced sufficient carbon dioxide to satisfy their nutritional requirements. Eagle stated that there was rapid acidification within less than 12 hours, necessitating daily changes of non-bicarbonate medium. Rapid acidification of the medium usually indicates either a dense cell population, rapidly growing cells or both, from which carbon dioxide and lactic acid are derived to acidify the medium.

In order for cells to survive in a non-bicarbonate buffer, the following are needed: a high inoculum, rapidly growing cells, a closed system, and no carbon dioxide trap. If these conditions are met, the cells usually are able to produce enough metabolic carbon dioxide to satisfy the nutritional needs for this compound. HeLa, rat hepatoma or L-cells grew slower in phosphite and Tris buffers than in bicarbonate, and when the caps of the culture vessels were loosened, the cells grew even slower (240). When bicarbonate was added, normal growth was restored. Out of six permanent strains of fibroblasts, derived from human, rabbit, and mouse, which were able to grow in a closed non-bicarbonate system, only one strain could grow in an open flask. This unusual capability is probably due to its capacity to produce and retain enough carbon dioxide for its own needs (241).

L-cells grown in non-bicarbonate media in a vessel containing a carbon dioxide trap showed no increase in number if the initial cell count was 5×10^5 cells per ml or fewer (198). Cells kept deficient of

carbon dioxide for periods of time up to 14 days did multiply after a lag of a few days if the carbon dioxide trap was removed. Carbon dioxide-deficient cultures showed progressively faster growth with increasing size of inoculum and approached normal growth when it reached 2×10^6 cells per ml. This response supports the interpretation that the growth of carbon dioxide-deficient cultures from inocula above 5×10^5 cells per ml is probably due to the development of a micro-environment of carbon dioxide from endogenous sources. In these cases the alkaline trap did not remove the carbon dioxide rapidly enough.

Some Physical and Chemical Characteristics of Carbon Dioxide and its Buffering Capacity

Dissolved carbon dioxide can exist in four forms: carbon dioxide; carbonic acid; bicarbonate; and carbonate. The partial pressure, pCO_2, of the unhydrated gas (carbon dioxide physically dissolved in water) differs from the total carbon dioxide in the aqueous phase in that it is only one of four forms of dissolved carbon dioxide. Water in equilibrium with normal air (pCO_2 of 0.03 per cent) has a pCO_2 of 0.03 per cent of an atmosphere, regardless of pH and bicarbonate concentration (152). Higher values may exist in a closed system or an open system not in equilibrium with surrounding air and within which carbon dioxide is generated. Laboratory and culture room air may contain as much as 0.08 to 0.16 per cent carbon dioxide due to a small unventilated room with a Bunsen burner (152).

The relationships between the forms of dissolved carbon dioxide are as follows:

$$2\,H^+ + CO_3^= \underset{(1)}{\rightleftharpoons} H^+ + HCO_3^- \underset{(2)}{\rightleftharpoons} H_2CO_3 \underset{(3)}{\rightleftharpoons} CO_2 + H_2O$$

The exchange reactions (1) and (2) are extremely fast as compared to reaction (3) (272). Watt and Paasche (272) determined that if all the carbon was in the hydrated form, it would take 90 seconds for it to be completely converted to carbon dioxide. In biological systems the enzyme carbonic anhydrase catalyzes reaction (3). The bicarbonate-carbonic acid system is an appropriate buffer for animal cells because of its suitable pK value, a fluid equilibrium with environmental carbon dioxide, and the compatibility of high concentrations of all components of the system with the viable cells.

Dissolved carbon dioxide and bicarbonate at equilibrium are related by the following equation (60): $pH = 6.1 + \log \frac{(HCO_3^-)}{(H_2CO_3)}$.

Therefore, above pH 6.1 more bicarbonate is present than carbonic acid. The concentration of carbon dioxide in bicarbonate buffers in equilibrium with a five per cent carbon dioxide in air mixture is 1.3×10^{-3} M at 35 degrees, and 1.7×10^{-3} M at 25 degrees (231). Gas purging in the pH range from five to seven with nitrogen or carbon dioxide-free air was reported to remove all inorganic carbon from the medium within minutes (272). In contrast to this, Ruiz-Amil et al. (197) reported that at pH 7.6 an amount of endogenous carbon dioxide estimated to be between 0.5 and 1.1 mM could not be removed by vacuum and gassing procedures.

Loomis (152) claimed that carbon dioxide can penetrate cell walls more easily than any other substance, including water. He stated that it diffuses across the epithelial membrane approximately 17 times faster than oxygen. This highly-liposoluble gas can penetrate cell membranes selectively, thus producing intracellular acidity even in alkaline solution (152).

Gluconeogenesis

Gluconeogenesis is defined as the synthesis of glucose and other hexose-containing polysaccharides from non-carbohydrate precursors containing five carbon atoms or less (143). Some of these molecules are Krebs cycle intermediates, pyruvate, lactate, glycerol, propionate, aspartate, glutamate, proline, arginine, ornithine, histidine, valine, isoleucine, threonine, tyrosine, phenylalanine, alanine, and serine (63, 137). Liver and kidney are the only mammalian tissues in which gluconeogenesis takes place at an appreciable rate (143, 137, 136).

Glucose must be formed because it is necessary for: the maintenance of certain cells, especially those of the blood and central nervous system (136, 174); precursors of amino sugars, uronic acids, and the ribose part of nucleic acids (136); tissues that can not obtain all their energy from fatty acid or ketone body oxidation (174); and an anaerobic energy supply of tissues such as muscle which are dependent on glycolysis (136). Gluconeogenesis normally proceeds at a modest rate (143). The rate of this process is adaptable to the glucose needs of the body, and depends on the nature of the food intake (136, 137). Gluconeogenesis is accelerated under the following conditions: fasting, or a low-carbohydrate diet (136, 143); heavy work (143, 136); diabetes (143); high blood levels of the adrenal-cortical steroid hormones--hydrocortisone and corticosterone (143); high blood levels of glucagon (237) and of the catecholamines--epinephrine and norepinephrine (64). Gluconeogenesis is important in conserving carbohydrate through the resynthesis of glucose from excessive quantities of several metabolites, particularly lactate and pyruvate produced by muscle and erythrocytes (63). Under prolonged starvation gly-

cogen reserves are used up, and amino acids derived from tissue proteins, as well as glycerol derived from adipose tissue serve as sources of carbon for gluconeogenesis (63).

Gluconeogenesis does not proceed by a direct reversal of glycolysis (the Embden-Meyerhof-Cori pathway). It bypasses four strongly endergonic glycolytic reactions, i.e. thermodynamically-unfavorable steps, by substituting alternate reactions that are strongly exergonic in the direction of glucose synthesis (233, 136). These gluconeogenic reactions are catalyzed by the following enzymes: pyruvate carboxylase, phosphoenolpyruvate carboxykinase (PEP-CK), fructose-1, 6-diphosphatase, and glucose-6-phosphatase (273, 136, 174). The above listed enzymes are considered the key gluconeogenic enzymes in that they catalyze pace-making reactions and are subject to metabolic controls (233). These enzymes are rate-limiting because their catalytic activities (less than 1 millimole/hour/gram net weight of liver) are lower than those of other gluconeogenic enzymes (233, 273). They govern one-way reactions, are involved in circumventing essentially irreversible reactions, and are localized chiefly or exclusively in organs capable of gluconeogenesis (273).

Of the gluconeogenic enzymes listed the writer is primarily concerned with those which fix carbon dioxide--pyruvate carboxylase and PEP-CK. Pyruvate carboxylase and PEP-CK are present in high concentrations in the glyconeogenic tissues, liver and kidney, and the Km values for their substrates are smaller than the normal tissue concentration of these substances (233). These two enzymes convert pyruvate to PEP by way of a two-step process called the abbreviated dicarboxylic acid shuttle (252). This pathway, which bypasses the glycolytic pyruvate kinase reac-

tion, is strongly exergonic, consuming both ATP and GTP. The conversion of pyruvate to PEP catalyzed by pyruvate kinase is unlikely in gluconeogenesis because: the rate of the reaction in the direction of PEP synthesis is low due to the energy barrier (280, 233); the apparent Km of the enzyme for pyruvate is 0.01 M, which is well above physiological levels (252, 233, 280); the activity of the enzyme is high in non-gluconeogenic tissues, such as brain, heart, and skeletal muscle, and low in the gluconeogenic tissues, the kidney and liver (233, 252, 254, 280); and the activity of the enzyme is lower under gluconeogenic than under glycolytic conditions (197, 135).

Another enzyme, PEP-synthetase, which catalyzes a one-step conversion of pyruvate to PEP has recently been discovered in E.coli (43), tropical grasses (101), photosynthetic bacteria (20), and Propionibacteria (61), but as yet it has not been found in animal tissues. It uses ATP (43) or ATP and inorganic phosphate (101) to catalyze the formation of PEP from pyruvate.

Gluconeogenesis will be discussed under the following headings: control of gluconeogenesis; gluconeogenesis and carbon dioxide fixation; effects of glucose, tryptophan, insulin, glucagon, catecholamines, and glucocorticoids.

Control of Gluconeogenesis

The pacemaker enzymes controlling the rate-limiting steps can be regulated by changing their concentrations through enzyme synthesis from precursors, which is called slow-coarse control, or by controlling the activity of an allosteric enzyme with no change in concentration--called rapid-fine control(136, 93). Two different hypotheses concerning

the regulation of gluconeogenesis are based on the concentrations of metabolites which in turn affect the synthesis or activity of the enzymes. The first hypothesis considers the concentration of acetyl-CoA to be an important regulator of gluconeogenesis. Another hypothesis considers the relative concentrations of ATP to ADP and ATP to AMP to act as the main regulators of gluconeogenesis.

Acetyl-CoA occupies a central position in cellular metabolism. It is the end-product of fatty acid oxidation as well as the starting substrate of fatty acid synthesis. In addition it combines with OAA to form citrate in the Krebs citric acid cycle. Acetyl-CoA is thought to control gluconeogenesis because without it pyruvate carboxylase is usually inactive (252, 147). But the intracellular concentrations of acetyl-CoA are usually high compared to the Km value for this allosteric effector (2×10^{-5}M for acetyl-CoA activation of pyruvate carboxylase) (276, 233). Therefore, pyruvate carboxylase would normally be present in an active state. Though glucagon causes an increase in gluconeogenesis in both fed and starved rats, only the fed rats' acetyl-CoA levels and pyruvate carboxylase activity increased (277, 93, 64). Thus pyruvate carboxylase is probably fully active in starved rats, and there can be an increase in gluconeogenesis without changes in acetyl-CoA levels or pyruvate carboxylase activity.

The energy status of the cell with regard to the adenine nucleotides plays an important part in gluconeogenesis. High ATP to ADP levels stimulate gluconeogenesis (174). ADP inhibits pyruvate carboxylase (98, 238) and AMP inhibits PEP-CK and glucose formation from lactate (78). Evidence that neither ATP nor ADP influences gluconeogenesis was obtained

when glucagon increased gluconeogenesis five fold while the ATP:ADP ratio decreased forty per cent (275).

It has not been shown definitely whether changes in the activities of enzymes involved in gluconeogenesis of mammals are the cause or result of altered rates of carbohydrate synthesis. Also, there are conflicting reports on whether the changing levels of metabolites are influenced by or cause the release of hormones or both.

Gluconeogenesis and Carbon Dioxide Fixation

In carp liver mitochondria, a five-fold increase in carbon dioxide fixation was caused by adding alanine (88). Alloxan diabetes in rats gave a seven to eight-fold increase over normal rats in the incorporation of the radioactivity into glucose from C-14 bicarbonate in 30 minutes (266). In vivo carbon dioxide fixation in rats was in the following order: alloxan diabetes > cortisol > normal unfed > adrenalectomized diabetic > normal (266).

The rate-limiting character of pyruvate carboxylase in gluconeogenesis in rat liver has been questioned since its activity has been found to be five times that of PEP-CK (167). Furthermore, gluconeogenesis was stimulated in perfused livers from starved rats by glucagon without a change in acetyl-CoA levels (167). The pyruvate carboxylase reaction may be influenced more by changes in the levels of substrates and co-factors than alterations of the level of total enzyme (210). Evidence for this possibility came from seeing no changes in pyruvate carboxylase activity when gluconeogenesis was increased in yeast (197). Likewise, when gluconeogenesis was induced by premature delivery of rats, pyruvate carboxylase was not induced (283). Feeding rats a low carbohydrate diet did not change

the activity of pyruvate carboxylase (143). However, fasting of rats and mice (107, 71) or chickens (212) doubled pyruvate carboxylase activity. Pyruvate carboxylase activity which is low in fetal dog liver parallels an increase in glucose production from bicarbonate after birth (173).

PEP-CK activity was high under gluconeogenic conditions in yeast (197), rat liver (143, 223), and guinea pig (178). Soluble PEP-CK activity which was low in the fetus increased after the birth of rats (10, 260) or premature delivery of rats (283) and this increase closely correlated with an elevation of glucose production from C-14 bicarbonate. The fetal dog also showed the same results as the rats after birth (173). The faster-growing rat liver tumors had very low PEP-CK activity and low gluconeogenesis, while the slower-growing tumors had higher PEP-CK activity and increased gluconeogenesis. A high correlation was found between the high PEP-CK activity and extremely high glycogen content of mollusks (226) and ciliated protozoa (220). In the flatworm, the increases in PEP-CK activity were the same as the increases in carbon dioxide incorporation into polysaccharides (189).

Effects of Glucose

In some cases glucose was shown to depress gluconeogenic enzyme activity, and in others to have no effect. Starvation caused a doubling of soluble PEP-CK activity in intact or adrenalectomized rats, but had no effect on mitochondrial PEP-CK (69, 177, 223). Refeeding the fasted rats with a high carbohydrate diet (284, 11) or adding actinomycin-D (195) restored the PEP-CK activity to normal. Fasting also enhanced the soluble but not the mitochondrial PEP-CK in the guinea pig (178, 144).

When glycerol, glucose, fructose, or galactose were fed to fasted rats, the liver PEP-CK activity was depressed (219). Induction of PEP-CK activity by premature delivery of rats is repressed by injecting actinomycin D, puromycin, glucose, galactose, mannose, fructose, lactate, pyruvate, or glycerol (128). Repression of the synthesis of PEP-CK by glucose may be accomplished indirectly through decreasing the release of glucagon (128). Glucose does not repress PEP-CK activity (128) that was increased by glucagon. Glucose repressed PEP-CK activity in ciliated protozoa (220), yeasts (247), and E.coli (116). Only the cytosol PEP-CK which was closely related to the high rate of gluconeogenesis was reduced by glucose in a ciliated protozoa (218). Glucose was more effective than puromycin in depressing elevated PEP-CK activity of fasted rats, and therefore, glucose might inhibit the synthesis and accelerate the rate of enzyme degradation (69). However, glucose per se is probably not a regulator because the correlation between enzyme activity and blood glucose concentration does not occur under all experimental conditions (69).

Other evidence that glucose itself is incapable of suppressing synthesis of gluconeogenic enzymes is that during starvation the blood glucose level of rats decreased while gluconeogenic enzyme activity stayed normal or increased (273). In fasted rats, there is no relation between PEP-CK activity and the concentration of liver glycogen (69, 219). It is believed that the drop or rise in blood sugar upon reaching critically low or high levels triggers the production of glucocorticoid hormones from the adrenal cortex and insulin from pancreatic beta cells, and that these hormones act as inducer and suppressor (273). Reports conflicting with this will be listed later in this chapter. Glucose and hydrocortisone

together had no effect on PEP-CK activity in rats even though the glycogen level was elevated 60 to 75 per cent over that of the normal rat (69).

Glucose in the body fluids is not a direct regulator or repressor of PEP-CK because in diabetic rats there is high PEP-CK activity even though there are high glucose levels (69, 273). Also, glucose levels remained high even when antibiotics blocked enzyme synthesis in diabetic rats (273). However, Young et al. (284) believed that by suppressing PEP-CK, carbohydrates affect the levels of storage polysaccharides. They stated that since mannoheptulose diabetes prevented a decrease of PEP-CK by carbohydrate, insulin was essential for carbohydrate-dependent repression of the enzyme. Others point out that it is likely that the metabolite that depressed PEP-CK is formed from glucose, but only in the presence of insulin (141). It was concluded that the depression of gluconeogenic enzymes after carbohydrate administration is initiated in some site away from the liver (219). Maybe a humoral factor, metabolite, or the concentration of circulating amino acids could increase the activity. Administering gluconeogenic amino acids caused increased PEP-CK activity even in fasted rats (219). Lack of glucose utilization in diabetes caused amino acids to be liberated from peripheral tissues (219).

Effects of Tryptophan

L-Tryptophan (2.4 mM) inhibited gluconeogenesis and PEP-CK in vivo in livers from fasted normal or fasted adrenalectomized rats (259, 234). Since quinolinic acid and other tryptophan metabolic products duplicated the inhibitory effects of tryptophan, it was postulated that tryptophan and certain of its metabolites inhibit gluconeogenesis by being converted to quinolinic acid (259). Quinolinic acid chelates divalent

metal ions and inhibits PEP-CK (259). The tryptophan effect is independ- of functioning adrenals because cortisol increased gluconeogenesis and particularly PEP-CK when tryptophan was not present. Conversely, tryptophan decreased gluconeogenesis when cortisol was not present (194). No effect was apparent when both tryptophan and cortisol were present together (194, 70). Paradoxically, tryptophan and quinolinic acid increased the in vitro assayable activity of PEP-CK (259, 68). Upon extraction, the inhibition appears to dissociate from the enzyme, leaving it in a metal-activated state twice as active as the enzyme from the normal rat with no tryptophan administered (68). Manganese in vitro in the presence of magnesium caused activation of PEP-CK similar to that caused by administered tryptophan (68). Only tryptophan, out of 19 amino acids administered to the intact animal, activated the supernatant PEP-CK in vitro, but when the tryptophan was administered in vitro, it had no effect on PEP-CK (68, 70).

Effects of Insulin

The glucose levels of alloxan diabetic rats are four to five fold higher than those in livers of normal rats (221, 177). Alloxan diabetes in rats caused a three to eight-fold increase in the incorporation of bicarbonate into glucose both in liver slices and in vivo in the liver (6, 266, 107). Insulin is a suppressor of the formation of gluconeogenic enzymes but it does not act directly on the liver (140, 69, 273, 141, 223). It affects the peripheral tissues, the product of which influence liver and kidney (141).

Pyruvate carboxylase activity was doubled in alloxan diabetic rats as compared to normal rats (265, 71, 107, 267) and when insulin was

administered the pyruvate carboxylase activity returned to normal (192). Insulin had no effect on increased activity of pyruvate carboxylase brought about by cortisol (140). In one paper, alloxan diabetes was reported to cause no change in pyruvate carboxylase activity of rats (143).

Rats made diabetic with mannoheptulose, pancreatectomy, or chronically diabetic with alloxan develop an elevated level of soluble liver PEP-CK (141, 223). Mannoheptulose (a 7-carbon ketose that blocks insulin release from the pancreas) causes a rapid increase in blood glucose and rat liver PEP-CK (144, 69). The amounts of guinea pig soluble, but not mitochondrial, PEP-CK are enhanced by mannoheptulose induced diabetes (144, 178). Alloxan diabetes in rats causes a four to six-fold increase in PEP-CK activity (107, 267, 69, 177, 144, 264).

Insulin had no effect on the PEP-CK of normal (177, 69, 223, 141) or fasted rats (141, 223). However, insulin did not depress the elevated PEP-CK in alloxan diabetic rats when carbohydrate was present (219, 223, 141). For the repression of PEP-CK in rat liver by carbohydrate, insulin was essential (284).

Effects of Glucagon and Catecholamines

Glucagon stimulates glucose production, pyruvate carboxylase activity (64, 93), and PEP-CK (64, 11, 223) activity in perfused livers from fed rats. In starved rats glucagon causes no change in pyruvate carboxylase activity (167, 237) although it does cause the PEP-CK activity to increase (64). In contrast, neither the supernatant nor mitochondrial fraction of guinea pig liver PEP-CK was affected by glucagon (144). Glucagon also increased fatty acid oxidation in rats (275, 93, 64) and the release of fatty acids may underlie the glucagon effect in gluconeogene-

sis. Injections of glucagon and the catecholamines, epinephrine and norepinephrine, into neonatal rat liver resulted in the development of PEP-CK, production of cyclic-3',5'-AMP and an increase in gluconeogenesis (128). Glucagon (275) and the two catecholamines also activated cyclic-3',5'-AMP in normal adult rats (64). The effects of the hormones on gluconeogenesis appeared to be mediated by cyclic AMP (64) which may act as the critical allosteric factor bringing about de-repression of PEP-CK (128). Gluconeogenesis is not stimulated by glucagon, epinephrine, or cyclic AMP in perfused livers from fasted-adrenalectomized rats (72) unless dexamethasone or cortisol is added in vivo or in vitro. Glucocorticoids alone have little effect on gluconeogenesis under these conditions. Also, livers from adrenalectomized rats show a normal rise in cyclic AMP after glucagon exposure. Thus the "permissive effect" of glucocorticoids on the action of glucagon or epinephrine consists of sensitization of a rate-limiting step in the gluconeogenic pathway to cyclic AMP (72).

Effects of Glucocorticoids

Cortisol (also called hydrocortisone) treatment of fasted rats results in a marked increase in the incorporation of C-14 bicarbonate into liver glycogen and glucose in vivo (6). C-14 bicarbonate incorporation into glucose in rats was increased four to five-fold in 30 minutes by cortisol (266). Triamcinolone (8×10^{-5} M), a fluorinated synthetic glucocorticoid, caused the C-14 bicarbonate incorporation into glucose to increase over that of the control rat (6). Glucocorticoids (adrenal steroids) caused a rise in blood glucose and liver glycogen two to three hours after administration in normal and adrenalectomized rats (59). This reflects enhanced liver and kidney gluconeogenesis as well as impaired

peripheral--skin, thymus, and adipose tissue--utilization of glucose (59). In livers from rats starved for 24 hours, cortisol at 50 mg per g of rat caused the glycogen to rise to 350 per cent of the control (114). Liver glycogen in the adrenalectomized rat increased from one to six g per cent with little change in blood glucose levels after 12 hours exposure to five mg hydrocortisone per kg (69). In the normal rat liver glycogen increased from five to ten g per cent with little change in blood glucose levels after 12 hours exposure to 25 mg hydrocortisone per kg (69). On the other hand, glucocorticoids have been reported to increase blood glucose levels in the rat (177, 105, 6). These observations are difficult to reconcile.

Soluble pyruvate carboxylase activity increased by 15 per cent in kidney cortex slices of an adrenalectomized rat following incubation with 2×10^{-5} M cortisol (140). Puromycin and cortisol together caused no effect on pyruvate carboxylase, and from these results, L'age et al.(140) concluded that glucocorticoids control de novo synthesis of the enzyme. Also, the soluble pyruvate carboxylase in kidney cortex slices from diabetic adrenalectomized rats treated with cortisol increased 50 per cent as did glucose synthesis (107).

Only a 30 per cent increase in pyruvate carboxylase activity and 70 per cent increase in glucose synthesis was seen with rat kidney cortex slices incubated with 2×10^{-5} M cortisol for one hour (108). Henning et al. (108) suggested a hormone-dependent induction or activation of pyruvate carboxylase as one of the primary actions of cortisol in regulating gluconeogenesis. In contrast to this, triamcinolone administered to neonatal rats did not affect pyruvate carboxylase activity (282). From the data of Foster and Lardy, and of Krebs, Shrago and Lardy (221) were un-

able to see a cortisol-sponsored increase in pyruvate carboxylase activity in rat liver mitochondria.

In kidney cortex and liver slices from diabetic adrenalectomized rats, cortisol caused a 50 to 60 per cent increase in glucose synthesis and 100 per cent increase in soluble PEP-CK (107). Triamcinolone administered at 400 micrograms per 100 grams rat led to increased protein catabolism and glucose production (6). Under the same conditions the liver PEP-CK activity was elevated 21 per cent in fasted rats and 42 per cent in fed rats (6). Liver PEP-CK activity was doubled by cortisol(177, 221) and cortisone (223) treatment of adrenalectomized rats. Cortisol in doses of five mg per 200 g of adrenalectomized rat caused the soluble liver PEP-CK activity to increase in three hours to a maximum of 140 per cent (69) and 200 per cent (144, 141). Then the enzyme level declined to normal by eight hours. The increased PEP-CK activity was a result of protein synthesis because it was blocked by ethionine, puromycin, and actinomycin D (141, 144). Adrenal cortical hormones are not essential for increasing PEP-CK activity because this enzyme responds to fasting or diabetes in the intact or adrenalectomized rat the same way (69, 144, 141).

Cortisol had no effect on the PEP-CK of rat liver mitochondria or microsomes (177, 144, 141). Neither the supernatant nor the mitochondrial PEP-CK of the guinea pig was increased by cortisol (144). Furthermore, triamcinolone did not induce PEP-CK in the neonatal rat liver (282). Gluconeogenesis can be stimulated by cortisol independent of de novo synthesis of enzymes. For example, cortisol stimulates gluconeogenesis (blood glucose and liver glycogen) in normal and adrenalectomized-starved rats even though PEP-CK induction is completely suppressed by actinomycin

D (195, 141, 196). Also, glucose and cortisol together had no effect on PEP-CK, but the glycogen level still increased 60 to 75 per cent in rats (69). Therefore, the primary action of glucocorticoids seems not to be induction of enzyme formation but to establish conditions which direct metabolites, such as amino acids and pyruvate, toward carbohydrate formation (141, 195). The secondary response might be elevation of enzymes along the path to glucose synthesis (172, 141, 195) due to induction or derepression by altered concentration of intermediates.

The incorporation of labeled alanine, pyruvate, glutamate, fructose, and glucose into liver glycogen of normal rats was increased seven to nine fold after a two hour treatment with prednisolone (215). It was concluded that the early effects of glucocorticoids are not on enzyme synthesis, but on the interaction of the hormone with the control points. Therefore, the primary effects of hormones may be concerned with a) changes in substrate availability; b) changes in the concentrations of metabolic signals; and c) interconversions of enzyme forms, since it was shown that the cortisol effect on glycogen synthesis in adrenalectomized rats occurred earlier than the increase in enzyme activity (174).

Adrenalcorticoids caused a release of amino acids from muscle and thymus (17, 6, 273) and a rise in the amino acid level in rats of 17 to 61 per cent after six hours and 34 to 87 per cent after 24 hours (273). Thus, a higher level of glucogenic amino acids can saturate pre-existing enzymes and cause an immediate increase in glucose without enzyme synthesis. Also, the rise in the pool of amino acids which serve as precursors for the synthesis of gluconeogenic enzymes may assist in de novo synthesis of enzymes (273). The corticoids may not help to transport amino acids

into the cell because triamcinolone does not stimulate alanine uptake, although it does cause an increase in glucose synthesis (105).

In rats the first observed effects of glucocorticoid action are to increase the rates of purine and protein syntheses in the liver and to inhibit them in the thymus and spleen (65). The cortisone acetate-induced (1.0 mg per 100 g rat) rise in liver glutamate, aspartate, and alanine, coincident with the fall in other amino acid levels, supports the view that these compounds are important intermediates in the gluconeogenic process (17). Glutamate is a product of one of the first induced enzymes (tyrosine-αKG transaminase) (65, 17). Since glutamate mimics cortisone in adrenalectomized rats in stimulating in liver the rates of purine and protein syntheses, increases in hepatically-generated glutamate might be one of the early events in the glucocorticoid promotion of metabolic processes in target organs (65).

Puzzling results were reported with prednisolone-increased glycolysis (increased glucose utilization and lactic acid production) after 24 hours in Chang adult human liver cells grown in tissue culture (186). The steroid augmented the incorporation of alanine into glucose and glycogen, and increased the glycogen content by 45 per cent at 20 hours. Glucocorticoids alone had little effect on gluconeogenesis in perfused livers from five-day fasted adrenalectomized rats (72). Under these conditions, the glucocorticoids were needed for their " permissive effect" on the action of glucagon or epinephrine through sensitization to cyclic AMP (45).

As can be seen, there are many conflicting reports on whether enzyme synthesis is needed for increased gluconeogenesis. Oddly, there

are many incidences of only the soluble enzymes changing under gluconeogenic conditions with no differences in the mitochondrial enzymes. Increases in enzyme activity may be due to increased enzyme biosynthesis or decreased enzyme degradation. Newsholme and Gevers (174) pointed out that the apparent inductions of enzyme synthesis described in the literature could be due to stabilization of enzyme protein against degradation. Possibly this enzyme stabilization is sensitive to actinomycin D and puromycin (which supposedly inhibit enzyme synthesis) and therefore an increase in enzyme degradation is seen. Both enzyme synthesis and degradation may be controlled processes, and hormones may directly or indirectly influence one or both of these processes. Newsholme and Gevers say that it is still premature to propose and discuss theories of glucocorticoid action in stimulating the increase in the synthesis of specific liver enzymes. They conclude that there was little information on enzyme degradation and that many aspects of hormone-stimulated protein synthesis were obscure.

Other Metabolic Pathways

Gluconeogenesis does not occur in rat adipose tissue due to the absence of fructose diphosphatase and glucose-6-phosphatase (30). However, the abbreviated dicarboxylic acid shuttle seems to be active because PEP is formed from pyruvate through a symmetrical 4-carbon intermediate (30). The reversal of glycolysis in mouse and rat adipose tissue is necessary for the formation of alpha-glycerophosphate which is needed for esterification of fatty acids (145, 30). The formation of glycerol from pyruvate was much lower in the adipose tissue of obese mice than non-obese (30). PEP-CK activity was 80 per cent lower and pyruvate carboxylase activity 30 per cent higher in obese than in normal mice (30).

More information on the relationship between carbon dioxide fixation and fatty acid synthesis will be seen under discussions on the enzymes acetyl-CoA carboxylase and malic enzyme. The role of carbon dioxide in pyrimidine and urea synthesis is discussed under "carbamyl phosphate synthetase." The synthesis of purines, porphyrins, and four-carbon compounds will also be dealt with under specific carbon dioxide fixing enzymes.

Carbon Dioxide Fixing Enzymes

Carbon dioxide-fixing enzymes found in heterotrophic organisms can generally be classified as those requiring ATP, reduced pyridine nucleotide, reduced ferredoxin, or no extra energy source (281, 179, 280). A reduced pyridine nucleotide is needed for the following enzymes: isocitrate dehydrogenase, malic enzyme, and phosphogluconate dehydrogenase (280, 281). Reduced ferredoxin is needed for two carboxylation reactions found only in bacteria (62): pyruvate synthetase and alpha-ketoglutarate synthetase. The enzymes requiring no extra energy source (also called enoyl carboxy-lyases) are: PEP-carboxykinase, PEP-carboxylase, PEP-carboxytransphosphorylase, ribulose diphosphate carboxylase, and aminoimidazole ribonucleotide carboxylase (281).

All the carboxylating reactions requiring ATP as an energy source are catalyzed by biotin-containing enzymes (281, 155, 179, 280, 274). Nearly all of the biotin enzymes have a molecular weight of approximately 700,000 (262, 280, 212). There are estimated to be about four moles of biotin per mole of enzyme (262, 280, 212). All of the reactions of biotin enzymes involve the direct participation of a coenzyme-A ester, except pyruvate carboxylase (280, 281, 212) and carbamyl phosphate synthetase (274).

The enzymes catalyze a two-step reaction. The first step results in the formation of an enzyme-biotin linked carbon dioxide complex from ATP, bicarbonate, and the enzyme (179, 280). The second step of the reaction involves an acceptor molecule receiving carbon dioxide on the carbon atom adjacent to the conjugate system of a CoA derivative (281, 179, 280).

Carboxylation by ATP-dependent biotin-containing enzymes (also called C-C bond forming carbon dioxide ligases) can take place on saturated or unsaturated derivatives of CoA (248). Carboxylations of saturated short-chain aliphatic derivatives of CoA on the carbon next to the thioester group are catalyzed by three enzymes: acetyl-CoA carboxylase converting acetyl-CoA to malonyl CoA; propionyl-CoA carboxylase converting propionyl-CoA to methyl-malonyl-CoA; and butyryl-CoA carboxylase converting butyryl-CoA to ethyl-malonyl-CoA. Carboxylations of alpha, beta-unsaturated aliphatic derivatives of CoA can take place at the alpha- or gamma-carbon. Two enzymes carboxylating the gamma-carbon are: beta-methyl-crotonyl-CoA carboxylase converting beta-methyl-crotonyl-CoA to beta-methyl-glutaconyl-CoA and crotonyl-CoA carboxylase converting crotonyl-CoA to glutaconyl-CoA. Two enzymes that carboxylate the alpha-carbon are: crotonyl-CoA carboxylase which converts crotonyl-CoA to ethylidine-malonyl-CoA, and beta-hydroxy-butyryl-CoA carboxylase, which converts beta-hydroxy-butyryl-CoA to alpha-carboxy-beta-hydroxy-butyryl-CoA. Although all of the reactions listed in this paragraph can take place in mammals, the gamma carboxylation is the most active (248).

Beta-Methyl-Crotonyl-CoA Carboxylase

Beta-methyl-crotonyl-CoA carboxylase (EC 6.4.1.4) is a biotin-containing enzyme that is inhibited by avidin (281, 179). It is found in

animals and bacteria and is in the pathway of leucine degradation (281). It forms beta-methyl-glutaconyl-CoA from ATP, bicarbonate, magnesium and beta-methyl-crotonyl-CoA (179). Some of the Km values for its components are: bicarbonate= 3×10^{-3}M, ATP = 0.8×10^{-4}M, and beta-methyl-crotonyl-CoA = 0.1×10^{-4}M (179).

Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyzes the formation of malonyl-CoA from acetyl-CoA, ATP, and bicarbonate (281). It participates in the process of adding two carbon units to growing saturated fatty acid chains (179). Carbon dioxide that is fixed by acetyl-CoA carboxylase into malonyl-CoA does not appear in the fatty acid chain because it is decarboxylated during the condensation process. The role of the enzyme is to by-pass an energetically unfavorable step (280). In rat mammary gland, acetyl-CoA carboxylase activity parallels fatty acid synthesis (115). It is the rate-limiting reaction in extra-mitochondrial synthesis of fatty acids in this organ (115). It is a biotin enzyme usually present in the soluble fraction of animal cells, yeast, and microorganisms (281).

Animal acetyl-CoA carboxylase has an absolute dependence upon citrate or closely related di- or tricarboxylic acids (179, 155, 115, 86, 165, 234). In contrast to previous reports, citrate was not required for activation of the enzyme in rat liver (239). It was pointed out that citrate probably serves only as a catalyst of the aggregation process, and the forces holding the protomeric units together in an aggregate do not require the permanent attachment of citrate (239). The rat liver enzyme was activated by prior incubation with magnesium (86) or by incubating at 37 degrees for three hours (239). The enzyme is inhibited by long

chain acyl-CoA derivatives in animals (155).

Unlike other biotin enzymes, chicken liver acetyl-CoA carboxylase has a molecular weight of four to eight million (87, 200). This catalytically active form is composed of filaments, and equilibrates with a small, inactive form whose molecular weight is 490,000 (87). Km values for the enzyme's components are: bicarbonate = 1×10^{-3}M, acetyl-CoA = 0.5×10^{-5} M, and ATP = 1.0 to 5.4×10^{-3} M in the presence of isocitrate (179).

Propionyl-CoA Carboxylase

Propionyl-CoA carboxylase (propionyl-CoA : carbon dioxide ligase (ADP), EC 6.4.1.3) is found mainly in the mitochondria of mammals and microorgansisms (179). It catalyzes the ATP dependent conversion of propionyl-CoA and bicarbonate to methyl-malonyl-CoA, and the function of propionyl-CoA carboxylase appears to be for the formation of succinyl-CoA for the Krebs cycle (179). It is a biotin-containing enzyme with a molecular weight of 700,000 (179). The pH optimum is 8.0 to 8.5, and its activity is increased by K^+, NH_4^+, Rb^+, and Cs^+ (82). The active species for carboxylation is bicarbonate (1). The Km values for bicarbonate are from 2 to 8×10^{-3}M (179, 58). Other Km values are: ATP = 0.5 to 0.8×10^{-4} M, and propionyl-CoA = 0.2 to 0.3×10^{-3} M (179).

Isocitrate Dehydrogenase

L-isocitrate dehydrogenase (EC 1.1.1.42) catalyzes three reactions: the reversible formation of isocitrate and TPN^+ from alpha-ketoglutarate, carbon dioxide, and TPNH; the irreversible decarboxylation of oxalosuccinate to alpha-ketoglutarate; and the irreversible reduction of oxalosuccinate with TPNH to isocitrate (187, 281). Oxalosuccinate synthesis from either direction has never been shown, and no enzyme-bound

OAS intermediate has been isolated (187, 193). Of the two isocitrate dehydrogenases in mammals (DPN- and TPN-linked) only the DPN-specific enzyme is concerned with regulating the decarboxylation of isocitrate (83, 203). The DPN-linked enzyme is found only in the mitochondria and catalyzes the irreversible decarboxylation of isocitrate (281, 201). The TPN-linked enzyme is present mostly in the cytoplasm of organisms (187, 281), except in rat brain where 35 per cent is found in the soluble position and 65 per cent in the mitochondrial fraction (201).

DPN-linked isocitrate dehydrogenases from the mitochondria of rat heart, insect flight muscle, and bovine heart are activated by ADP (83, 37). In one case, yeast DPN-specific enzyme was reported to need AMP (187) for activation, and in another case, the enzyme did not need it (103). The DPN-linked enzyme in Neurospora crassa is inhibited by high concentrations of alpha-ketoglutarate and activated by citrate (203).

TPN-linked isocitrate dehydrogenase from bovine heart is not affected by ADP, ATP, DPN^+, or DPNH (37). The maximum velocity in the direction of decarboxylation is only four times that for the reverse reaction (42). The reaction in the direction of carboxylation was assayed at pH 7.4 with alpha-ketoglutarate, NADPH, HCO_3^-, and $MgCl_2$ (193). Km values for the various components are as follows: bicarbonate = 9 mM, alpha-ketoglutarate = 2.5 to 13 x 10^{-5} M, TPNH = 1.4 to 9.2 x 10^{-6} M, isocitrate = 0.45 to 2.6 x 10^{-6} M, and TPN^+ = 0.1 x 10^{-6} M (42, 187). The physiological ratio in cells of $TPNH/TPN^+$ is more than 20 (42).

Isocitrate dehydrogenase could function metabolically in the direction of carboxylation to form isocitrate which, in turn, could be converted to citrate, and then cleaved to OAA and acetyl-CoA. All of these

enzymes are in the soluble part of the cell (280). Acetyl-CoA could be used for fatty acid synthesis and OAA could be used for glucose synthesis by gluconeogenesis (42, 280). In lactating rat mammary gland slices, it was estimated that 20 to 30 per cent of the glutamate metabolized via the Krebs cycle in the presence of glucose was by the backward pathway, i.e. through carboxylation of alpha-ketoglutarate and eventually incorporated into fatty acids (156). In perfused rat liver, it was estimated that 40 to 60 per cent of alpha-ketoglutarate carbon was contributed to gluconeogenesis and fatty acids by the reversed Krebs cycle (50).

Isocitrate dehydrogenase seemed to be involved in gluconeogenesis in the dog because its increased activity after birth paralleled glucose production from bicarbonate (173). Carboxylation of alpha-ketoglutarate was increased three to four-fold by diabetes in the rat liver (263). The major substrate of carbon dioxide fixation in lobster, frog, and rabbit nerves is alpha-ketoglutarate (262). The importance of the carboxylation of alpha-ketoglutarate in the nervous system may be for the control of citrate and therefore the levels of acetyl-CoA and acetyl choline (262).

Phosphogluconate Dehydrogenase

Phosphogluconate dehydrogenase (EC 1.1.1.44) catalyzes the reversible formation of 6-phosphogluconate and NADP from NADPH, carbon dioxide, and D-ribulose-5-phosphate (113). The enzyme is widely distributed among animals, plants, and bacteria (281). It is not certain if it functions metabolically in the direction of carboxylation because there is no evidence that hexoses are synthesized via carbon dioxide fixation into 6-phosphogluconate (281, 280).

PEP-Carboxylase

PEP-carboxylase (EC 4.1.1.31, orthophosphate: oxalacetate carboxylase (phosphorylating)) is found in plants and microorganisms (161, 281, 280). It catalyzes the irreversible carboxylation of PEP to OAA and Pi (161). Its ΔF° is -7.2 K cal per mole, and bicarbonate or carbonate is the active species (161, 44). Estimates of its molecular weight lie between 265,000 (230) and 350,000 (161). The enzyme's pH optimum lies between 8.0 and 9.0 (161, 206, 230), and with acetyl-CoA present in E.coli, it is lowered to pH 7.0 (47).

PEP-carboxylase plays an important role in E.coli (47) and Salmonella typhimurium (206, 157), in replenishing the supply of OAA for the Krebs cycle. The enzyme from E.coli is strongly inhibited by aspartate, asparagine, fumarate, and malate, and is activated by acetyl-CoA (175, 176). The enzyme from Salmonella typhimurium is activated by acetyl-CoA (157), FDP (205), CDP, GTP, and CMP (157). PEP-carboxylase from Tetrahymena pyriformis--a ciliated protozoan--is not stimulated by acetyl-CoA nor inhibited by aspartate (218).

For PEP-carboxylase, acetyl-CoA, the Km of which is 1.4×10^{-4} M, stimulated the reaction rate 30-fold in E.coli and lowered the Km for PEP from 5.5×10^{-3} M to 6.4×10^{-4} M (230). Acetyl-CoA is thought to exert its stimulating effect through interaction with an allosteric site on the enzyme (230). The Km for PEP in Salmonella typhimurium is 12 mM and is lowered to 1 mM with CDP or GTP (206). Other Km values for PEP run from 1.7×10^{-4} M (281) to 5.5×10^{-4} M (161). The enzyme requires divalent metal ions with either Mg^{++} or Mn^{++} being active (26, 230). Km values for Mg^{++} range from 3 to 9.8×10^{-4} M (161, 26). It has a higher affinity for

bicarbonate than most carbon dioxide-fixing enzymes with the Km values about 2 to 3 x 10^{-4} M (281, 161). The Km values for bicarbonate with the following enzymes are: isocitrate dehydrogenase, 9 x 10^{-3} M (42); acetyl-CoA carboxylase, 1 x 10^{-3} M(179); and malic enzyme, 1.3 x 10^{-4} M(118). Avidin does not inhibit the enzyme (26, 27).

PEP-Carboxytransphosphorylase

PEP-carboxytransphosphorylase (EC 4.1.1.38 --pyrophosphorylase: oxalacetate carboxy-lyase) is found only in propionic acid bacteria (148, 280). The enzyme catalyzes the formation of OAA and pyrophosphate from PEP, carbon dioxide, and inorganic phosphate (148, 229). The ionic equilibrium constant for OAA formation is 9 x 10^{-7} (279), and the rate of carboxylation is 7 times as fast as that of decarboxylation (148). The molecular weight of the enzyme is 430,000 (148), and it is not a biotin enzyme (229, 228). It has a broad pH optimum from 6.1 to 8.0 (148). Mg^{++} or Mn^{++} is needed (148, 229). Some Km values for the substrates and cofactors are: PEP = 1.7 to 5 x 10^{-4} M, Pi = 12 x 10^{-4} M, Mg^{++} = 1.2 to 2.1 x 10^{-3} M and Mn^{++} = 1.4 to 5 x 10^{-4} M (148, 228). The Km value for bicarbonate was listed as 2.9 x 10^{-4} M (228) and as 4 to 10 x 10^{-3} M, depending on the pH (148).

Ribulosediphosphate Carboxylase

Ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39), also called carboxydismutase (179), is rarely found in animal cells. It was found in rabbit lymphocytes, mouse tumor cells, and in human erythrocytes (66). It catalyzes the formation of two moles of 3-phosphoglycerate from ribulose-1,5-diphosphate and carbon dioxide (183, 281).

Aminoimidazole Ribonucleotide Carboxylase

Phosphoribosyl-aminoimidazole carboxylase (EC 4.1.1.21) puts carbon dioxide into the 6 position of the purine ring in the de novo synthesis of purine nucleotides (281). It carboxylates 5-amino-imidazole ribonucleotide.

Pyruvate Synthetase and Alpha-ketoglutarate Synthetase

These two carbon dioxide-fixing enzymes are ferredoxin-dependent, and are part of the reductive carboxylic acid cycle (62). This cycle has been shown only in anaerobic bacteria, and it functions mainly in the synthesis of amino acids (62). It reverses two reactions of the Krebs cycle which in aerobic cells are essentially irreversible.

Alpha-ketoglutarate synthetase catalyzes the formation of alpha-ketoglutarate from succinyl-CoA, carbon dioxide, and reduced ferredoxin. In the photosynthetic bacterium Chlorobium thiosulfatophilum, all the radioactivity from C-14 bicarbonate was found in carbon-1 of glutamate (21). Succinate, ATP, and CoA together may substitute for succinyl-CoA (62, 21).

Pyruvate synthetase is found in both non-photosynthetic, anaerobic bacteria and photosynthetic bacteria (20). It catalyzes the formation of pyruvate from acetyl-CoA, carbon dioxide, and reduced ferredoxin. Acetyl-phosphate or acetate plus ATP will substitute for acetyl-CoA in Chromatium--an anaerobic photosynthetic bacterium (22). The label from C-14 bicarbonate was found in the carbon-1 of pyruvate and alanine (4).

Carbamyl Phosphate Synthetase

Carbamyl phosphate, the product of the enzymic reaction, plays an important role in the biosynthesis of pyrimidines and in the urea cycle.

The urea cycle gets rid of excess ammonia and also forms arginine. The synthesis of carbamyl-phosphate, which is required in both arginine and pyrimidine nucleotide synthesis, is carried out by a single enzyme in E. coli and mushrooms(185). There is feedback inhibition of this enzyme's activity by only one end-product (UMP), but ornithine decreases the feedback inhibition of UMP in both these organisms (185). The E.coli enzyme utilizes glutamine, bicarbonate and two moles of ATP to irreversibly form carbamyl phosphate (2, 274). It does not need N-acetylglutamate, and ammonia may substitute, although less effectively, for glutamine (2). E.coli carbamyl phosphate synthetase is a biotin enzyme (274). Another bacterial enzyme, carbamate kinase, catalyzes the reversible formation of carbamyl phosphate from one mole each of NH_4^+, HCO_3^-, and ATP (3).

Two carbamyl phosphate synthetases are found in rat liver and the carbamyl phosphate pools are separated in mammals (90). The N-acetylglutamate-dependent enzyme can only use ammonia as a nitrogen donor. Along with ornithine transcarbamylase it is found in the mitochondria and plays a role in arginine and urea synthesis (35, 90). Both enzymes increase rapidly after 17 days of gestation (90). The glutamine-dependent enzyme along with aspartate transcarbamylase is found in the soluble fraction of the cell. Both enzymes are important in pyrimidine synthesis and they decrease in activity in rat liver from 17 days of gestation until birth (90). Ammonia also can substitute for glutamine as nitrogen donor, but its Km value is 100 times higher (90).

In freshly prepared rat liver mitochondria, N-acetylglutamate has no effect on the irreversible carbamyl phosphate synthetase reaction. But after aging or sonication of the mitochondria, citrulline synthesis becomes

dependent upon N-acetylglutamate (36). N-acetylglutamate probably unmasks an active site on the enzyme or alters the enzyme surface. Glutamine was the main ammonia donor and two moles of ATP were required.

The mouse spleen enzyme does not require N-acetylglutamate, and UTP is the only pyrimidine nucleotide that inhibits it (244). Glutamine or ammonia will work and the Km value for NH_4^+ is 1.8 mM and for glutamine, 0.3 mM. Orotic acid inhibits the incorporation of C-14 bicarbonate into carbon-2 of uracil in Ehrlich ascites cells (92).

Malic Enzyme

Malate enzyme (L-malate: NADP oxidoreductase-decarboxylating EC 1.1.1.40) is widely distributed among animals, plants, and microorganisms (199, 281, 280, 120). The enzyme catalyzes the following three reactions in pigeon liver (119):

1) L-malate + $NADP^+$ $\rightleftharpoons^{Mn^{++} \text{ or } Mg^{++}}$ HCO_3^- + pyruvate + $NADPH_2$

2) OAA $\xrightarrow[NADP^+]{Mn^{++} \text{ or } Mg^{++}}$ pyruvate + HCO_3^-

3) pyruvate + $NADPH_2$ $\xrightarrow{Mn^{++} \text{ or } Mg^{++}}$ L-lactate + $NADP^+$

But the rate of pyruvate reduction in reaction (3) is only one per cent of the activity for oxidative decarboxylation of L-malate in reaction (1). The optimum pH for reaction (1) is 7.2 to 7.6 (189, 171, 261), and for reaction (2), 4.5 (281). The rate of malate decarboxylation compared to that of OAA decarboxylation is 1.24 to 1 (120). The rest of the discussion will concern only reaction (1). Optimum pH for the pigeon liver enzyme varied greatly with the concentration of the substrate (199). At 10^{-4} M malate the pH optimum was 7.2, and at 10^{-1} M malate, it was 8.5 to 9.0.

It is generally accepted that malate enzyme functions as a decarboxylase for lipogenesis. It is not involved in carbon dioxide fixation during gluconeogenesis. Evidence for these two views is based on the fact that the enzyme varies reciprocally with gluconeogenic enzymes under gluconeogenic conditions, and increases under conditions favoring fatty acid synthesis (141, 280, 120). Adrenalectomy or the presence of glucocorticoids had little effect on the rat liver enzyme (143, 141, 223, 144). The malic enzyme is not present in rat liver in high enough amounts to account for malate formation during pyruvate conversion to glucose (143, 120, 223, 144). Its activity in rats was decreased by diabetes and increased by insulin (278, 141, 223, 144). The enzyme's activity in rats falls during fasting, and rises, together with lipogenesis upon refeeding a high carbohydrate diet (223, 284, 144, 9, 278, 141, 143). Induction of the enzyme is suppressed by fat (141, 284).

The apparent Km for pyruvate with pigeon liver enzyme varies from 6.4 to 20 mM (118, 236), and this appears to be above the physiological concentration (233). Furthermore, the apparent Km for bicarbonate is 13 mM (118), a similarly high value (233, 252, 280). Malic enzyme is located almost exclusively in the supernatant fraction of the cell in the liver and kidney of rats (166, 38, 284) and other animals (94). The exceptions are in the flatworm where all activity if found in the mitochondria (189), and the rat brain with 25 per cent soluble and 75 per cent mitochondrial malic enzyme (201). Both the soluble and mitochondrial enzymes of the rat brain have the same Km's and activity per gram as the liver enzyme. Since gluconeogenesis takes place in the mitochondria and lipogenesis in the extra-mitochondrial fraction of pigeons and rats, the malic enzyme is

probably not essential for gluconeogenesis. In addition, yeast cells which have no malic enzyme can grow on pyruvate (247).

The distribution of malic enzyme in rat tissues suggests that it functions to provide NADPH for synthetic purposes, such as fatty acid and steroid synthesis (278, 243, 141, 143). The product of the decarboxylation reaction, pyruvate, is easily converted to acetyl-CoA which is also needed in fatty acid synthesis along with NADPH (243, 284). Rat adipose tissue has a high concentration of malic enzyme (143, 9, 278). The adrenal cortex, where NADPH is used for steroid synthesis, is rich in this enzyme (278). The bovine adrenal cortex has a mitochondrial enzyme whose total activity is 1/5 that of the cytosol, and the specific activity ½ that of the cytosol (225). Since the (V max) decarboxylation over the (V max) carboxylation is 105 for the mitochondrial enzyme and 2.5 for the cytoplasmic enzyme, it was concluded that the mitochondrial malic enzyme is the source of NADPH for steroid synthesis (225, 224).

Thermodynamically the malic enzyme favors decarboxylation of malate (73, 223). It is thought to control the level of C-4 dicarboxylic acids, when they are abundant, by decarboxylating malate to pyruvate and carbon dioxide (243, 202). In two types of yeast the enzyme activity was high during gluconeogenesis when malate, aspartate, or acetate were substrates (197).

One of the latest articles in 1968 by Wada et al.(261), in contrast to others, stated that the physiological function of malic enzyme is to convert pyruvate to malate by utilizing NADPH. They based this on the finding that the administration of pyruvate, but not malate to rats resulted in a doubling of the liver enzyme activity. They thought that

malic enzyme facilitated the operation of the Krebs cycle by regulating malate concentration in the cytoplasm.

E.coli contains also a DPN-linked malic enzyme. Since aspartate caused a two to three-fold increase in the DPN-linked enzyme activity in E.coli, the DPN-linked enzyme probably plays a role in the decomposition of malate (243). OAA inhibited the E.coli TPN-linked enzyme 95 per cent, while it inhibited the DPN-linked enzyme only 37 per cent (123). The TPN-specific enzyme from E.coli was also inhibited by acetyl-CoA (202). Acetate repressed the enzyme in Pseudomonas putida (121). In pigeon liver TPN^+ binds in competition with TPNH, and the enzyme has nearly the same affinity for each (117, 118). The following are non-competitive inhibitors in pigeon liver: malate and TPNH; malate and bicarbonate; and TPN and bicarbonate (118). Uncompetitive inhibitors in pigeon liver are: pyruvate and malate; and TPN^+ and pyruvate (118). In the adult rat, thyroxine increases malate enzyme activity in the liver but not in the brain (106).

The pigeon liver enzyme has a molecular weight of 280,000 (120). Its specific activity in the decarboxylating direction is 0.21 micromoles per min per mg of protein. Rat kidney and liver had similar specific activities to pigeon liver (254). The specific activity in rat adipose tissue was 19 ± 7 micromoles per min per g of protein (284). The equilibrium of the malate enzyme catalyzed reaction lies toward the synthesis of malate with a ΔF° of -1.8×10^3 calories(281). The Km values for the enzyme from pigeon liver are for TPNH = 2.1 micromolar, TPN^+ = 1.4 micromolar, and malate = 86 micromolar (118). The apparent Km for malate is 2.85 mM for the mitochondrial enzyme and 0.47 mM for the supernatant medium enzyme from the bovine adrenal cortex (224). Mg^{++} or Mn^{++} will cata-

lyze the reaction in pigeon liver (199). In pigeon liver, malate synthesis was at the same rate for 0.033 mM Mn^{++} as 3.3 mM Mg^{++} (236). But 7 mM Mn^{++} and 7 mM Mg^{++} gave the same rate (236).

Pyruvate Carboxylase

Pyruvate carboxylase (Pyruvate: carbon dioxide ligase (ADP), EC 6.4.1.1) is considered a link between fat and carbohydrate metabolism (71). It is stimulated by acetyl-CoA which is a substrate for fatty acid synthesis and a catabolic product of the oxidation of fatty acids and pyruvate. Its product, OAA, can combine with acetyl-CoA to form citrate in the Krebs citric acid cycle or break down to PEP for the start of the gluconeogenic pathway. Its role in gluconeogenesis and the effects of hormones and metabolites on it were discussed earlier in the section on gluconeogenesis. In the kidney the function of pyruvate carboxylase may be to ensure adequate levels of OAA for ketone body oxidation via the Krebs cycle (147). Ketone bodies are the main energy source in the kidney, and there is less dependence on carbohydrate metabolism.

Two partial reactions make up the total reaction. The formation of the enzyme-biotin-carbon dioxide complex requires ATP, Mg^{++}, bicarbonate, and acetyl-CoA, and transfer of carbon dioxide from this intermediate requires only pyruvate addition (169, 214). C-14 pyruvate exchange with OAA is independent of any added reaction components with the chicken liver mitochondrial enzyme (214). ATP exchange with P^{32}-ortho-phosphate requires acetyl-CoA, Mg^{++}, bicarbonate, and ADP with this same enzyme. Pyruvate carboxylase from chicken liver catalyzes an exchange of ADP with ATP which is insensitive to avidin inhibition, cold inactivation, or other reaction components, and depends only on Mg^{++}(213). Acetyl-1^{14}C-CoA

does not contribute any labeled carbon to OAA (253), and functions only in the carboxylation reaction (75, 46) as an activator. In yeast the carboxylation reaction is practically irreversible (153). Decarboxylation proceeds at only 10 per cent of the carbon dioxide fixation rate in chicken liver (212).

Pyruvate carboxylase is not very stable. The yeast enzyme lost 80 per cent of its activity after 24 hours at 22 degrees or 0 degrees (197), and 50 per cent of its activity in one to two days at -20 degrees (46). The activity of the crude rat kidney cortex enzyme dropped after 1½ hours at 23 degrees or at 0 degrees in the presence of 1.6 mM GSH (107). With no glutathione at 0 degrees the activity decreased immediately, and all three preparations at four hours had only 20 to 35 per cent of initial activity. The chicken liver enzyme is inactivated after five hours at a temperature of two degrees to 14 per cent of the original activity (212). This cold-lability is not observed in initial extracts, but it appears after the first purification step (252, 212). Sucrose at 1.5 M (212, 252), acetyl-CoA, urea, or sodium dodecyl sulfate (233, 210) protects the enzyme against the cold. Cold inactivation can be partially reversed in 10 to 15 minutes up to 70 per cent by a temperature of 23 degrees (252). The reactivation at 23 degrees depends on the conditions used and the protein concentration from sheep kidney (147) and chicken liver (212).

There is probably no physiological significance of cold inactivation (212). Pyruvate-OAA exchange, ATP-Pi exchange, and the overall reaction are sensitive to cold inactivation in the chicken liver mitochondria (214). ATP-ADP exchange is insensitive to cold inactivation of the enzyme (213). The chicken liver enzyme at 23 degrees exists as an active tet-

ramer with a molecular weight of 660,000 (257, 212). It has a $s^{\circ}_{20,w}$= 14.8S and a minor component whose $s^{\circ}_{20,w}$= 6.75S (208,212). At two degrees, the tetramer dissociates to give four subunits ($s^{\circ}_{20,w}$= 6.75S) with a molecular weight of 165,000 (212, 257). Each subunit contains one biotin and one Mn^{++} molecule (212). Rewarming to 23 degrees restored the catalytic activity and tetramer structure (257). Treatment of the yeast enzyme with maleic anhydride inactivates and dissociates the enzyme in a manner which suggests a tetramer-dimer-monomer conversion (285).

Chicken liver mitochondria incorporated $^{54}Mn^{++}$ into pyruvate carboxylase (211). The enzyme contains firmly-bound manganese at four moles per mole of enzyme (211, 208, 212). Bound manganese plays a role in the second partial reaction of the carboxylation of pyruvate by the enzyme-biotin-CO_2 complex (169). It was proposed from nuclear magnetic resonance spectrum studies that bound manganese facilitates the carboxylation of pyruvate by increasing the nucleophilic character of the methyl group of pyruvate and the susceptibility of the 1'-N-carboxybiotin intermediate to nucleophilic attack (168). Avidin blocked one of the three cis ligand positions of bound manganese (208).

Free Mg^{++} functions in the first partial reaction in the formation of the enzyme-biotin-CO_2 complex (169). Mg^{++} apparently has two roles in the reaction mechanism by forming a complex with ATP to form the true Mg-ATP-substrate and forming a complex with the enzyme to activate the enzymic reaction (169). Manganese ion is half as active as Mg^{++} in yeast (153), and its Km value for the chicken liver enzyme is 75 times higher than that of Mg^{++}(127). ATP can not be replaced by other nucleotide triphosphates with the enzyme from yeast (28) or sheep kidney (147).

Ranges for the Km values for different components of enzymes from yeast (197), rat kidney (216), sheep kidney (125, 14, 147), and chicken liver (212, 213, 127) were as follows: pyruvate from 4 to 8 x 10^{-4} M; ATP from 0.29 to 2.4 x 10^{-4} M; bicarbonate from 0.62 to 2.7 x 10^{-3} M; Mg^{++} from 0.25 to 4.2 x 10^{-3} M; and acetyl-CoA from 1.9 to 4.1 x 10^{-5} M. The active species of carbon dioxide is bicarbonate (44, 7, 147). The optimal concentration of bicarbonate was 20 to 30 mM (166).

All pyruvate carboxylase purified from mammals and birds is found to be essentially inactive in the absence of an acyl CoA (210, 147). However, it was noted by Scrutton and Mildvan in 1968 (208) that the addition of 0.2 mM acetyl-CoA decreased by almost 30 per cent the rat of decarboxylation of OAA by pyruvate carboxylase. Aspergillus niger's enzyme shows maximum activity in the absence of acyl-CoA (18). The enzymes from Chromatium (74, 73) and yeast (28, 285, 75, 153, 46, 197) were active without acetyl-CoA, but adding acetyl-CoA doubled their activity. Propionyl-CoA and crotonyl-CoA could replace acetyl-CoA with the sheep kidney enzyme (147). In chicken liver either formyl-, acetyl-, propionyl-, crotonyl-, or iso-butyryl-CoA could activate pyruvate carboxylase (210, 127). Acyl-CoA esters (C_2-C_{18}) activate the enzyme from yeast with C_{16}-C_{18} being the most effective (285).

At least two moles of acetyl-CoA interact with the allosteric enzyme in a cooperative effect. This is apparent because a plotting of 1/velocity versus $1/(\text{acetyl})^2$ produces a straight line for the enzyme from yeast (28), and sheep kidney (14). A lysine residue of the enzyme is believed to be involved in the interaction, because acetyl-CoA protected the sheep kidney enzyme from inhibition by 1-F-2,4-dinitrobenzoate (126).

Since acetyl-CoA protects the enzyme against cold-inactivation and affects avidin inhibition, it is proposed that acetyl-CoA acts as an allosteric effector (210, 233). Acetyl-CoA might be needed for the maintenance of subunit interactions which are required for stabilization of the catalytically active site. Acetyl-CoA accelerated yeast pyruvate carboxylase activity independent of ATP and pyruvate concentrations, and lowered the Km for bicarbonate (46). Activation by acetyl-CoA is an effective feedback control system for the synthesis of OAA from pyruvate (233, 136). If an organism has no OAA, pyruvate can be converted to acetyl-CoA which activates pyruvate carboxylase to form OAA. OAA can condense with acetyl-CoA to form citrate, or it can form PEP. It is of interest to note that the Km's for acetyl-CoA of citrate synthetase and pyruvate carboxylase are about 0.02 mM (136). If there is enough acetyl-CoA for the Krebs cycle, there is enough to activate pyruvate carboxylase.

Other activators are Rb^+ and NH_4^+ in yeast (28). Potassium stimulates activity two to four fold in yeast (197) and Aspergillus niger (285). Adding malate, succinate, alpha-ketoglutarate, or citrate led to large increases of bicarbonate incorporation with pyruvate carboxylase in rat kidney mitochondria (166). It was thought that the increased carbon dioxide fixation was not due to increased enzyme activity, but to dilution of radioactive OAA by a large pool of pre-existing four carbon dicarboxylic acids. In rat kidney mitochondria, C-14 bicarbonate incorporation for the pyruvate carboxylase assay was greatly increased by alpha-ketoglutarate or glutamate (164). Removal of alpha-ketoglutarate by adding NH_3 resulted in increased carboxylation of pyruvate (110). Fumarate, malate, alpha-ketoglutarate, succinate, and citrate had no effect on OAA

synthesis in sheep kidney (147).

Contrary to the results described in the previous paragraph, pyruvate carboxylase from sheep kidney was non-specifically inhibited by citrate, fumarate, malate, and alpha-ketoglutarate (157). Alpha-ketoglutarate (10mM) inhibited pyruvate carboxylation by about 80 per cent in rat liver homogenates (98). Malonyl-CoA inhibited the enzyme from rat kidney (165) and chicken liver (210). Fluorocitrate strongly inhibited pyruvate utilization and C-14 bicarbonate incorporation in rat kidney mitochondria (165). The inhibition by citrate was due to its stimulation of acetyl-CoA carboxylase to form malonyl-CoA. The ratio of acetyl-CoA to malonyl-CoA levels was suggested as a control of pyruvate carboxylase (210).

Aspartate (28) and OAA inhibited pyruvate carboxylase from yeast (182). Oxalate inhibited the yeast enzyme by removing Mg^{++} (197, 153). The yeast enzyme was also inhibited by Ca^{++}, Na^{+}, and Li^{+} (28). Concentrations of pyruvate greater than 6.7 mM, Mg^{++} greater than 10 mM or bicarbonate greater than 30 mM inhibited rat kidney pyruvate carboxylation (166). There was evidence that pyruvate carboxylase from chicken liver contained few if any disulfide bridges (212), and that free thiol groups were not required for activity with sheep kidney enzyme (147). But since mercury salts were very inhibitory, free sulfhydryl groups were considered essential with enzymes from chicken liver (127) and yeast (75, 28). Since alanine, PEP, and lactate did not inhibit the sheep kidney enzyme, it was thought that the presence of a keto oxygen on the alpha-carbon is essential for binding to the enzyme (147). Compounds with large substituents on the beta-carbon of pyruvate inhibited the enzyme (147, 216).

ADP inhibits pyruvate carboxylase in chicken liver (213, 127) and in rat liver the inhibition is not overcome by adding ATP (16, 270). ADP inhibition was overcome in rat liver by adding inorganic phosphate which removed ADP by oxidative phosphorylation (270). In rat kidney mitochondria, lack of inorganic phosphate inhibited pyruvate carboxylation (166). Therefore, inorganic phosphate and ADP are important for the regulation of pyruvate carboxylase. The ratio of ADP to ATP levels would be able to control pyruvate carboxylase activity (127).

Biotin is firmly bound to pyruvate carboxylase at four moles per mole of chicken liver enzyme (211, 208, 212). Avidin, an egg white protein, can inhibit pyruvate carboxylase by binding to biotin (179). Avidin inhibited the enzyme from chicken liver (127) and yeast (197) about 90 per cent, and preincubation of avidin with biotin caused no inhibition. Preincubation of avidin with biotin did not always stop all inhibition because there was still a 60 per cent loss of sheep kidney enzyme activity caused by avidin preincubated with excess biotin (147). Acetyl-CoA from 0.02 to 0.2 mM increased the inactivation of the chicken liver enzyme by avidin (210). Also, the rate of inactivation by avidin was increased by pyruvate or OAA and decreased by oxalate (169).

ATP as well as CTP, GTP, UTP, ITP, and TTP protected chicken liver pyruvate carboxylase against avidin inactivation (213). However, ATP and Pi exchange, pyruvate-^{14}C and OAA exchange, and formation of the enzyme-biotin-CO_2 complex with the chicken liver enzyme were inhibited by avidin (214).. The enzyme-ATP complex was not inactivated by avidin, and the ATP-ADP exchange was insensitive to avidin (213). Biotin was implied as part of the active site when the isolated enzyme-biotin-CO_2 complex

was identified as 1'-N'-carboxybiotin (214). Excess avidin changed the sedimentation coefficients of 14.1S and a minor 6.75S to 17.3S and a minor component of 24.4S (208). Inactivation of the enzyme by avidin is accompanied by an interaction between avidin and bound manganese with it blocking one of the three cis ligand positions of bound manganese (208).

Pyruvate carboxylase is mainly found in liver and kidney (147) where gluconeogenesis is high, and is low or absent in tissues such as brain, heart, and skeletal muscle that have low gluconeogenesis (233). It is also located chiefly in the mitochondria of liver and kidney from frog, beef, rabbit, mouse, rat, and sheep (127). The rabbit brain and heart had little activity, and the spleen and skeletal muscle had none (127). The enzyme was found exclusively in the mitochondria of rat brain (201) and mouse liver (71). In both the fetal and adult rat liver, only 10 per cent was located in the cytoplasm (10). However, the rat liver enzyme, found mostly in mitochondria and nuclei, could be extracted from 40 to 70 per cent into the soluble fraction (107, 216, 209). Glutamic dehydrogenase--a mitochondrial enzyme--was not extracted. Maybe pyruvate carboxylase is not in the mitochondria, but is absorbed by nuclear (216) or outer mitochondrial membranes (209).

The pH optimum for the enzyme ranges from 7.4 to 7.9 for chicken liver (127, 212) to pH 8.4 for yeast (197) and sheep kidney (147). Specific activity of the crude enzyme was about 40 micromoles/minute/gram from yeast (28), sheep kidney (147), and rabbit (127). Chicken (212) and rat (10) liver enzymes had high specific activities.

Phosphoenolpyruvate-Carboxykinase

PEP-CK (GTP: oxalacetate carboxy-lyase (transphosphorylating)

EC 4.1.1.32) was called OAA carboxylase until 1956 (281, 139, 255). The enzyme from pig liver catalyzed Mn^{++} and ITP or GTP dependent OAA and C-14 bicarbonate exchange (32). This exchange is inhibited by PEP and GDP (33). These results supported a reversible two-step reaction sequence. In the first step, which is rate-limiting, PEP and IDP or GDP combined with the enzyme to form a complex. This complex combined with bicarbonate in the second step to form OAA and ITP or GTP.

The main metabolic function of PEP-CK is not that of carboxylation, but rather decarboxylation (280, 33). It acts exclusively as a gluconeogenic enzyme, i.e. decarboxylating, in E.coli (116). Its role in gluconeogenesis and the effects upon it by hormones and metabolites were discussed previously in the section on gluconeogenesis. A mutant of E. coli which did not contain PEP carboxylase but did contain PEP-CK could grow only when supplied Krebs cycle intermediates (7). This indicated that carbon dioxide fixation did not occur through PEP-CK in E.coli. The rate of the reaction with PEP-CK from pig liver (33) and a ciliated protozoan (218) is at least 10 times as fast in the decarboxylation direction than the carboxylation. Some investigators considered its Km values for bicarbonate, which were 16 to 25 mM, to be too high for carboxylation (33, 44). But lower Km values for bicarbonate of 2 to 5 mM have been reported (281, 24), and the physiological bicarbonate concentration is about 20 mM for mammalian plasma (149). In chicken liver, the PEP-CK reaction is readily reversible (256). No pyruvate kinase is present in the adult liver fluke, so PEP-CK might have to play a carbon dioxide fixing role (190). In two references carbon dioxide was listed as the active form for PEP-CK (45, 44) and another listed bicarbonate (33).

Adenosine polyphosphate was the only active nucleotide for PEP-CK in yeast (281, 24, 25), a ciliated protozoan (218, 220), and a photosynthetic bacterium (249). Guanosine and inosine nucleotides are active with animal PEP-CK (281) such as that from chicken liver (139), guinea pig liver (112), and pig liver (32). In ten invertebrates (226) and a liver fluke (190), IDP was most effective. The flatworm can use either ITP or GTP (189).

PEP-CK activity is much higher with Mn^{++} than Mg^{++} in liver fluke (190), pig liver (32), and yeast (24). In vitro manganese activated PEP-CK in the presence of Mg^{++} the same as administered tryptophan in rats (68). Magnesium was thought to complex the nucleotide and Mn^{++} to activate the enzyme (68). Metal ions may be involved in the formation of a Mg-ITP chelate with an optimal Mg^{++}: ITP ratio of three (178). It was noted that Mn^{++} chelates carbon dioxide (25). Yeast PEP-CK showed an absolute requirement for divalent metal ion (24). However, no metal ion was needed as an activator for decarboxylation catalyzed by yeast PEP-CK (25). PEP formation with guinea pig liver mitochondrial PEP-CK was more active with Mn^{++} than Mg^{++} at pH 7.4 and more active with Mg^{++} than Mn^{++} at pH 8.0 (112). The soluble PEP-CK was more active with Mn^{++} than Mg^{++} at all pH's.

Free sulfhydryl groups are essential for catalytic activity of PEP-CK because glutathione caused a reversal of p-chloromercuribenzoate inhibition in pig liver (32) and yeast (24). Protection of sheep kidney PEP-CK against inactivation by sulfhydryl group reagents was afforded by the following compounds in order of effectiveness: IDP > ITP > IMP > inosine (13). PEP formation with mitochondrial but not soluble PEP-CK from

guinea pig liver was inhibited by AMP (112). But the soluble PEP-CK activity was inhibited when the ratio of metal to ITP is greater than one.

PEP-CK is found in plants, microorganisms, and mainly in animal gluconeogenic tissues--liver and kidney (254, 233, 280). It has low activity in rat brain, heart, skeletal muscle (254), and adipose tissue (11). Almost all of the PEP-CK from adult rat liver (144, 284, 69, 221) and adipose tissue (11) is located in the soluble fraction of the cell. Mouse (127), hamster (143, 178), and flatworm (189) PEP-CK is almost all in the cell supernatant fraction. In guinea pig, 25 (143, 178, 112) to 90 (144) per cent of the PEP-CK is found in the soluble fraction, with the remainder being found in mitochondria and nuclei. The adult liver fluke contains the enzyme in both the mitochondrial and supernatant fractions (190). Rabbit liver contains no soluble PEP-CK; 3/5 is located in the mitochondria and 2/5 in the nuclei (178). PEP-CK is contained only in the mitochondria of pigeon liver (77) and kidney (127).

A ciliated protozoan--Tetrahymena pyriformis--has PEP-CK evenly distributed between the mitochondria and cytosol, but only the cytosol enzyme is closely related to the rate of gluconeogenesis (218). The 17-day fetal rat liver has 90 per cent of its PEP-CK in the mitochondria and nuclei, and this is just the opposite of the adult rat (10). Activity in the soluble fraction increases 25-fold in the first two days after birth, and this parallels the increase in gluconeogenesis. The PEP-CK of both the mitochondria and supernatant fraction from fetal rat liver (10) and guinea pig liver (112) had similar kinetic characteristics.

Km values for PEP ranged from 0.1 to 0.4 mM (112, 33, 281, 10). Km values for IDP or GDP were from 0.03 to 0.16 mM and for ITP or GTP

from 0.16 to 0.58 mM (33, 112). There was a broad span of Km values for Mn^{++} from 1 x 10^{-6} M (68) to 0.6 mM (33,112). The molecular weight for PEP-CK was found to be between 54,000 and 80,000 (68, 32, 283, 10). Optimal pH values for PEP-CK in the carboxylation direction were 5.2 to 5.4 for yeast (247, 29), 5.9 for liver fluke (190), 6.2 for flatworm (189), 6.5 for sheep kidney (13), 6.6 to 7.0 for pig liver (32, 33), 7.0 for rat liver (10), and 7.4 for guinea pig (112).

CHAPTER III

EXPERIMENTAL PROCEDURES

CELL CULTURE METHODS

Cell Characteristics

The only organism used in these studies was an established line of mouse cells, NCTC clone 929 (strain L, Earle) (204). The primary strain from which this line is derived was started *in vitro* in 1940 from normal subcutaneous connective tissue of an adult C 3H strain mouse (57).

The L-cells grow in monolayers on a glass surface. All glassware had been sterilized by autoclaving. For most of this work the cells were grown in stoppered T-60 flasks or screw-capped 250 ml milk dilution bottles. The cells covered a flat surface area of 55 cm^2 per milk dilution bottle in the stationary phase. If the milk dilution bottles were put on a slowly-rotating wheel, they attached to all four sides for a total surface area of 220 cm^2.

The cells were transferred every seven days from one vessel into four new vessels. Four days after the transfer of cells the 10 ml of medium per stationary bottle was replaced with fresh medium. After seven days incubation at 37 degrees the single monolayer of cells was harvested under aseptic conditions by scraping the cells from the glass surface with a rubber-covered bent glass rod. Clumps of cells were broken up and suspended in the medium by repeated aspiration through a 10 ml pipette. Thirty ml of fresh medium was added and 10 ml of the resulting suspension

was distributed to each of four flasks. The initial cell population of from 2 to 4 x 10^6 cells per surface usually reached 10 to 25 x 10^6 cells after seven days.

Growth Media

The cells were grown in a defined synthetic medium called XXI-42. This medium was identical in composition to medium MD 705/1 (129) except for the following additions in mg/100 ml: L-serine 6.4 and phenol red 1.0; and substitutions in mg/100 ml: L-valine 10.0, L-leucine 10.0, L-methionine 8.0, and L-isoleucine 7.5. In certain experiments Tris-, TES-, or HEPES-buffers were used in place of HCO_3^- in the medium. The Tris-medium was identical to medium XXI-42 except for the following changes per 100 ml medium: 121 mg of Tris was added and HCO_3^- was deleted, 68.3 mg of KH_2PO_4 instead of 8, and 71.0 mg of Na_2HPO_4 instead of 30. TES-medium was identical to medium XXI-42, except that TES was added at 229 mg per 100 ml instead of bicarbonate and an additional 156 mg NaCl was added. The HEPES-medium was identical to medium XXI-42 except that HEPES was added at 238 mg/100 ml in place of HCO_3^- and an additional 156 mg NaCl was added. A phosphate-buffered saline (PBS) solution was sometimes used for short-term incubation of the cells. The composition of PBS (31) was 22.8 mg $NaH_2PO_4 \cdot H_2O$, 133 mg Na_2HPO_4, 900 mg NaCl, 45 mg KCl, and 20.4 mg $MgCl_2 \cdot 6\ H_2O$ in a total volume of 100 ml. All media were filter-sterilized through 0.45 micron pore-size filters and stored at 5 degrees until needed. Glutamine, because of its instability, was not put into solution until just before use.

Methods Used in C-14 Bicarbonate Incorporation Studies

The $NaH^{14}CO_3$ was prepared by acidifying barium carbonate-C-14

with perchloric acid, collecting the CO_2 in dilute NaOH, and titrating the solution to pH 7.4. The $NaH^{14}CO_3$ had a specific activity between 10.6 millicuries and 52.5 millicuries per millimole. All C-14-bicarbonate incorporation experiments were started with four-day-old cells. The used medium was decanted and replaced with experimental medium. Both Tris (bicarbonate-free) medium and the normal medium, to which bicarbonate had been added, were used. Air, made carbon dioxide-free by passage through an Ascarite trap, had been bubbled through the Tris medium for at least 30 minutes. The media were adjusted to pH 7.4. Carbon-14-bicarbonate was added to the medium in concentrations of 8.5 to 98 microcuries per flask just before filter sterilization. The normal medium contained 267 micromoles of bicarbonate per flask and the radioactive bicarbonate that was added ranged from 0.8 to 2.0 micromoles. Each flask of cells was washed with 5 ml of non-radioactive medium before adding the experimental medium. The cells were exposed to C-14 bicarbonate for intervals ranging from 24 to 96 hours.

At the end of the experimental incubation period the flask was inverted so that the medium drained away from the cells. The medium was acidified by injecting 0.5 ml of 5 per cent perchloric acid with a hypodermic needle through the rubber vial seal. The gaseous system was flushed for at least 15 minutes with air, and the carbon dioxide was trapped by bubbling the exhaust through 10 to 15 ml of a mixture of two parts absolute ethanol to one part 2-aminoethanol. Then the trap solution was drained into a 25 ml volumetric flask and the trap apparatus washed with absolute ethanol. The acidified medium was transferred to another vessel and frozen. Five ml of 0.9 per cent sodium chloride was added to the flask and

everything was washed except the cells. Two more portions of 0.9 per cent sodium chloride were used to rinse the whole bottle, and all three washings were combined.

Methods Used in Chemical Fractionation of the Cells

The cells were fractionated using a modified Schmidt and Tannhauser procedure (207). The cell layer was extracted with three five ml portions of 10 per cent trichloroacetic acid. The cell layer was then washed three times with five ml of 0.9 per cent sodium chloride. At this step the cells were either assayed for protein or extracted further. Lipid components were removed from the cell layer by serial extractions with three ml of each of the following: 80 per cent ethanol, 100 per cent ethanol, 25 per cent chloroform in ethanol, and dry ether. The duration of each extraction was about five minutes and all fractions were combined.

Digestion of the cell layer which still contained protein and nucleic acids was done by exposing the cells to three ml of 0.3 N KOH overnight at room temperature. The solution was neutralized with 1 N HCl, and acidified with 0.9 ml of 50 per cent trichloroacetic acid (TCA). The solution was kept at five degrees overnight and the DNA and protein were removed by centrifugation. The supernatant solution, which contained RNA, was decanted and the pellet was washed twice with 0.9 ml of five per cent TCA at 70 degrees. These washings were combined with the supernatant liquid. The protein-DNA pellet was assayed for protein and radioactivity. All other fractions: carbon dioxide, used medium, TCA extract, lipid extract, and RNA hydrolysate were assayed for radioactivity.

Methods Used in Preparing Cell Extracts and in Isolating Mitochondria

Cells that were to be used for enzyme assays were usually exposed

to the experimental media for three days, from day four to day seven. Nutritional components were added to or deleted from the medium in order to determine the effect of the particular variable on the activity of certain enzymes. Many of the nutritional changes were expected to influence gluconeogenesis. For example, the effect of lower than usual (27.8 mM) glucose concentration or no glucose at all in the medium was examined. The effect of the absence of carbon dioxide and its buffering derivatives from the medium was tested by replacing bicarbonate with TES. In these experiments a carbon dioxide trap containing 1.5 ml of 20 per cent KOH was employed in order to remove all vestiges of carbon dioxide. The carbon dioxide trap was a glass vial, 60 x 17 mm, containing filter paper and cotton and glued to the inside of the bottle cap. The initial bicarbonate concentration in medium XXI-42 is 26.7 mM.

Certain adrenocorticoid hormones were added to the medium in order to observe their effects on the gluconeogenic enzymes. Hydrocortisone (HC) or deoxycorticosterone acetate (DOC) at 10^{-6} to 10^{-7} M were used in medium XXI-42. To prepare the 5×10^{-3} M steroid stock solutions, 18 mg of HC or 19.4 mg of DOC were dissolved in one ml of 95 per cent ethanol, and these solutions were made up to ten ml with water.

Cells were havested by adding 0.25 per cent trypsin in 0.85 per cent sodium chloride to the medium. One to two ml of trypsin was sufficient for a stationary bottle and 1.5 to 3.0 ml was enough for a wheel bottle. The cell layer was removed from the glass surface in two to five minutes by shaking at 25 degrees or 37 degrees. The suspension of cells was immediately transferred to an Erlenmeyer flask in an ice water bath. A one ml aliquot of the mixture was withdrawn for cell counting using a

hemocytometer. Another two ml was removed and the cells were spun down with a clinical centrifuge for later protein determination. This cell pellet was washed twice in cold 0.85 per cent sodium chloride. The remainder of the cell suspension was centrifuged for 15 minutes at 1000 rpm in an International refrigerated centrifuge, Model PR1. The supernatant liquor was discarded and the cells were resuspended in cold 0.85 per cent sodium chloride. The cells were washed twice in cold saline and then transferred to a Potter-Elvehjem homogenizer or to a plastic tube for freeze-thawing.

Freezing and thawing disrupted the mitochondria and consequently this technique was not used when mitochondrial systems were being studied. A small amount of water or 10^{-4} M Clelands Reagent in 10^{-3} M Tris at pH 7.2 was added to the cells. Quick freezing was done in an ethanol-dry ice mixture. Thawing was done by putting the tube in 25 degree water. Freezing and thawing were repeated four more times in order to disrupt the cells. The mixture was centrifuged at 1600 X gravity (4,200 rpm) for 20 minutes in a Spinco Model L centrifuge using a fixed angle 40 rotor. The supernatant medium was pipetted off and frozen for enzyme assays.

Intact mitochondria were prepared by homogenizing the cells in a chilled Potter-Elvehjem all-glass homogenizer for three to four minutes. The cells were homogenized in two to four volumes of cold 0.88 M sucrose at pH 7.2. The hypertonic sucrose method was adapted from Schneider and Hogeboom (250). The homogenate was centrifuged at 1600 X gravity (4,200 rpm) for 10 minutes in a Spinco Model L centrifuge using a fixed angle 40 rotor. The supernatant medium was pipetted off and saved. The sediment was resuspended with four ml of cold 0.88 M sucrose and spun again at

1600 X gravity for 10 minutes. The two supernatant fractions were combined and spun at 29,000 X gravity (21,000 rpm) for 20 minutes using the same 40 rotor. The supernatant fraction containing soluble enzymes was pipetted off and saved. The packed mitochondria were resuspended in nine ml of 0.88 M sucrose and spun again at 29,000 X gravity for 20 minutes. The supernatant fraction was pipetted off and discarded. The mitochondria were ready for enzymic analysis.

SEPARATION OF CHEMICAL COMPOUNDS

Ion Exchange Column Analyses

Ion exchange columns were used to fractionate the used growth medium, trichloroacetic acid extracts of the cells, and the enzyme assay reaction mixture. An anion exchange column was used to separate organic and amino acids. Five grams of Dowex-1-chloride resin (200 to 400 mesh, AG1-X8) was converted to the acetate form and poured into an 11 mm inner diameter column to a height of 75 mm. The column was assembled on a Gilson fraction collector and the sample was applied. The column was washed twice with three ml of water and then a linear acetic acid gradient elution was set up as follows: 3 N acetic acid flowed into a 250 ml flat bottom aspirator bottle containing 200 ml water and a stirring magnet. This was attached to the top of the resin column that had been overlaid with 7 ml of water. All connections were air tight. The flow rate was adjusted to 0.5 ml per min and approximately 5 ml fractions were collected. At tube number 32, the gradient system was removed and 6 N acetic acid was fed into the resin column. At tube number 42, 4 N HCl replaced the acetic acid elution system. The volume of each collected fraction was measured with a serological pipette. Samples of 0.1 ml from the ap-

propriate fractions were taken for radioactivity, amino acid, lactic acid, and keto acid assays.

The first fraction through the Dowex-1-acetate column contained neutral and basic components. This fraction was further resolved on a cation exchange column (Dowex-50 W (100-200 mesh, 8 per cent cross-linked) resin in the hydrogen form). Two water-jacketed columns were employed for this purpose. The longer of these had an inner diameter of 10 mm and contained 75 g of resin which filled it to a height of 107 cm. The shorter column had an inner diameter of 11 mm and contained 16 g of resin which filled it to 180 mm. After the sample was applied, the column was washed with three ml of water. Two and one-half ml of 1 N HCl was added to the resin and the column was attached to a reservoir containing 1 N HCl. At tube number 44 a gradient elution was started in which 4 N HCl flowed into a 250 ml mixing chamber containing 1 N HCl. The mixture was fed to the column and at the same time the water jacket was heated to fifty degrees. This cation exchange procedure was adapted from Moore et al. (111). Samples of 0.1 ml were taken from each tube for amino acid and radioactivity analyses.

The advantage of using acetic acid and hydrochloric acid as eluents for ion-exchange columns is that they are volatile and thus can easily be removed. Eluent fractions from the ion-exchange columns were transferred to small Erlenmeyer flasks and frozen in dry ice. The top was covered with gauze and the contents were lyophilized by attaching to a vacuum pump with a KOH and cold finger trap. The powder was taken up in small amounts of water, transferred to a small vial, and again lyophilized.

Paper Chromatographic Analyses

The dried fractions from the ion exchange column could be identified and further separated by paper chromatography. They were dissolved in 0.1 to 0.5 ml of water and spotted in duplicate on chromatography paper (Whatman No. 1, size 49 x 46 cm), along with known standards of amino acids and organic acids. The papers were spotted and developed by one-dimensional descending chromatography in each of three solvent systems (n-butanol: acetic acid: water (12:3:5) at 23 degrees for 10 hours, phenol at 23 degrees for 24 hours, and absolute ethanol: 28 per cent ammonia: water (16:1:3) at five degrees for 17 hours). The chromatograms were then dried, and radioautographs were made by exposing them to no-screen X-ray film for one to four weeks. After this, the strips were cut into sections which corresponded to the standards and counted for radioactivity in a liquid scintillation system. Organic acids were detected by spraying with bromcresol green (BCG) at a concentration of 1 mg per ml of 95 per cent ethanol. Contrast between the yellow spots and the background was accentuated by exposing the BCG-sprayed paper to dilute vapors of ammonia. Amino acids were located by spraying the paper with 0.5 per cent ninhydrin in butanol and heating until the spots appeared.

Amino Acid Analysis

Amino acids were also separated using a Beckman Model 120 C amino acid analyzer by the method of Benson et al. (15b). A Nuclear-Chicago Model 6770 liquid scintillation flow system was attached to the analyzer in order to detect radioactivity. Trichloroacetic extracts of the cells were extracted three times with five ml of ethyl ether to remove TCA and taken to dryness under vacuum in a desiccator containing KOH and P_2O_5.

The used medium was diluted in lithium citrate sample-dilutor buffer before applying to the amino acid analyzer column.

CHEMICAL IDENTIFICATION PROCEDURES

Lactic Acid Determination

Lactic acid was identified by paper chromatography and quantitated colorimetrically or enzymatically. The Barker-Summerson colorimetric test, as revised by Umbreit *et al.* (251), was used. For the enzymatic analysis a lactic dehydrogenase (LDH) kit from California Biochemical Corporation was used.

Keto-Acid Determination

Alpha-ketoglutarate was identified by paper chromatography and quantitated by the phenylhydrazone test (250). The alpha-ketoglutarate fraction from the Dowex-1-acetate column was purified by extraction as the undissociated acid for 48 hours with ethyl ether using a Kutscher-Steudel liquid-liquid extractor. Three ml of the redissolved ether extract, containing five to 35 micrograms of alpha-ketoglutarate, was used in the phenylhydrazine assay.

Pyruvic acid was assayed for by the phenylhydrazone test as above or by a shortened version of it. To a 1.5 ml sample containing up to 0.2 micromoles of pyruvate was added 0.5 ml of 2,4-dinitrophenylhydrazine (250 milligrams per liter of 3 N HCl). After incubating for 10 minutes at 37 degrees, one ml of 2.5 N NaOH was added and the solutions were mixed. The optical density was read ten to thirty minutes later at 510 millimicrons.

Glucose Determination

The glucose concentration was determined by the glucose oxidase-

peroxidase method using Glucostat reagent from Worthington Biochemical Corporation.

Glycogen Determination

Glycogen was assayed-for in L-cells that had been harvested and washed with 0.85 per cent sodium chloride. This method was adapted from Valentine (258). To 0.3 ml of cell pellet was added 0.3 ml of 60 per cent NaOH. The tubes were covered with glass marbles and put into a boiling water bath for 45 minutes. They were then chilled in an ice bath and 10 ml of 100 per cent ethanol was added. The supernatant liquid was decanted, leaving a white polysaccharide precipitate. The pellet was washed twice with five ml of 95 per cent ethanol and dissolved in 1.5 ml of warm water. Three ml of concentrated sulfuric acid containing 0.2 per cent anthrone (freshly made and kept in an ice bath) wad added slowly with mixing to the tubes in the ice bath. The tubes were boiled for 10 minutes and then cooled in an ice bath. The optical density was read at 620 millimicrons. A standard curve from six to sixty micrograms of glycogen was used.

Amino Acid Determination

Amino acids were determined by the ninhydrin assay procedure from Clark (41). A 0.1 ml sample of eluate from the Dowex column, from which the HCl or acetic acid had been removed by vacuum, was analyzed. A 0.6 ml aliquot of the ninhydrin reagent was added to the 0.1 ml sample and mixed well. The tubes were boiled for 20 minutes and then cooled quickly in a water bath. To each tube was added 3.2 ml of 50 per cent aqueous n-propanol with thorough mixing. The optical density was read at 570 millimicrons after 10 minutes. If proline was the amino acid being measured its optical density was read at 440 millimicrons.

Protein Determination

The procedure of Lowry et al.(154) was used to determine the amount of protein in a sample. Bovine serum albumin was used as the standard.

Carbon Dioxide Determination

Carbon dioxide concentration in a solution was determined with a Natelson Microgasometer, Model 600. Sodium carbonate, dried at 100 degrees and dissolved in carbon dioxide-free water, was used as the standard. Only 0.03 ml of sample was needed and it was acidified with 0.03 ml of 1 N lactic acid in a closed system. The pressure of the carbon dioxide that was released was measured. The pressure was again measured after absorbing the carbon dioxide in 0.03 ml of 3 N NaOH. From the change in pressure, the carbon dioxide content was calculated.

Assay For Avidin

Avidin was assayed for spectrophotometrically by its binding with methyl red (4'-dimethylaminoazobenzene-2-carboxylic acid) or HABA (2(4'-hydroxyazobenzene) benzoic acid). Free HABA had an absorption maximum at 348 millimicrons and avidin causes a decrease in the 348 millimicron band and a new absorption band to appear at 500 millimicrons (85). Avidin also causes new absorption maxima to appear at 548 and 577 millimicrons with methyl red. Optical density readings were taken at the different wave lengths with avidin and the dyes, separately and together, in order to obtain their maximum absorption.

Radioactivity Measurements

Radioactivity was detected or measured by radioautography using X-ray film and by a flow liquid scintillation spectrometer as mentioned

earlier. All other radioactivity measurements were done with a liquid scintillation spectrometer, Packard Model 314 E. Flat-spectrum settings were used with the spectrometer in order to minimize quenching (271). The radioactivity was determined using 15 ml of counting solution in 20 ml low potassium glass counting vials. The counting solution contained 10 ml of 0.3 per cent p-terphenyl and 0.005 per cent dimethyl-POPOP [1,4-bis(2-(4-methyl-5-phenyloxazolyl))-benzene] in toluene plus 4.3 ml of absolute ethanol, and 0.7 ml of 2-aminoethanol.

CHEMICAL DEGRADATION PROCEDURES

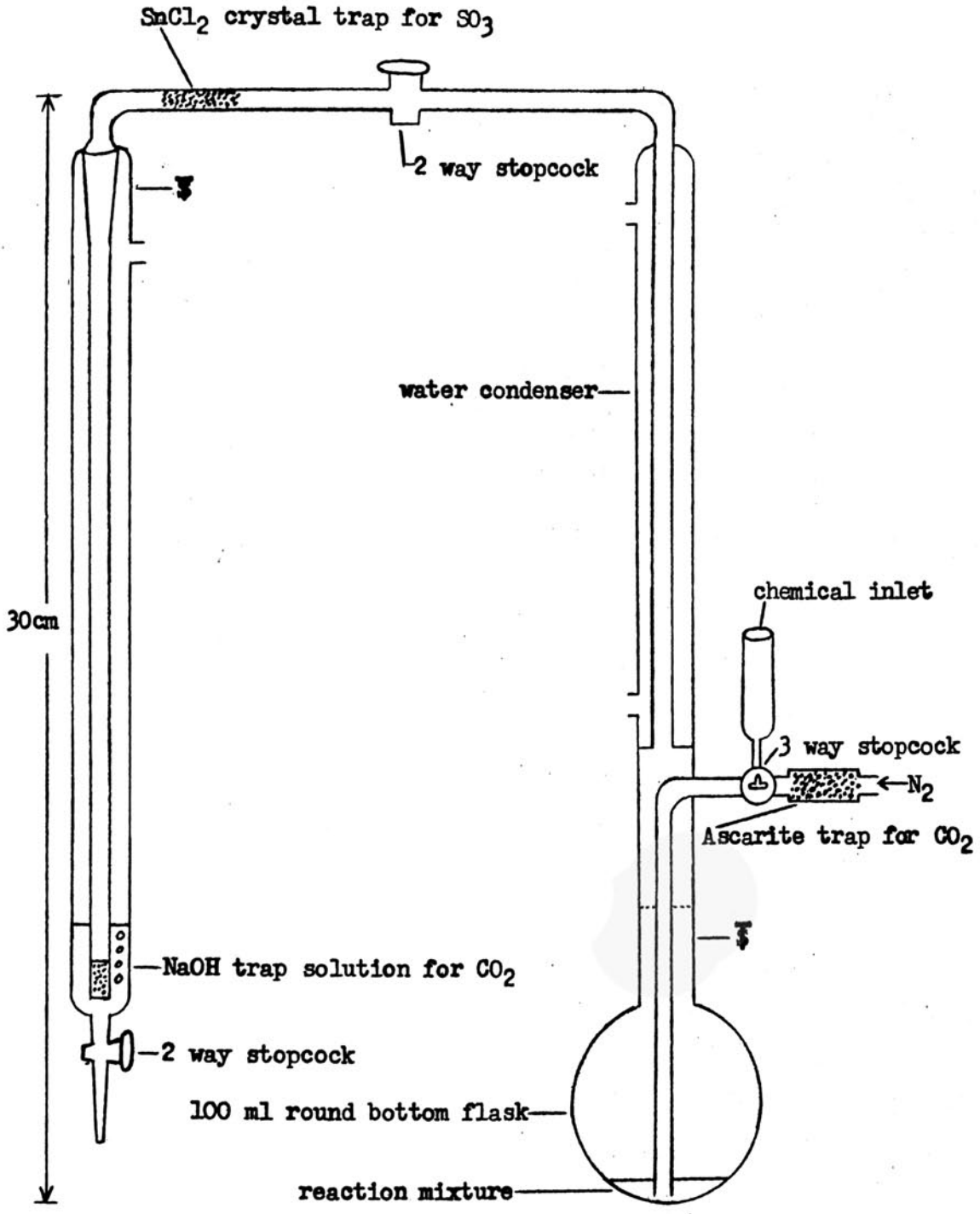
Decarboxylation of Lactate

The lactic acid fraction from the anion exchange column was acidified with five to ten ml of 5 N HCl. The fraction was extracted with ethyl ether for 48 hours using a Kutscher-Steudel continuous liquid-liquid extractor. After the ether was evaporated with an air stream the lactic acid residue was dissolved in dilute HCl and was steam distilled until 20 volumes of distillate was collected. The undistilled fraction containing lactic acid was titrated to pH 7 using 0.1 N NaOH.

Carbon-one of lactate was decarboxylated by a method similar to that of Aronoff (5). Lactate, totaling about 0.3 millimoles, was added to a 100 ml flask and taken to dryness under vacuum in a desiccator. The flask was chilled in ice and attached to the apparatus illustrated in Figure 1. Nitrogen (made carbon dioxide-free by passage through an Ascarite trap) was flushed through the system for 15 minutes. Fifteen ml of carbon dioxide-free 0.2 N NaOH was added quickly to the carbon dioxide trap. Then 0.45 mmoles of potassium dichromate in 10 ml of 9 N sulfuric acid was added to the reaction flask. The ice bath was removed and re-

FIGURE 1

DIAGRAM OF COMBUSTION APPARATUS



placed with a boiling water bath. The reaction vessel was heated for 20 minutes and the system flushed for another 30 minutes with carbon dioxide-free nitrogen. The NaOH trap was drained into a 25 ml volumetric flask and washed with carbon dioxide-free water. This was used for the formation of barium carbonate. The residue of the reaction mixture contained acetic acid and unreacted lactic acid. The two components were separated by steam distillation. The volatile acetic acid was titrated and assayed for radioactivity. The lactic acid which is not steam-distillable was titrated and assayed for radioactivity.

Formation of Barium Carbonate

The 25 ml of solution containing the NaOH-trapped carbon dioxide was quickly transferred to a large test tube and placed in a 90 degree water bath for five minutes. Five ml of 0.5 M ammonium chloride and barium chloride was added to the tube and to a tube used as blank that was filled with 25 ml of the NaOH trap solution. The tubes were cooled in ice water and the precipitate of barium carbonate was filtered onto a small millipore filter (25 mm with 0.45 micron pores). The paper had been previously heated under a heat lamp, dried in a desiccator, and accurately weighed. The precipitate was washed with carbon dioxide-free water. The paper and barium carbonate sample were thoroughly dried and weighed.

Barium Carbonate Conversion to Carbon Dioxide

The filter paper with barium carbonate was placed in a flask connected to a water-cooled condenser and a carbon dioxide trap containing 10 ml of absolute ethanol and five ml of 2-aminoethanol. Ten ml of 5 M perchloric acid was added to the flask and the system flushed for one hour with nitrogen. The 15 ml of trap solution was drained into a 25 ml volu-

metric flask and the trap washed with absolute ethanol. An aliquot was was measured for radioactivity. The specific radioactivity of the carbon dioxide was determined.

Complete Combustion of Lactate and Glutamate

Complete combustion of lactate and glutamate was done by persulfate oxidation using a method similar to that of Aronoff (5). Approximately 0.1 millimoles of lactate or 0.05 millimoles of glutamate was added to a 100 ml flask and taken to dryness under vacuum in a desiccator. Fifteen ml of carbon dioxide-free water was added and the flask chilled in ice. The solution was made acidic with a few drops of 18 N H_2SO_4, using a trace of Congo red as an indicator. The flask was attached to the apparatus illustrated in Figure 1. The system was swept with carbon dioxide-free nitrogen for 15 minutes with the flask still in ice. Fifteen ml of carbon dioxide-free 0.2 N NaOH was added to the carbon dioxide trap. After adding 250 mg of potassium peroxydisulfate 1.5 ml of 5 per cent silver nitrate, that was made up in carbon dioxide-free water, was added. The ice bath was removed and replaced with a 30 degree water bath, the temperature of which was raised to 70 degrees over 15 minutes. After 20 minutes the temperature was increased to 80 to 90 degrees and maintained there with occasional shaking of the flask until the solution cleared. After simmering for another 10 minutes the system was swept with carbon dioxide-free nitrogen for 30 minutes. The trap solution was drained into a 25 ml volumetric flask and the trap washed with carbon dioxide-free water. Barium carbonate was formed as explained earlier.

Carbon-One Decarboxylation of Glutamic Acid

The number one carbon of glutamic acid was removed either by

ninhydrin or chloramine-T. The chloramine-T method was adapted from Dakin (51). Chloramine-T (12 micromoles) and glutamic acid (four micromoles) were mixed together in 2.0 ml of solution in a 15 ml flask. This flask was attached to an apparatus with a water-cooled jacket and a carbon dioxide trap containing 10 ml of absolute ethanol and five ml of 2-aminoethanol. Nitrogen was flushed through the system for five minutes and the flask was heated to 50 degrees for 20 minutes while maintaining the nitrogen flow.

The ninhydrin reaction was similar to that described by Calvin et al. (23). Approximately 0.2 mmole of glutamic acid was added to a 100 ml flask and taken to dryness under vacuum in a desiccator. The flask was chilled in an ice bath and six ml of 0.15 M citrate buffer, pH 2.5, made from carbon dioxide-free water, was added. After the system was flushed with carbon dioxide-free nitrogen for 15 minutes, 15 ml of 0.2 NaOH (carbon dioxide-free) was added to the carbon dioxide trap. Ninhydrin (300 mg) was added and the flask placed in a boiling water bath for 10 minutes. It was shaken occasionally and flushed continuously with nitrogen until 30 minutes after the reaction had gone to completion. The trap solution was drained into a 25 ml volumetric flask and the trap washed with carbon dioxide-free water. The carbonate was then converted to its barium salt.

Carbon-Five Decarboxylation of Glutamic Acid

The Schmidt degradation, as adapted by Cutinelli et al. (49), removed carbon number five of glutamic acid. Twenty-five micromoles of glutamic acid was added to a 100 ml flask and vacuum evaporated to dryness. It was put into an ice bath and attached to an apparatus containing a water-cooled jacket and carbon dioxide trap. Nitrogen (carbon diox-

ide-free) was flushed through the system for 15 minutes. Fifteen ml of carbon dioxide-free 0.2 N NaOH was added to the trap. One ml of cold concentrated sulfuric acid and one ml of cold chloroform were added to the reaction vessel. After adding 5 mg of sodium azide, the reaction flask was heated at 48 degrees for three hours. Nitrogen flow was maintained during this period. The trap solution was drained into a 25 ml volumetric flask and barium carbonate was collected as described previously.

ENZYMATIC ASSAY METHODS

General Methods Used in Carbon Dioxide Fixation Experiments

The following procedure was used to assay for these carbon dioxide-fixing enzymes: pyruvate carboxylase, PEP-carboxykinase, PEP-carboxylase, PEP-carboxytransphosphorylase, malate enzyme, diphosphoribulose carboxylase, isocitrate dehydrogenase, and phosphogluconate dehydrogenase. All assays used the incorporation of radioactive bicarbonate into an acid-stable product as a measurement of enzyme activity. Reagents such as TES buffer, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, cysteine-HCl, reduced glutathione, pyruvate, alpha-ketoglutarate, and glutamate were kept frozen until needed. Because of their instability all other components were made freshly. In each case the reaction mixture was put together and its pH adjusted to 7.4. It was then distributed to 12 x 75 mm test tubes in an ice bath. A control vessel, lacking the main substrate, was used to correct for endogenous activity.

The soluble enzymes at 0.2 ml per tube or the mitochondrial suspension at 0.3 ml per tube were added to the reaction mixture and the tube was tightly covered with a rubber vial seal. The mitochondrial suspension

was 0.88 M with respect to sucrose, and after it was added to the reaction mixture the sucrose concentration was 0.26 M. The soluble enzymes were in solutions of 0.88 M sucrose, water, or 10^{-4} M Tris plus 10^{-5} M Cleland's reagent. In some cases the soluble enzymes had been dialyzed at five degrees against 10^{-5} M Cleland's reagent in 10^{-4} M Tris at pH 7.4 and then concentrated by lyophilization.

The reaction mixture (0.95 ml) was transferred from the ice bath to a 30-degree water-shaker bath. After five minutes 0.05 ml of a solution containing five microcuries of C-14 $NaHCO_3$ was injected using a Hamilton syringe through the vial seal into the reaction mixture. The vial seal was covered with stop-cock grease to obviate any pinhole leaks and the tube incubated at 30 degrees for 30 minutes with shaking. The radioactive bicarbonate had a specific activity between ten and fifty microcuries per micromole and the amount added was between 0.1 and 0.5 micromoles.

To end the reaction, 0.2 ml of 20 per cent trichloroacetic acid was injected into the reaction mixture. Nitrogen was bubbled through the acidified reaction mixture for five minutes and the unreacted carbon dioxide was trapped by bubbling it through five ml of 2-aminoethanol in ten ml of absolute ethanol. Then non-radioactive carbon dioxide was bubbled through the reaction mixture for 20 minutes, and nitrogen for another five minutes. The trapped carbon dioxide was drained into a 25 ml volumetric flask, and the trap was washed with absolute ethanol.

The reaction mixture was refrigerated overnight and then the protein was centrifuged down. Duplicate 0.1 ml aliquots were taken from the supernatant fraction and put into two counting vials. Fifteen ml of scin-

tillation counting solution was added to one vial. The other vial was taken to dryness overnight in an evacuated desiccator containing P_2O_5 and KOH. Water (0.1 ml) was added to the dried residue. After the residue was dissolved, 15 ml of scintillation counting solution was added to the vial. Both vials, along with a vial containing an aliquot from the trapped carbon dioxide, were counted for radioactivity in a liquid scintillation counter.

Since acid was used to stop the reaction the carbon dioxide fixation product would have to be acid-stable. Oxaloacetate is the product of many carbon dioxide-fixing reactions but it is very labile, even at neutral pH and room temperature (104, 198, 153, 255). Therefore, all the reactions that formed OAA were coupled with 10 units of malic dehydrogenase (MDH) and 2.5 micromoles of NADH to convert the OAA to an acid-stable product, malate. Following the oxidation of NADH spectrophotometrically was not useful as a gauge of the progress of the reaction because of interference by other NADH using enzymes present in the crude preparation.

Glutamate and glutamate-oxaloacetate transaminase were used in some cases to convert OAA into acid-stable aspartate. Attempts were made to stop the reaction by adding two mg of 2,4-dinitrophenylhydrazine in 0.4 ml of 6N HCl. The resulting OAA-hydrazone was extracted four times with two ml of ethyl-acetate and the extract taken to dryness under an air stream in a counting vial. After adding 0.1 ml of water and 15 ml of counting solution the radioactivity was determined using a scintillation counter. However, the hydrazone strongly quenched the scintillation process so that this method was not useful. Also, adding 0.5 mg of 2,4-dinitrophenylhydrazine in 0.1 ml (that had been dissolved in 6N HCl and

neutralized) to a PEP-CK assay system was ineffective.

The buffer TES was selected for use with nearly all enzyme reactions because it has a suitable pk_a of 7.5, does not participate in biological reactions, and does not precipitate cations (84).

The enzymes were not purified because of the difficulties in getting sufficiently large amounts of them from mammalian cells grown in tissue culture. Also, some of the enzymes that were assayed are very unstable. To guarantee that the reaction components had access across the mitochondrial membrane the activities of the normal mitochondrial preparations were compared with those of freeze-thawed mitochondria. Since no great differences in activities were seen between these two preparations it was concluded that the reaction components were entering the intact mitochondria. Furthermore, it was reported that PEP (174), pyruvate, ATP, and HCO_3^- (143) readily penetrate the mitochondria.

In some cases, 48 units of hexokinase and 10 micromoles of glucose were used to remove ATP, GTP, or ITP to see if they were activators or inhibitors of an enzyme. Inhibition by avidin was used to check for biotin enzymes. Avidin from Nutritional Biochemicals Corporation at 2150 units per gram was used. One mg of avidin was added to 0.2 ml of enzyme along with 0.1 ml of 0.85 per cent sodium chloride containing 0.5 mg of avidin. The enzyme was incubated with avidin at room temperature for at least 10 minutes before using.

Assay for Pyruvate Carboxylase

The general procedure listed earlier was followed. The following components were used: 10 micromoles each of sodium pyruvate, $MgCl_2$, ATP, and cysteine • HCl; acetyl-CoA, 0.2 micromoles; TES (pH 7.4) 100

micromoles; NADH, 2.5 micromoles; and MDH, 10 units. Since carbon dioxide incorporation was used instead of optical density measurement, there was no interference from lactic dehydrogenase. Endogenous acetyl-CoA carboxylase and carbamyl-phosphate synthetase activities were corrected for by using a pyruvate-free control.

Assay for Phosphoenolpyruvate Carboxykinase

The general procedure listed earlier was followed. The following components were used: 10 micromoles of PEP, reduced glutathione, and $MnCl_2$; IDP, five micromoles; TES (pH 7.4), 100 micromoles; NADH, 2.5 micromoles; and MDH, 10 units. Lactic dehydrogenase, although expected to be present in the crude extract, was sometimes added in order to trap endogenous pyruvate that might otherwise be used by pyruvate carboxylase.

Assay for Phosphoenolpyruvate Carboxylase

The general procedure presented earlier was used. The following components were added: PEP, 10 micromoles; $MgCl_2$, 10 micromoles; TES (pH 7.4), 100 micromoles; NADH, 2.5 micromoles; and MDH, 10 units.

Assay for Phosphoenolpyruvate Carboxytransphosphorylase

The general procedure presented earlier was used. The following components were used: PEP, 10 micromoles; $MgCl_2$, 10 micromoles; phosphate, 10 micromoles; TES (pH 7.4), 100 micromoles; NADH, 2.5 micromoles; and MDH, 10 units.

Assay for Malate Enzyme

The general procedure which was presented previously was used. The following components were added: pyruvate, 10 micromoles; $MnCl_2$, 10 micromoles; NADPH, one micromole; and TES (pH 7.4), 100 micromoles.

Assay for Ribulosediphosphate Carboxylase

The general procedure that was presented earlier was used. The following components were used: 10 micromoles of sodium ribose-5-phosphate, ATP, and $MnCl_2$; and TES (pH 7.4), 100 micromoles.

Assay for Isocitrate Dehydrogenase

The general procedure that was presented earlier was followed. The following components were used: alpha-ketoglutarate, 10 micromoles; $MnCl_2$, 10 micromoles; NADPH, one micromole; and TES, pH 7.4, 100 micromoles.

Assay for Phosphogluconate Dehydrogenase

The general procedure presented earlier was used. The following components were used: sodium ribose-5-phosphate, 10 micromoles; $MnCl_2$, 10 micromoles; NADPH, one micromole; and TES (pH 7.4), 100 micromoles.

Assay for Carbamyl Phosphate Synthetase

The procedure of Anderson and Meister (3) was used. The following components were mixed together to a volume of 0.95 ml per 12 x 75 mm test tube in an ice bath: potassium phosphate buffer (pH 7.6), 100 micromoles; $MgCl_2 \cdot 6H_2O$, 10 micromoles; N-acetylglutamate, five micromoles; glutamine, five micromoles; ammonium chloride, five micromoles; ATP, 10 micromoles; and 0.2 ml of enzyme. A blank with all the components, except ATP was also run. The test tube was capped with a rubber vial seal and it was incubated for five minutes at 30 degrees. The reaction was started by injecting 0.05 ml of radioactive bicarbonate into the tube with a syringe. After 30 minutes the reaction was stopped by injecting 0.1 ml of a freshly-made solution containing 0.7 N ammonium hydroxide and 2.7 N potassium hydroxide. The tube was incubated at 37 degrees for 10 minutes. This con-

verted carbamyl phosphate to cyanate. Cyanate was converted to urea by adding 0.4 ml of 4 M ammonium chloride (pH 8.5) and heating at 100 degrees for 10 minutes. Aliquots were put on a 9 mm column containing 6 ml of Dowex 1-x8 (200-400 mesh) in the hydroxide form. Radioactive urea was eluted with 10 ml of water, and the radioactive bicarbonate remained bound to the resin. Aliquots were counted for radioactivity in the liquid scintillation system.

Assay for Pyruvate Kinase

Attempts to assay for pyruvate kinase were made by coupling the reaction of PEP and ADP with glucose, hexokinase, NADP, and glucose-6-phosphate dehydrogenase and measuring the reduction of NADP at 340 millimicrons. The assay was not successful because of the high endogenous reductions of NADP by the crude enzyme solution.

Assay for Succinate Oxidase

This assay was used to check to see if the so-called mitochondrial fraction actually contained mitochondria. There were not enough mitochondria to measure oxygen changes with a Gilson oxygraph from a mixture of mitochondria, succinate, ADP, and inorganic phosphate. Electron micrographs of mitochondria stained by sodium phosphotungstic acid showed intact mitochondria. Succinate oxidase was assayed by combining 25 micromoles of Tris (pH 7.4), one micromole of sodium succinate, one micromole of triphenyltetrazolium chloride, enzyme, and a pinch of phenazine methosulfate in a volume of 0.05 ml. A blank contained everything except succinate. The tubes were put into a beaker in a desiccator containing a little water and a suction was pulled until bubbles appeared in the water. It was incubated at 37 degrees for one hour. Acetone (0.5 ml) was added

to each tube and the protein centrifuged down. The optical density of the formazan product was read at 480 millimicrons.

CHAPTER IV

RESULTS AND DISCUSSION

INCORPORATION OF CARBON DIOXIDE IN L-CELLS

Effects of Low Concentrations of Carbon Dioxide on the Growth of L-Cells

Bicarbonate is present at a high concentration in medium XXI-42 which was usually employed for the growth of L-cells in this study. It is included in the medium in high concentrations principally as a buffer, but if it is deleted in favor of another buffering system, the rate of cellular growth decreases. In order to determine whether carbon dioxide is an essential constituent of the growth medium, L-cells were exposed to bicarbonate-free media that were buffered with either Tris or TES. The characteristics of growth and C-14 bicarbonate incorporation in these two culture media were determined and compared to those in the normal medium.

L-cells were grown for four days in bicarbonate-rich medium and then refed with bicarbonate-free, Tris-buffered medium. During 66 hours exposure to the Tris medium these cultures grew only poorly, and ended up with 26 per cent less protein than those retained in the bicarbonate-buffered medium. Similarly, cultures that were refed with TES-buffered medium and incubated for 72 hours contained 19 per cent fewer cells than the normal cultures. The effects of carbon dioxide deficiency could be exaggerated by including a carbon dioxide trap in the culture vessel. Under these conditions the cultures that were refed with TES medium fared very poorly.

After 48 hours they contained 63 per cent as many cells as the bicarbonate-buffered control cultures and after 72 hours, 38 per cent. In fact, the cell population actually decreased relative to that determined at the time of refeeding. Even though bicarbonate was not intentionally added to the Tris- and TES- media, it was found to be present to a greater or lesser extent as a result of uptake of the gas from air during the process of media preparation and as a result of normal metabolic activities of the cells during the incubation period. A KOH trap was used to rid the culture system of the carbon dioxide that is derived from these two sources.

In contrast to cells that were grown in bicarbonate medium, cells that were incubated for two weeks in TES medium along with a KOH trap failed to grow and exhibited abnormal morphology. However, when the cells in the TES-buffered medium were refed with either 12 ml of bicarbonate medium or 12 ml of TES medium plus 2 ml of bicarbonate medium, the normal appearance of the cells was rapidly restored and growth resumed. From these results it is concluded that carbon dioxide is indeed necessary for cell survival and growth. The poor growth and other associated effects that are observed in the TES-buffered media must not be due to the toxicity of TES.

Distribution of Incorporated Carbon Dioxide in the Culture System

In order to determine the extent to which L-cells incorporate carbon dioxide, the cells were exposed to C-14 bicarbonate in the growth medium for various periods of time. The amounts of C-14 bicarbonate that were incorporated into metabolic products are shown in Table 1. Although these values appear to be small, the amounts actually incorporated must be somewhat greater. Some of the radioactive carbon dioxide that is fixed into organic products may be liberated again as carbon dioxide by decarboxyla-

tion of the labeled product. Furthermore, dilution of the radioactive carbon dioxide by its non-radioactive counterpart, derived from metabolic sources, would result in an apparent gradual decrease in the rate of carbon dioxide uptake.

Carbon dioxide incorporation by L-cells was studied in the bicarbonate-buffered and non-bicarbonate-buffered media. The initial concentration of bicarbonate in the normal medium is approximately 27 mM. Some carbon dioxide (1 to 2 mM) remained in the newly-made Tris medium even after it was gassed with nitrogen. This amount was close to that reported by Ruiz-Amil et al. (197). Since the added C-14 bicarbonate was less than 0.3 mM, the specific activity of the radioactive bicarbonate in the Tris medium was 12 to 20 fold as high as that in the bicarbonate medium. Surprisingly, the consumption of radioactive bicarbonate from the Tris medium was less than that from the bicarbonate medium (Table 1). The cells in bicarbonate-buffered medium appear to incorporate much more carbon dioxide than those in the Tris medium.

Under the conditions used in these experiments, and in certain others reported in the literature (131), L-cells were observed to be in the exponential growth phase during the day that followed refeeding (from day 4 to day 5) and to grow somewhat more slowly in the subsequent two days (from day 5 to day 7). The older, slower growing cells incorporate carbon dioxide at a rate only 1/5 to 1/10 that of the faster growing cells (Table 2). During growth the specific activity of the added radioactive bicarbonate decreased, due mainly to the production on non-radioactive bicarbonate from endogenous metabolic sources. However, since the normal medium contained a large amount of non-radioactive bicarbonate to begin with,

TABLE 1

CARBON DIOXIDE FIXATION BY NCTC CLONE 929 MOUSE CELLS (STRAIN L) DURING GROWTH IN CHEMICALLY DEFINED MEDIA

Period of Incubation	$^{14}CO_2$ Incorporated into Metabolic Products*	
	Tris Buffered	HCO_3^- Buffered
Hours	%	%
48	0.59	0.77
66	0.92	1.35
96	--	2.76

* The metabolic radioactive products included those found in the used growth medium, acid-soluble, and acid-insoluble fractions of the cells. Each cell monolayer contained approximately 8 mg. of protein.

TABLE 2

INCORPORATION OF C-14 BICARBONATE INTO METABOLIC PRODUCTS OF L-CELLS

Units of Incorporation	Exposure Time in Hours *		
	0-24	24-48	48-72
Moles per cell per hour x 10^{15}	12	2.2	1.1
Micromoles per g of cell protein per hour	26	5.4	2.8

* The cells were exposed to radioactive bicarbonate starting with day 4 of a 7-day growth cycle. Cells from day 4 to day 5 were in an exponential growth phase, while those from day 5 to day 7 were growing at a decreasing rate.

much more metabolic carbon dioxide had to be produced in this system than in the low-bicarbonate Tris-buffered medium to bring about a significant change in the specific activity of the bicarbonate. In other words, the specific radioactivity of the bicarbonate in the Tris-buffered medium would be expected to decrease more rapidly than that of the normal medium. Quantitative aspects of carbon dioxide fixation are made complex, even in these relatively simple experimental conditions, by progressively changing specific radioactivity of the bicarbonate in the medium, the effects of rapidly changing bicarbonate concentrations of the metabolic activities of the cells and the differential rates of growth of the cells in the low- and high-bicarbonate systems.

The incorporation of C-14 bicarbonate, as shown in Table 2, is more rapid during the first 24 hour period than during the next 24 hours. The rate of carbon dioxide fixation roughly parallels that of cell multiplication. It appears as though carbon dioxide fixation is either essential for rapid growth or is a result of it.

L-cells were exposed to radioactive bicarbonate in the bicarbonate- and Tris-buffered media for periods of time up to 96 hours, and the distribution of the products of carbon dioxide fixation between the medium and cells was determined as shown in Table 3. There was very little difference in the distribution of the isotope between the Tris- and bicarbonate-buffered cultures. Furthermore, the fraction of the incorporated radioactivity located in the acid-soluble portion of the cell remained at about 7 per cent from 48 to 96 hours of incubation. The acid-soluble extract contains only 1/7 to 1/9 as much radioactivity as is found in the medium. Since only a small amount of radioactivity was found to collect in the acid-soluble ex-

TABLE 3

THE METABOLIC FATE OF CARBON DIOXIDE INCORPORATED INTO L-CELLS DURING GROWTH IN CHEMICALLY DEFINED MEDIA

Growth Medium	Period of Incubation with $^{14}CO_2$	Distribution of Incorporated Carbon Dioxide Within the Culture		
		Used Medium	Cellular Constituents	
			Acid-soluble	Acid-insoluble
	Hours	%	%	%
Tris Buffered	48	69	7	24*
	66	53	8	39
HCO_3^- Buffered	48	65	7	28*
	66	56	8	36
	96	45	6	49

*The radioactivity that was incorporated from carbon dioxide into the acid-insoluble fraction of the cells was found to be distributed approximately evenly among the combined protein and DNA, the RNA, and lipid fractions of the 48-hour cultures in either Tris- or bicarbonate-buffered medium. The cells were fractionated by the Schmidt-Tannhauser method as described on page 55.

tract most of the products of carbon dioxide fixation must be readily incorporated into the acid insoluble cell constituents or must be transported to the nutrient environment.

The amount of radioactivity found in the used growth medium at 48 hours was large but it decreased with lengthened exposure time. In contrast to this, the radioactivity in the acid-insoluble fraction of the cells increased continuously with exposure time. After 48 hours incubation with radioactive bicarbonate the acid-insoluble part of the cells was fractionated by the Schmidt-Tannhauser method into the combined protein and DNA, RNA, and lipid fractions as described on page 55. Among these three fractions the radioactivity was found to be distributed approximately evenly.

The growth medium constitutes a large pool of free amino acids and other nutrients with which some of the radioactive products formed by the cell could equilibrate. Thus, within the cell, the products of carbon dioxide fixation would be diluted by their non-radioactive counterparts from these other sources. In the surrounding medium the specific activity of the radioactive metabolite would increase as long as the rate of carbon dioxide was maintained. However, as the rate of carbon dioxide fixation decreases and the pool of metabolites of the medium continues to feed the cells, the amount of radioactivity in the medium would be expected to decrease somewhat.

On the other hand many of the high molecular weight cellular constituents, such as proteins, lipids, and DNA are relatively stable and probably retain most of the radioactivity that has been incorporated into them. Therefore, it is reasonable to expect the extent of the incorpora-

tion of radioactive products into the cells to increase with increased exposure time.

Fractionation of the Used Growth Media

The used growth media were resolved into several radioactive fractions on an anion-exchange column, Dowex-1 acetate, by gradient elution with increasing concentrations of acetic acid. The radioactivity of each of the eluted fractions was measured and the composition of them was ascertained by paper chromatography in three solvent systems and radioautography. The keto acids, amino acids, and lactic acid were identified and/or quantitated by colorimetric tests.

Glutamic acid contains 33 per cent and aspartic acid 2 per cent of the radioactivity found in the used growth medium (Table 4). These percentages for the two amino acids are not affected by the length of incubation nor by the different buffers. There appear to be no great differences between radioactive products formed in either Tris or bicarbonate media (Tables 4 and 5) except, possibly, for the 66 hour lactic acid fraction (IV) and combined malic and succinic acid fractions (V).

The rates of production of radioactive lactic acid and total lactic acid follow anti-parallel patterns with increasing exposure time. Radioactive lactate acid (IV) is present in relatively high concentrations at 48 hours but in lower concentrations at 66 and 96 hours (Table 4). In these studies, as in those of others (82b), total lactate concentration was observed to gradually increase with increasing exposure time.

Substantially more radioactive alpha-ketoglutarate (VII) was found in the used medium after 66 or 96 hours incubation than after 48 hours (Table 4). These differences may be due to time-related changes in

TABLE 4

ANION-EXCHANGE FRACTIONATION OF THE USED GROWTH MEDIUM

Eluted Radioactive Fractions*	INCUBATION PERIOD AND CULTURE MEDIUM				
	48 hours		66 hours		96 hours
	Tris buffered	HCO_3^- buffered	Tris buffered	HCO_3^- buffered	HCO_3^- buffered
	%	%	%	%	%
I	18	18	23	22	27
II	32	34	35	30	33
III	2	2	3	2	2
IV	45	41	--	16	8
V	2	3	10	5	9
VI	--	--	--	2	2
VII	2	2	28	23	20

* The fractionation was carried out on Dowex-1 acetate by a linear gradient elution with increasing concentrations of acetic acid. Fraction I contains neutral and basic substances; II contains only glutamic acid; III contains only aspartic acid; IV contains only lactic acid; V contains malic and succinic acids; VI contains 5-carboxypyrrolidone; and VII contains mainly alpha-ketoglutaric acid.

the specific activity of the carbon dioxide or to altered metabolic rates associated with the successive intervals of incubation. For example, the younger cells might incorporate bicarbonate more rapidly, exchange certain cytoplasmic components with the medium more rapidly, or metabolize the product of carbon dioxide fixation more slowly than the older cells. If the cells release a radioactive product into the surrounding medium without reincorporating it, it will accumulate in the medium. In this regard, other authors have reported conflicting results, some finding no loss of alpha-keto acids by L-cells (227) and others observing leakiness of L-cells to keto acids (52).

If the newly-formed radioactive product does equilibrate with a large pool of unlabeled product, that radioactive product is more apt to be detected. The probability of it being metabolized further to other radioactive products or being decarboxylated to release the previously fixed C-14 as carbon dioxide would diminish. This would be the case if any of the newly-formed radioactive amino acids, e.g. glutamate or proline, diffuse across the cell membrane to become diluted by the large extracellular pool of unlabeled amino acids. The radioactive products which diffuse from the cell only slowly or equilibrate with a relatively small pool of non-radioactive compounds will probably be found in only small amounts in the medium. Examples of compounds that are not present in the growth medium and would probably be metabolized by the cell as soon as they are formed are citrate, OAA, malate, succinate, and fumarate. On the other hand, if a radioactive product leaks from the cells into the medium and the cells are unable to reincorporate it, the size of the growth medium pool would not affect its utilization.

Other acidic radioactive products that were detected in the growth medium are malic acid, succinic acid, and 5-carboxypyrrolidone. The first fraction to be eluted from the anion exchange column contained neutral and basic substances. The proportion of this fraction in the used growth medium increased from 18 per cent at 48 hours to 27 per cent at 96 hours (Table 4). This fraction was further resolved by cation-exchange chromatography on Dowex-50-H^+ by elution with increasing concentrations of HCl (Table 5). Radioactive proline comprised 40 to 52 per cent of fraction I, or 11 per cent of the total carbon dioxide products detected in the growth medium. Asparagine contained 33 to 40 per cent of the radioactivity in fraction I, or 8 to 11 per cent of the total radioactivity in the growth medium. A neutral fraction, the radioactive constituents of which were not identified, constituted 13 to 21 per cent of fraction I, or three to five per cent of the radioactive substances of the growth medium. The neutral fraction was ninhydrin negative. It is apparent that a large number of metabolites are formed by the cells, part of the substance of which is derived from carbon dioxide by way of carbon dioxide fixation reactions.

A series of analyses carried out on an amino acid analyzer essentially confirmed the above findings obtained through the use of Dowex-1 and Dowex-50 chromatography (Table 6). Traces of radioactive alanine and aspartate were found only after 72 hour exposure of the cells to the medium containing C-14 bicarbonate. Table 8 gives the specific activity of each radioactive amino acid in the growth medium. The specific radioactivity of asparagine appears quite high when compared to the other amino acids, probably because its concentration in the medium is low. The re-

TABLE 5

CATION-EXCHANGE RESOLUTION OF FRACTION I

Eluted Radioactive Fractions	Incubation period and culture medium		
	66 hours		96 hours
	Tris buffered	HCO_3^- buffered	HCO_3^- buffered
	%	%	%
(a)	21	13	20
(b)	33	36	40
(c)	46	52	40

The fractionation was carried out on Dowex-50 in the hydrogen form by elution with increasing concentrations of HCl. Fraction (a) is made up of neutral substances; (b) contains mainly asparagine; and (c) contains proline only.

TABLE 6

DISTRIBUTION OF THE RADIOACTIVITIES AMONG AMINO ACIDS IN THE GROWTH MEDIUM FOLLOWING EXPOSURE OF L-CELLS TO C-14 BICARBONATE

Radioactive Amino Acid	Per Cent of Total Radioactive Amino Acids* Exposure Time in Hours		
	24	48	72
Aspartate	--	--	2**
Asparagine	28	12	4
Glutamate	52	64	65
Proline	20	24	27
Alanine	--	--	2**

* Values were calculated from 2 experiments and each experiment was run in duplicate. The amino acids comprised 40 to 55 per cent of the total radioactive products of the used growth medium.

** The radioactive product was found in only one experiment.

TABLE 7

DISTRIBUTION OF THE RADIOACTIVITIES AMONG AMINO ACIDS IN THE TRICHLOROACETIC ACID EXTRACT FOLLOWING EXPOSURE OF L-CELLS TO C-14 BICARBONATE

Radioactive Amino Acid	Per Cent of Total Radioactive Amino Acids * Exposure Time in Hours		
	24	48	72
Aspartate	32	18	10
Asparagine	8	8	5
Glutamate	42	54	57
Proline	14	20	28
Unidentified Amino Acid**	4	--	--

* Values were calculated from 2 experiments and each experiment was run in duplicate. The amino acids comprised 26 to 36 per cent of the total radioactive products found in the trichloroacetic acid extract.

** The unidentified amino acid peak was close to that of citrulline.

TABLE 8

CHANGES IN THE SPECIFIC RADIOACTIVITIES OF AMINO ACIDS IN THE USED GROWTH MEDIUM FOLLOWING EXPOSURE OF L-CELLS TO C-14 BICARBONATE

Radioactive Amino Acid	cpm·mg cell protein^{-1}·micromole amino acid^{-1}* Exposure Time in Hours		
	24	48	72
Aspartate	--	--	138**
Asparagine	3477	3138	2435
Glutamate	341	431	509
Proline	337	330	421
Alanine	--	--	69**

* Values were calculated from 2 experiments, and each experiment was run in duplicate. Radioactivity for each amino acid was expressed with respect to its total concentration.

** The radioactive product was observed in only one experiment.

TABLE 9

CHANGES IN THE SPECIFIC RADIOACTIVITIES OF AMINO ACIDS IN THE TRICHLOROACETIC ACID EXTRACT FOLLOWING EXPOSURE OF L-CELLS TO C-14 BICARBONATE

Radioactive Amino Acid	cpm·mg cell protein^{-1}·micromole amino acid^{-1}* Exposure Time in Hours		
	24	48	72
Aspartate	2030	1202	1373
Asparagine	2638	1161	1079
Glutamate	935	920	1070
Proline	479	451	681
Unidentified Amino Acid	**	--	--

* Values were calculated from 2 experiments and each experiment was run in duplicate. Radioactivity for each amino acid was expressed with respect to its total concentration.

** The value for the micromoles in solution could not be determined, and the unidentified amino acid peak was close to that of citrulline.

sults from both Tables 6 and 8 indicate that the total and specific radioactivities increase for both glutamate and proline from 24 to 72 hours. The radioactivity of asparagine, unlike that of glutamate and proline, decreased after 24 hours.

Fractionation of the TCA Extract

Tables 7 and 9 record the distribution of radioactive amino acids in the trichloroacetic acid (TCA) extract of the cells. The most noticeable difference between the distribution of radioactive amino acids in the TCA extract and the growth medium is the high concentration of labeled aspartate in the extract. Radioactive alanine was found in only the growth medium.

The amount of radioactive asparagine in the growth medium at 24 hours is much higher than that in the TCA extract (compare Tables 6 and 7). The specific radioactivities of aspartate and asparagine in the TCA extract decreased rapidly from 24 to 48 hours and leveled off from 48 to 72 hours (Table 9). In contrast, the specific activities of glutamate and proline in the TCA extract remained constant from 24 to 48 hours and then increased in specific activity from 48 to 72 hours (Table 9).

The specific radioactivities of glutamate and proline in the cellular extracts were $1\frac{1}{2}$ to $2\frac{1}{2}$ times as high as those in the extracellular growth medium (Tables 8 and 9). The specific radioactivity of aspartate in the cells was 10 times as high as in the growth medium. On the other hand, the specific radioactivity of asparagine in the cell exceeded that of the growth medium by a factor of only $1\frac{1}{2}$ to 3. The fresh growth medium contains large amounts of glutamate, proline, and aspartate, but no asparagine. Even though proline and glutamate are available in the growth

medium, the cells continue to synthesize them. In fact the levels of both of these amino acids gradually increase while the level of aspartate decreases although slowly. Since the ratios of the specific radioactivities of glutamate, proline, and asparagine in the cell to those in the surrounding medium are close to one, there appears to be rapid exchange of these amino acids across the cell membrane. However, the much higher specific radioactivity of aspartate in the cell than in the medium indicates that very little radioactive aspartate leaves the cell. The property of L-cells to retain the aspartate which they have produced and to exchange other amino acids across the cell membrane was discovered earlier (131).

The ratios of the total amounts of free glutamate (45:1) and proline (25:1) in the medium to those in the cells stayed constant over a 3-day period. The ratio of free aspartate in the medium to that in the cells increased from 45:1 at 24 hours to 75:1 at 72 hours. In contrast the ratio of asparagine in the medium to that in the cells dropped from 26:1 at 24 hours to 7:1 at 48 and 72 hours. Aspartate levels decreased more slowly in the medium than in the cells. On the other hand, asparagine levels dropped faster in the medium than in the cells. These results, along with the radioactivity results (Tables 6-10), indicate that both asparagine and aspartate are rapidly formed from bicarbonate during the first 24 hours after feeding, but, of these two, only asparagine is quickly secreted into the medium. After the first 24 hours the cells rapidly consume the intracellular radioactive aspartate and the extracellular labeled asparagine.

The amino acids comprised 26 to 36 per cent of the total radioactive products in the TCA extract, and 40 to 55 per cent of the total radioactivity in the used growth medium. The lower percentage of radio-

active amino acid products and the higher percentage of radioactive non-amino acid products in the TCA extract than in the growth medium suggest that radioactive products other than the amino acids were excreted into the medium at a slower rate than amino acids. Also, most of the non-amino acid radioactive products, such as malate, citrate, succinate, OAA, and fumarate, would not undergo isotopic dilution in the medium because of the lack of pools of the compounds in the medium.

A TCA extract of cells that had been exposed to C-14 bicarbonate in normal medium for 48 hours was fractionated by anion exchange chromatography. The eluted C-14-labeled fractions were identified by correlating the radioactive peaks with chemically identifiable peaks corresponding to known components of the used growth medium (Table 4). The radioactivity of the TCA extract was found to be distributed as follows: neutral and basic substances, 20 per cent; glutamate, 13 per cent; aspartate, 9 per cent; lactate, 4 per cent; succinate and malate, 13 per cent; and a peak containing some alpha-ketoglutarate and mostly citrate, 40 per cent. The TCA extract contains proportionately much less radioactive glutamate and lactate than the growth medium (Table 4). On the other hand, the TCA extract contains proportionately more radioactive aspartate, succinate, malate, and citrate than the growth medium (Table 4). It appears that the cell does not lose radioactive aspartate and Krebs cycle intermediates other than alpha-ketoglutarate to the medium.

Degradation of Lactate and Glutamate

In order to shed some light on which pathways of carbon dioxide fixation operate in L-cells, two radioactive products of the fixation process, lactate and glutamate, were isolated from the medium, purified,

and degraded by chemical means. The specific radioactivity of each of these two compounds was determined by complete combustion. In parallel experiments carbon-one was removed by decarboxylation and the specific radioactivity of the carbon dioxide thus formed was determined. The fraction of the radioactivity of the product that was found in carbon-one was calculated from the relative specific radioactivities. As presented in Table 10 all of the radioactivity of glutamate was found to be located in carbon-one. About 90 per cent of the radioactivity of lactate was found in carbon-one. There were no distinct differences in the distribution of the radioactivity in either lactate or glutamate between Tris- and bicarbonate-buffered cultures.

From the distribution of the radioactivity in the two compounds some information on the possible pathways of carbon dioxide fixation in the L-cells was obtained. This is depicted in Figure 2. In step (1) pyruvate is carboxylated to malic acid by the malic enzyme or to OAA by pyruvate carboxylase. OAA can also be formed from the carboxylation of PEP through the agency of PEP-CK, PEP carboxylase, or PEP carboxytransphosphorylase. All of these fixation reactions would form 4-carbon dicarboxylic acids labeled in position-4. In order to explain the occurrence of carbon-1-labeled lactate one would postulate its derivation from carbon-1-labeled OAA via pyruvate (step (3)).

Carbon-4-labeled malate or OAA can end up with half the isotope in carbon-1 by rapid equilibration with a symmetrical molecule such as fumarate or succinate as indicated in step (2). Since the lactate that was formed in these experiments had 90 per cent of its radioactivity in carbon-1, there must have been rapid isotope exchange between the 4-carbon

TABLE 10

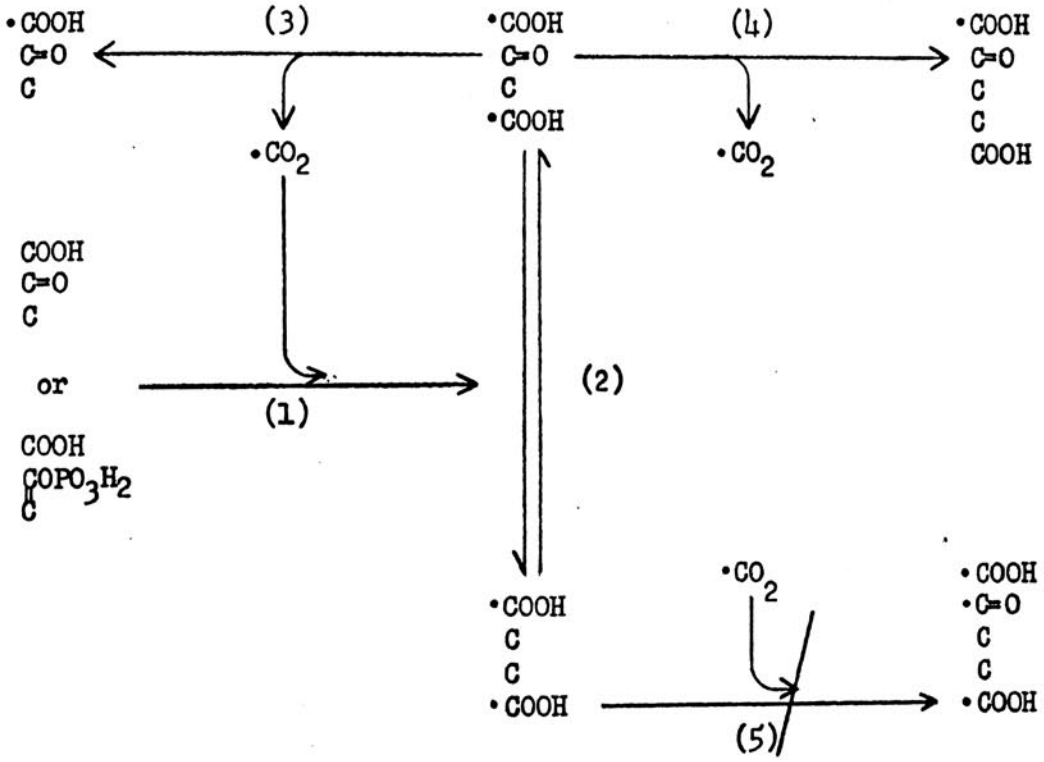
PARTIAL CHEMICAL DEGRADATION OF LACTATE AND GLUTAMATE FORMED FROM THE PRODUCTS OF $^{14}CO_2$ FIXATION

Product	Distribution of Carbon-14		
	48 hr cultures		96 hr cultures
	Tris Buffered	HCO_3^- Buffered	HCO_3^- Buffered
	%	%	%
COOH HCOH CH_3	91 9	86 14	87 13
Lactate*			
COOH H_2NCH CH_2 CH_2 COOH	102 0	105 0	
Glutamate			

* The values for lactate were determined from duplicate experiments.

FIGURE 2

CARBON DIOXIDE FIXATION IN L-CELLS



dicarboxylic acid and a symmetrical intermediate which effectively randomized the position of the label between carbon-one and four.

Studies in rats (254, 11) and Chlorella pyrenoidosa (109) have shown that four-carbon dicarboxylic acids, formed from C-14 bicarbonate, do equilibrate through a symmetrical intermediate. After being exposed to C-14 bicarbonate, JAI sarcoma (162) and rabbit nerve cells (262) synthesized aspartate and asparagine, equally labeled in carbon-1 and 4. Similarly, in rats (104, 270) C-14 bicarbonate was found to be incorporated equally into carbon-1 and 4 of malate. The activities of malate dehydrogenase and fumarase in the kidney and liver of rat are more than 100 times as great as those of the malate enzyme, pyruvic carboxylase and PEP-CK (137).

Evidence from the radioactivity pattern of glutamate, in which carbon-1 is labeled exclusively, demonstrates that alpha-ketoglutarate may have been formed by way of pathway 4 (Figure 2). Transamination or reductive amination would be responsible for the conversion of alpha-ketoglutarate to glutamate. Pathway-four involves several steps in the Krebs cycle including the condensation of OAA with acetyl-CoA to form citrate, the isomerization of citrate to isocitrate, and the decarboxylation of isocitrate to form alpha-ketoglutarate. In this process carbon-4 of OAA ends up as carbon-1 of alpha-ketoglutarate. Thus 4-^{14}C-OAA would not have to randomize through fumarate in order to form radioactive alpha-ketoglutarate or glutamate.

A carboxylation reaction that is ruled out by the exclusive location of the label at carbon-1 of glutamate is step (5) in Figure 2. If the enzyme that would catalyze this carboxylation reaction, alpha-ketoglu-

tarate synthetase, were present and functioning in the L-cells, the radioactivity from bicarbonate would end up in positions 1, 2, and 5 of alpha-ketoglutarate and glutamate. The actual labeling pattern eliminates also the possible reversal of the succinic acid thiokinase catalyzed reaction in the Krebs cycle.

Another carbon dioxide fixation reaction that would eventually result in the labeling of carbon-1 of lactate would be the carboxylation of alpha-ketoglutarate through the agency of isocitrate dehydrogenase (reversal of step (4), Figure 2). This reaction sequence would produce citrate which could be cleaved to yield OAA with the label in carbon-1. Lactate labeled in carbon-1 could then be formed from the OAA. In addition the carboxylation of ribulose-1, 5-diphosphate by ribulosediphosphate carboxylase could result in the labeling of carbon-1 of 3-phosphoglycerate. This latter compound would be converted by glycolysis to lactate labeled in carbon-1.

About 10 per cent of the radioactivity in lactate was not found in carbon-1 (Table 10). A possible carbon dioxide fixation reaction that would lead to the accumulation of a small amount of radioactivity in carbon-3 of lactate is the carboxylation of ribulose-5-phosphate by phosphogluconate dehydrogenase. The product, 6-phosphogluconate, could be reduced to glucose 6-phosphate which would be labeled in carbon-1. After proceeding through glycolysis the label would end up in carbon-3 of lactate. However, pyruvate would also be labeled in carbon-3 by this sequence and acetyl-CoA formed from pyruvate would be labeled in position two. Through the Krebs cycle the acetyl-CoA would result in the labeling of OAA in carbon two and three. If none of the Krebs cycle intermediates were drained off,

subsequent revolutions of the cycle would result in loss of all the isotope as carbon dioxide.

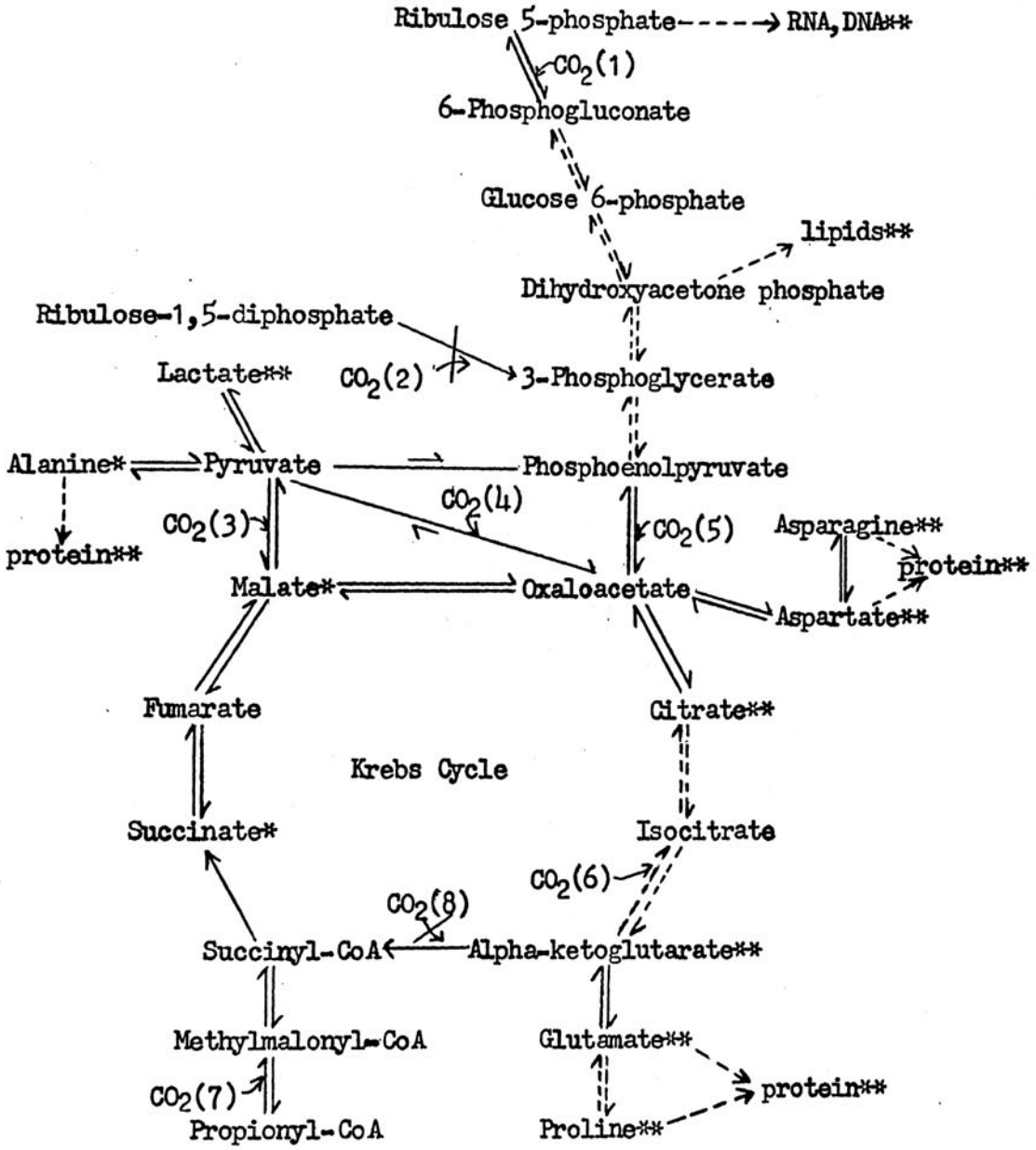
The 4-carbon dicarboxylic acids of the Krebs cycle could become labeled in carbons 1 and 4 through the carboxylation of propionyl-CoA by propionyl-CoA carboxylase and subsequent conversion of the resulting intermediate, methylmalonyl-CoA, to succinyl-CoA.

Some of the metabolic pathways involving the many radioactive products derived from C-14 bicarbonate are shown in Figure 3. Not shown in the figure is the formation of carbamyl phosphate by carbamyl phosphate synthetase. Carbamyl phosphate could be utilized in the Krebs-Henseleit cycle to form ornithine, citrulline, and urea and in another pathway to form pyrimidines. Also absent from the figure is the carboxylation reaction utilizing aminoimidazole ribonucleotide carboxylase in the de novo synthesis of purine nucleotides. In the fractionation of cells, some radioactivity from bicarbonate was found in the DNA and RNA fractions (Table 3). This would imply that either or both of the previously listed carbon dioxide fixation enzymes needed for the formation of purines and pyrimidines are functioning. The deoxyribose and ribose portions of the nucleic acids could become labeled by carbon dioxide fixation reactions. If gluconeogenesis accounts for a significant amount of hexose synthesis, carbon dioxide fixation would result in labeling of the deoxyribose and ribose portions of the nucleic acids. However, under the conditions of these experiments the probability of this happening is not good.

The radioactivity that was found in the lipid-extractable part of the cell may be derived from alpha-glycerophosphate. This compound is formed from dihydroxyacetone phosphate, which could ultimately be labeled

FIGURE 3

PATHWAYS OF CARBON DIOXIDE FIXATION IN L-CELLS



* indicates a minor isolatedradioactive product
** indicates a major isolated radioactive product
Dotted lines denote more than one reaction is involved in the pathway between two compounds. Pathways of carbon dioxide fixation not shown involve the following enzymes: acetyl-CoA carboxylase, carbamyl phosphate synthetase, and aminoimidazole ribonucleotide carboxylase.

by almost all of the carboxylation reactions shown in Figure 3. Another potential source of lipid radioactivity could come from acetyl-CoA which had been formed from pyruvate labeled in either position 2 or 3. A carbon dioxide fixation reaction that could result in the label in carbon-3 of pyruvate is carboxylation of D-ribulose 5-phosphate as explained earlier in step (1) in Figure 3. Carbon dioxide fixed by acetyl-CoA carboxylase into malonyl-CoA does not appear in the fatty acid chain because it is decarboxylated during the condensation process.

ENZYME CHARACTERISTICS

Assays for Enzymes

Although the labeling patterns of certain metabolic products that were synthesized by the L-cells during exposure to C-14 bicarbonate provide suggestive evidence concerning the pathways of carbon dioxide fixation which are functioning in the cells, further evidence was needed to verify which of these enzymes was actually present. Qualitative and quantitative evidence was sought for the presence of the following carbon dioxide fixing enzymes: malic enzyme, pyruvate carboxylase, PEP-CK, PEP-carboxylase, PEP-carboxytransphosphorylase, carbamylphosphate synthetase, phosphogluconate dehydrogenase, ribulosediphosphate carboxylase, and isocitrate dehydrogenase.

Assays were not run for pyruvate synthetase, alpha-ketoglutarate synthetase, propionyl-CoA carboxylase, or aminoimidazole ribonucleotide carboxylase. As shown from the isotopic labeling experiments that were discussed previously, alpha-ketoglutarate synthetase does not appear to be active or present in L-cells. Both alpha-ketoglutarate synthetase and pyruvate synthetase have been found only in anaerobic bacteria (21, 62).

Isotopic labeling showed that aminoimidazole ribonucleotide carboxylase is probably present and active in L-cells. This is expected in L-cells because of their ability to synthesize their entire purine complement and because of its known importance in the de novo synthesis of purines in other animal cells (281). Labeling experiments indicated that propionyl-CoA carboxylase could be functioning in L-cells. It is known to occur in animal cells (179). Acetyl-CoA carboxylase was also not sought, but because of its importance in fatty acid synthesis, it is probably active in L-cells.

The endogenous carbon dioxide content of the reaction mixtures was determined by the Natelson Microgasometer to be 2 mM, and this value was used in determining the specific activity of the C-14 carbon dioxide of all reaction mixtures. The values obtained in all enzyme assays were corrected for endogenous activity by subtracting the activity shown by the substrate-free control.

Isolation of Mitochondria

Intact mitochondria were isolated from the cells as explained in Chapter III. The integrity of the isolated mitochondria was checked with an electron microscope. Electron micrographs showed the mitochondria to be whole and to have diameters of approximately 0.78 microns. To further verify that the fraction contained mitochondria, succinic oxidase activity of it was assayed. Succinic oxidase activity is usually associated with the mitochondrial part of the cell. The specific activity of succinic oxidase in the mitochondria was 10 times as high as that of the supernatant fraction. There was little difference in succinic oxidase activity between frozen and non-frozen (intact) mitochondria.

To guarantee that the reaction components had access across the mitochondrial membrane the activities of the normal mitochondrial preparations were compared with those of freeze-thawed mitochondria. Since appreciable differences were not seen in the activities of malic enzyme, pyruvate carboxylase, or PEP-CK between these two kinds of preparations it was concluded that the reaction components were able to enter the intact mitochondria. Furthermore, it was reported that PEP (174), pyruvate, ATP, and bicarbonate (143) readily penetrate the mitochondria.

Extraction of Cell Protein

The protein concentration of seven-day-old L-cells averaged 2 x 10^{-10} g/cell and varied only slightly when different culture media were used. Between 24 and 33 per cent of the cell protein was extracted into the combined supernatant fraction and mitochondria that were used for enzyme assays. The protein content of the cytosol was about 10 times as high as that of the mitochondria.

Characteristics of Pyruvate Carboxylase in the Crude Cell Homogenate

Carbon dioxide fixation by pyruvate carboxylase is indicated as step (4) in Figure 3. The assay reaction for pyruvic carboxylase was run for 30 minutes as described on page 72. The reaction velocity was found to be constant over a two hour period.

No activity was seen if the enzyme had been boiled for 10 minutes before the assay, if the reaction medium was made acidic with trichloroacetic acid, or if no enzyme was used. Thus, the activity that was observed in this system must have been due to enzymatic catalysis. The pH optimum of pyruvic carboxylase was found to be 6.9, the activity at pH 7.4 being about 70 per cent of that at pH 6.9. No activity was found

above pH 8.2.

The substrate and cofactor requirements were determined by deleting components from the complete reaction mixture and measuring the activity of the dialyzed enzyme. The data are presented in Table 11. Pyruvate, Mg^{+2}, and ATP are all essential. In order to be certain that all endogenous ATP had been eliminated hexokinase and glucose were added to the assay system from which ATP was deleted. Since leaving out ATP resulted in very low activity, it is reasonable to conclude that there was little interference from the malic enzyme. Likewise, since the omission of pyruvate resulted in low activity, there could have been little interference from carbamyl phosphate synthetase and acetyl-CoA carboxylase in the fixation of radioactive bicarbonate. All pyruvate carboxylase assays were corrected for substrate-free endogenous activity.

Surprisingly, pyruvate carboxylase activity doubled when acetyl-CoA was not present (Table 11). All pyruvate carboxylase preparations purified from mammals and birds are essentially inactive in the absence of acetyl-CoA (147, 210). However, it was noted by Scrutton and Mildvan in 1968 (208) that the addition of 0.2 mM acetyl-CoA decreased the rate of decarboxylation of OAA by pyruvate carboxylase by almost 30 per cent. The enzyme from Aspergillus niger shows maximum activity in the absence of acyl-CoA (18). The crude enzyme preparation would be expected to contain acetyl-CoA carboxylase which could form malonyl-CoA from acetyl-CoA. Malonyl-CoA is a potent inhibitor of pyruvate carboxylase from rat kidney mitochondria (165) and chicken liver (210). Because citrate synthetase would probably be present in the enzyme preparation the product of the pyruvate carboxylase reaction, OAA, could combine with acetyl-CoA to form

TABLE 11

SUBSTRATE AND COFACTOR REQUIREMENTS FOR PYRUVATE CARBOXYLASE ACTIVITY

Assay System *	N**	% of Control Activity ***
- Pyruvate	>80	<5
- Acetyl-CoA	4	201
- Mg^{+2}	2	0
- ATP	2	37
- ATP + 10 mM Glucose + HK	5	5

HK = 48 units of hexokinase

*The complete assay system contained 10 mM sodium pyruvate, 10 mM $MgCl_2$, 10 mM ATP, 10 mM cysteine·HCl, 0.2 mM acetyl-CoA, 2.5 mM NADH, 0.1 M TES (pH 7.4), 10 units of MDH, 5 microcuries of C-14 $NaHCO_3$ (2mM), and cytosol dialyzed enzyme (1mg).

**N = number of experiments

***Each value was corrected for endogenous activity without added pyruvate.

citrate. Radioactive citrate was found in the used reaction mixture as will be mentioned later. Citrate could be decarboxylated rapidly through the Krebs cycle thus losing the radioactive carbon dioxide that was fixed. Also, fluorocitrate strongly inhibited pyruvate carboxylase in rat kidney mitochondria through its stimulation of acetyl-CoA carboxylase to form malonyl-CoA (210). Therefore, acetyl-CoA might be inhibiting the pyruvate carboxylase reaction for the above described reasons. Furthermore, it might not be a necessary cofactor for the enzyme from L-cells.

When the coupling system of malate dehydrogenase (MDH) and NADH was replaced with glutamate-oxaloacetate transaminase and 10 mM glutamate, pyruvate carboxylase activity was found to be only 6 to 50 per cent of that with MDH and NADH. Since glutamate addition to the MDH and NADH system resulted in little change in activity, glutamate, itself, was not inhibitory. The product of the transaminase reaction, alpha-ketoglutarate, has been reported to inhibit pyruvate carboxylase from sheep kidney (157) and rat liver (98). Perhaps, pyruvate carboxylase from L-cells is inhibited by alpha-ketoglutarate which is formed from glutamate when glutamate-oxaloacetate transaminase is present.

The carbon dioxide normally present in the reaction system was about 2 mM. When more bicarbonate was added, the incorporation of carbon dioxide was observed to increase. Thus it appears that the Km value for bicarbonate is greater than 2 mM. As mentioned in Chapter II, Km values reported for bicarbonate ranged from 0.62 to 2.7 x 10^{-3} M.

Analyses of the used reaction mixtures by anion exchange and paper partition chromatography showed the main radioactive products to be citrate, alpha-ketoglutarate, and a little OAA. If pyruvate carboxylase

were the only enzyme present, OAA would be the only radioactive product. Malate is expected to accumulate because NADH and MDH were added. However, the enzyme preparation that was used probably contains citrate synthetase and many other enzymes. Thus the newly-formed OAA might readily condense with acetyl-CoA, which is present in the assay system, to form citrate. With a crude enzyme system from chicken liver (253) or rat liver (10, 71) the main product in the assay for pyruvate carboxylase was citrate.

Preincubation of the enzyme with a commercial preparation of avidin from Nutritional Biochemical Corporation did not result in any change in its activity. If an enzyme contains biotin it is usually inhibited strongly by binding of avidin to the biotin (155). The avidin that was used in these studies was assayed by two accepted procedures and found not to be active (85). The assay, in which avidin is supposed to form new absorption maxima with two dyes, was described in Chapter III. A recent paper (274) reported that E.coli carbamyl phosphate synthetase was not inhibited by avidin from Nutritional Biochemicals Corporation but was inhibited by avidin from two other sources. Consequently, the results obtained from the avidin inhibition studies do not purport to show that pyruvate carboxylase from L-cells does not contain biotin.

Avidin inhibits pyruvate carboxylase from chicken liver (127) and yeast (197), and the enzyme from all sources is considered to contain biotin (208, 211). The crude enzyme preparation might contain enzymes that are capable of releasing bound avidin (a protein) from the biotin enzyme. Several of the commonly occuring nucleoside polyphosphates (CTP, GTP, UTP, ITP, TTP, and ATP) protected pyruvate carboxylase against

inactivation by avidin (200). Therefore, the avidin that was used could have been inactive or, if active, could have been hydrolyzed from the enzyme. The enzyme could have been protected from avidin inhibition by other components of the system. Alternatively, the enzyme might not even contain biotin.

Cell extracts that had been frozen for two months retained only 40 per cent of the initial pyruvate carboxylase activity. Pyruvate carboxylase from other sources is even more unstable (197, 46). Variations in the pyruvate carboxylase activity that result from the exposure of L-cells to nutritional changes and hormonal additions will be presented later.

Pyruvate carboxylase was found to be located almost exclusively in the cytosol fraction of L-cells. Table 14 shows that 99 per cent of its activity was found in the soluble part of the cell, and only one per cent in the mitochondria. Also, the relative distribution of the enzyme between the two subcellular fractions varies only slightly under different experimental conditions (Tables 14 and 18). This observation is in direct contrast to those of others, as mentioned in Chapter II, who reported that the enzyme from many sources is located chiefly in the mitochondria (127). But 40 to 70 per cent of the rat liver enzyme, which supposedly is found mostly in mitochondria and nuclei, was found in the post mitochondrial soluble fraction of cytoplasm (107, 209, 216). It was pointed out that pyruvate carboxylase might not normally occur in the mitochondria. It could simply be adsorbed by nuclear (216) or outer mitochondrial membranes (209).

Characteristics of Phosphoenolpyruvate-Carboxykinase in the Crude Cell Homogenate

Carbon dioxide fixation with PEP-CK is shown as step (5) in Figure 3. The usual assay reaction for phosphoenolpyruvate-carboxykinase (PEP-CK) was allowed to run for 30 minutes as described on page 73. The reaction velocity was found to be constant over a two-hour-period.

No PEP-CK activity was detected if the enzyme had been previously boiled for 10 minutes, if the reaction medium was made acidic with trichloroacetic acid, or if no enzyme was used. Consequently, it is reasonable to infer that the activity in these assays for PEP-CK was due to catalysis by the intact enzyme.

In several experiments ADP or GDP was substituted for IDP without significant change in the PEP-CK activity (Table 12). Thus, it seems as though the enzyme is able to use any one of the three nucleotide diphosphates in carboxylating PEP. A nucleotide diphosphate is needed for the reaction. Deletion of IDP resulted in a decrease in PEP-CK activity to 20 per cent of the control (Table 12). It is unusual for all three of these nucleotide diphosphates to be active with PEP-CK from one source. Adenosine polyphosphate was the only active nucleotide for PEP-CK of yeast (24, 25, 281) and a ciliated protozoan (218, 220). Guanosine and inosine nucleotides are active with animal PEP-CK (281) such as that from chicken liver (139), guinea pig liver (112), and pig liver (32). Since most of these studies were conducted on purified enzymes the results reported on their nucleotide requirements are probably more definitive than those on the crude extracts used here.

The crude enzyme preparation would be expected to contain pyru-

TABLE 12

SUBSTRATE AND COFACTOR REQUIREMENTS FOR PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY

Assay System*	N**	% of Control Activity***
- PEP	>80	<1
- IDP	4	20
- IDP + 10 mM GDP	4	85
- IDP + 10 mM ADP	4	110
- Mn^{+2}	2	8
- Mn^{+2} + 10 mM Mg^{+2}	2	105

*The complete assay system contained 10 mM PEP, 10 mM glutathione, 10 mM $MnCl_2$, 5 mM IDP, 2.5 mM NADH, 10 units of MDH, 0.1 M TES (pH 7.4), 5 microcuries of C-14 $NaHCO_3$ (2mM), and dialyzed cytosol enzyme (1 mg).

**N = number of experiments

***Each tube was corrected for endogenous activity without added PEP present.

vate kinase, which could catalyze the formation of pyruvate and ATP from PEP and ADP. Pyruvate carboxylase could then fix carbon dioxide with the newly-formed pyruvate and ATP. Attempts were made to assay for pyruvate kinase, but, as described in Chapter III, there was too much interference from other enzymes for a successful assay. Lactate dehydrogenase activity was measured in the enzyme preparation. Pyruvate that would be formed from the added PEP by way of the pyruvate kinase reaction could be converted to lactate by LDH and endogenous NADH.

PEP-CK activity doubled when kexokinase and glucose were added to the assay system. Together, the hexokinase and glucose trap endogenous ATP as well as ATP which is formed from the added ADP by way of pyruvate kinase. Utter and Kurahashi (256) also found that the synthesis of OAA from PEP, which is catalyzed by PEP-CK from chicken liver in the presence of ADP or IDP was doubled by adding hexokinase and glucose. Furthermore, no PEP-CK activity was detected when 10 mM ATP was added to the assay system that also contained ADP. From these observations ATP appears to inhibit PEP-CK activity. PEP-CK from a photosynthetic bacterium was also inhibited by ATP (249). Since ATP did not increase PEP-CK activity but completely inhibited it, very little of the measured PEP-CK activity could have been due to carboxylation of pyruvate by pyruvate carboxylase. Also, the pyruvate kinase reaction requires ATP, whereas IDP was used most of the time for assaying PEP-CK activity.

An absolute requirement of PEP-CK for a divalent metal ion is revealed in Table 12. Either Mn^{+2} or Mg^{+2} will work. PEP-CK from pig liver (32), yeast (24), and liver fluke (190) is much more active with Mn^{+2} than Mg^{+2}. With the enzyme from rat adipose tissue Mg^{+2} would not

substitute for Mn^{+2} (11). Mitochondrial PEP-CK from guinea pig liver was more active with Mn^{+2} than Mg^{+2} at pH 7.4 and more active with Mg^{+2} than Mn^{+2} at pH 8.0 (112).

Phosphoenolpyruvate is essential for PEP-CK activity. Table 12 shows that without PEP the activity of PEP-CK was only one per cent of the activity when it was present. All PEP-CK assays were corrected for the substrate-free endogenous activity.

Although preincubation of the enzyme with the commercial avidin preparation caused little change in PEP-CK activity, PEP-CK could not be eliminated as a biotin-containing enzyme for the reasons described in the previous section concerning the effects of avidin on pyruvate carboxylase. However, no one has yet shown PEP-CK from any source to contain biotin or to be inhibited by avidin (281).

When the coupling system of NADH and MDH was replaced with 10 mM glutamate and glutamate-oxaloacetate transaminase, C-14 carbon dioxide fixation with PEP-CK increased two to five fold. With the enzyme from bakers' yeast both coupling systems gave the same results (29). The increased C-14 carbon dioxide incorporation with the glutamate and glutamate-oxaloacetate transaminase trapping system might be due to dilution of the radioactive product by large unlabeled pools rather than to increased enzyme activity. As a result less of the radioactive product would be decarboxylated with the consequent loss of fixed C-14 carbon dioxide. Added glutamate would be converted to alpha-ketoglutarate as a result of the transamination reaction. Subsequent metabolism of the alpha-ketoglutarate could result in the production of OAA which can dilute the radioactive OAA. Aspartate, the product of glutamate-oxaloacetate trans-

aminase reaction, is less likely to be metabolized than malate, the product of the MDH and NADH coupling system.

Carbon dioxide was usually present in the assay mixture at 2 mM. Adding more bicarbonate increased the incorporation of carbon dioxide. It appears that the Km value for bicarbonate was greater than 2 mM. Reported Km values for bicarbonate for this system range from two to five mM (24, 281) to 16 to 25 mM (33, 44).

The pH optimum for PEP-CK from L-cells lies below 6.0. The enzymatic activity was still increasing below pH 6.0, but this lies outside the range of effectiveness of the TES buffer which has a pK_a of 7.5. Activity at pH 6.0 was 10 times as high as that at pH 7.4. Optimal pH values for PEP-CK in the carboxylation direction were 5.2 to 5.4 for yeast (29, 247), 5.9 for liver fluke (190), 6.2 for flatworm (189), 6.5 for sheep kidney (13), 6.6 to 7.0 for pig liver (32,33), 7.0 for rat liver (10), and 7.4 for guinea pig (112). Thus, the enzyme from L-cells seems to resemble that of the lower organism more than the higher animals in this respect.

The enzyme kept at five degrees for 24 hours was only half as active as that kept in the frozen state for the same period. Furthermore, the frozen enzyme was only 60 per cent as active as the lyophilized enzyme. Therefore, in the crude extract, the enzyme is fairly unstable in the liquid state, more stable when frozen, and most stable in the dried state. Anion exchange and paper chromatographic analyses of the used reaction mixtures showed that the radioactive products included mainly malate and some OAA. These are the expected radioactive products.

Phosphoenolpyruvate-carboxykinase derived from L-cells that were

grown under "normal" conditions was found to be distributed almost evenly between the mitochondria and cytosol (Table 13). When the cells were exposed for various periods of time to low-glucose media or to media containing no glucose more enzyme than usual was found in the cytosol (Table 17). Steroid hormones had varying effects upon the relative distribution (Table 13) of the enzyme. These variations will be discussed later. Almost all of the PEP-CK from adult rat liver (69, 144, 221, 284) and adipose tissue (11) is located in the soluble fraction of the cell. That of mouse liver (127), hamster liver (143, 178), and flatworm (189) is almost all in the non-mitochondrial supernatant fraction. In guinea pig liver, 25 (112, 143, 178) to 90 (144) per cent of the PEP-CK is found in the soluble fraction, with the remainder being found in mitochondria and nuclei. Rabbit liver contains no soluble PEP-CK; 60 per cent is located in the mitochondria and 40 per cent in the nuclei (178). PEP-CK is contained only in the mitochondria of pigeon liver (77) and kidney (127).

Characteristics of the Malic Enzyme in Crude Extracts of L-Cells

Carbon dioxide fixation with malic enzyme is indicated by step (3) in Figure 3. In assaying the malic enzyme the reaction was generally run for 30 minutes as described on page 73. The reaction velocity was found to be constant over a two hour period.

No activity could be detected if the extract was boiled for 10 minutes before using, the reaction mixture was made acidic, or no extract was used. Thus, undenatured malic enzyme is needed in order to catalyze the reaction.

The endogenous activity, measured in absence of added pyruvate, was usually no more than one per cent of the activity when all components

were present. When dialyzed extract was employed with no NADPH, there was no activity. The specificity of the malic enzyme for NADPH eliminates any possibility of interference from pyruvate carboxylase, which also carboxylates pyruvate, but which requires ATP. Only three per cent of the control activity was seen in experiments with the dialyzed preparation when no Mn^{+2} was added. Therefore, all of the expected reaction components are necessary for malic enzyme activity in these L-cell extracts. All malic enzyme assays were corrected for pyruvate-free endogenous activity.

The pH optimum for the malic enzyme from L-cells was 6.5. The activity at pH 6.5 was double that at pH 7.4 and it decreased to zero at pH 8.2.

Preincubation of the enzyme with avidin caused little change in activity. But for the reasons given in the pyruvate carboxylase section about avidin the malic enzyme could not be eliminated as a biotin-containing enzyme. However, no one has yet shown malic enzyme from any source to contain biotin or to be inhibited by avidin (124).

The enzyme lost little of its activity by freezing it for two months. Lyophilizing it and reconstituting it in the same amount of water had little effect on the activity. The enzyme retained 90 per cent of its activity after being left at five degrees for one day. From these observations the malic enzyme appears quite stable.

Carbon dioxide was normally present in the assay mixture at 2 mM. Adding more bicarbonate increased the incorporation of carbon dioxide. It appears that the Km value for bicarbonate is greater than 2 mM. A Km value of 13 mM for bicarbonate has been reported for the pigeon liver enzyme (118).

Analyses of the used reaction mixtures showed the main radioactive products to be aspartate, malate, OAA, and alpha-ketoglutarate. If malic enzyme were the only enzyme present, malate would be the expected product. However, since the enzyme preparations that were used contain other enzymes, the newly-formed malate could be converted to aspartate, OAA, and other compounds.

Malic enzyme is located almost exclusively in the cytosol of L-cells (Table 15). Additions of cortisol or deoxycorticosterone to the media did not affect the relative distribution of the enzyme between the two sub-cellular fractions. Malic enzyme from the liver and kidney of rats (38, 166, 284) and other animals (94) is also found almost entirely in the post-mitochondrial supernatant fraction of the cell. Variations in the activity of the malate enzyme that resulted from exposure of the cells to sterol-containing media will be discussed later.

Characteristics of Carbamyl Phosphate Synthetase in the Crude Cell Homogenate

Carbamyl phosphate synthetase was assayed for only with the supernatant fraction of the cell. Its specific activity at pH 7.4 was 0.83 micromoles of carbon dioxide fixed per hour per g of protein. The endogenous activity with no ATP was only 10 per cent of the activity when all components were present. Since this enzyme is active in L-cells, the radioactivity that was found previously in the RNA and DNA fractions of these cells could have been due in part to the activity of this enzyme. Carbamyl phosphate is utilized in the synthesis of pyrimidine nucleotides.

Characteristics of Isocitrate Dehydrogenase in the Crude Cell Homogenate

The isocitrate dehydrogenase reaction in shown as step (6) in

Figure 3. Isocitrate dehydrogenase activity was measured only in the cytosol, and was found to be quite active even in preparations that had been frozen for over 9 months. Its specific activity at pH 7.0 was 27.8 micromoles of carbon dioxide fixed per hour per g of protein. The activity with no alpha-ketoglutarate present was only one per cent of that when all components of the system were present. When NADPH was deleted the activity of the non-dialyzed enzyme was only 22 per cent of the activity when NADPH was present. NADH did not substitute for NADPH. Therefore, both alpha-ketoglutarate and NADPH appear to be necessary for activity.

Pyruvate caused 93 per cent inhibition of carbon dioxide fixation by isocitrate dehydrogenase and alpha-ketoglutarate inhibited malate enzyme by the same amount. Pyruvate and NADPH are substrates for the malic enzyme which was previously shown to be present in the crude enzyme preparation. Pyruvate probably does not inhibit isocitrate dehydrogenase directly. Malic enzyme could easily form malate which, in turn, could be converted to isocitrate by the Krebs cycle. Therefore, even though both enzymes would be fixing carbon dioxide they would be forming a common product. Accumulation of the product, isocitrate, might inhibit both enzymatic reactions.

The TPN-linked isocitrate dehydrogenase is present mostly in the cytoplasm of organisms (187, 281). Though isocitrate dehydrogenase functions mainly in the direction of decarboxylation in the Krebs cycle, it can form isocitrate from alpha-ketoglutarate under similar conditions (42). Isocitrate could be converted to citrate, and then cleaved to OAA and acetyl-CoA. Acetyl-CoA could be used for fatty acid synthesis and OAA could be used for glucose synthesis by gluconeogenesis (42, 280).

It was estimated that in lactating rat mammary gland slices, 25 per cent of the glutamate metabolized via the Krebs cycle proceeded by the backward pathway mentioned above (156). In perfused rat liver, it was estimated that 50 per cent of the carbon of alpha-ketoglutarate was contributed to gluconeogenesis by the reversed Krebs cycle (50).

Characteristics of Phosphogluconate Dehydrogenase in the Crude Cell Homogenate

Phosphogluconate dehydrogenase was found to be present in the cytosol and to be able to catalyze the fixation of carbon dioxide. The specific activity of the enzyme was 13.3 micromoles of carbon dioxide fixed per hour per g of protein. When NADPH was deleted the activity of the nondialyzed enzyme was only 25 per cent of that when all components were present. When ribose 5-phosphate was left out the activity was found to be only five per cent of that of the complete system. It was assumed that the enzyme preparation contained phosphopentose isomerase in order to convert ribose 5-phosphate to ribulose-5-phosphate. Carbon dioxide fixed by phosphogluconate dehydrogenase could end up in carbon-3 of lactate. In isotope labeling experiments with bicarbonate, 90 per cent of the radioactivity in lactate was located in carbon-1. A feasible way for the label from bicarbonate to end up in carbon-3 is by operation of phosphogluconate dehydrogenase reaction and subsequent conversion of the product to lactate via glycolysis. This reaction is shown in step (1) in Figure 3. Although this possibility exists, its occurrence in vivo is quite improbable because there is no evidence in animals that hexoses are synthesized via carbon dioxide fixation into 6-phosphogluconate (281).

Carbon Dioxide Fixing Enzymes Not Found in L-Cells

Neither PEP-carboxylase nor PEP-carboxytransphosphorylase were found in the cytosol of L-cells. This is not surprising since PEP-carboxytransphosphorylase has been found only in propionic acid bacteria (148, 180) and PEP-carboxylase has been found only in plants and microorganisms (161, 280, 281).

Ribulosediphosphate carboxylase was not found in the cytosol. The assay for this enzyme was similar to that for phosphogluconate dehydrogenase except that ATP replaced NADPH. It was assumed that ribose 5-phosphate kinase would be in the enzyme preparation in order to convert ribose 5-phosphate to ribulosediphosphate. This assay procedure has been used previously (66). After subtracting the value for the ATP-deficient control no activity was found. There appeared to be slight activity when the value for the ribose phosphate-free control was used as the blank. But this slight activity was probably due to carbamyl phosphate synthetase activity with ATP, bicarbonate, and probably ammonium ions in the non-dialyzed enzyme preparation. The absence of ribulosediphosphate carboxylase from L-cells was not unexpected because the enzyme is rarely found in animal cells (66). The non-involvement of this enzyme in L-cells is indicated as step (2) in Figure 3.

Alpha-ketoglutarate synthetase was not assayed-for, but as mentioned earlier the labeling patterns of the isotope from C-14 bicarbonate found in glutamate indicated that this enzyme did not appear to be functioning in L-cells. The enzyme has been located only in anaerobic bacteria (21, 62). The non-involvement of it in L-cells is shown as step (8) in Figure 3.

Comparisons of the Carbon Dioxide Fixing Enzymes in L-Cells

From the observed pH optima, specificity for substrates and co-factors, distributions between mitochondria and cytosol, response to various hormonal and nutritional changes, and diverse effects of inhibitors and activators, each of the several carbon dioxide fixing enzymes was found to be distinguishable. There appeared to be little interference by other enzymes for each enzymatic assay. The specific activities of carbon dioxide fixing enzymes found in the crude extracts of L-cells under normal conditions are shown in Table 19.

An attempt was made to compare the total activities of pyruvate carboxylase, malic enzyme, and PEP-CK in the L-cells. Assuming that the intracellular pH is the same as that of the medium, 7.4, the total activity of the malic enzyme in the cell would be five to six times that of the PEP-CK and about four times that of pyruvate carboxylase. If the enzymes are compartmentalized within the cell at their optimal pH's and their activities corrected to values that would be obtained at the optimum pH's, the malic enzyme would have about the same total activity as PEP-CK, a value that would be three to five times as high as that of pyruvate carboxylase. To attempt to translate the activities of enzymes determined in cell-free extracts to actual *in vivo* activities may be misleading. Thus, caution should be exercised in using these values. Enzymatic assays are often run at pH's and temperatures quite different from those in the cell. Components in the assay mixtures are probably not at optimal concentrations, nor is there any certainty that the enzyme systems in the cell operate under optimum conditions. The extraction of the enzyme might cause a decrease or increase in its activity.

EFFECTS OF CORTISOL AND DEOXYCORTICOSTERONE ON L-CELLS

Since the activities of pyruvate carboxylase (71, 107) and PEP-CK (143, 223) have been reported to increase under gluconeogenic conditions in rats, it was decided to examine L-cells for variations in the activity of these enzymes under simulated gluconeogenic conditions. Sufficient glucose is normally present in the growth medium for L-cells, and it was thought that gluconeogenesis would be functioning slowly, if at all. However, in perfused rat liver, gluconeogenesis was not inhibited by high levels of glucose (63). Cortisol has been reported to cause slight increases in pyruvate carboxylase activity in rat kidney (107, 108, 140) but no changes in the rat liver enzyme (221). Soluble PEP-CK from rat liver (69, 141, 144) was increased by cortisol, while mitochondrial PEP-CK was unaffected (141, 144, 177). Steroid hormones are not usually present in the artificial media that are used to grow L-cells and it is highly unlikely that the cells produce them. Whether the cells, which more than 20 years ago had been isolated from a mouse by Earle, respond to cortisol and deoxycorticosterone was examined. The cells were exposed to one of the hormones in the medium for three days before the cells were harvested and a cell-free extract was prepared for enzymic assays.

Effects of Cortisol and Deoxycorticosterone on the Carbon Dioxide Fixing Enzymes of L-Cells

After three days exposure of L-cells to 10^{-7} or 10^{-6} M hydrocortisone (HC) the mitochondrial PEP-CK activity decreased slightly (Table 13). However, by application of the t-test (4b) both of these values were found not to differ significantly from the control value. On the other hand, the PEP-CK activity in the cytosol of cells exposed

TABLE 13

EFFECTS OF HYDROCORTISONE AND DEOXYCORTICOSTERONE ON THE PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY OF MITOCHONDRIA AND THE CYTOSOL

Media Additions (M)		N*	Mitochondria Sp. Act.**		Mitochondria Rel.Dist.***	Cytosol Sp. Act.**		Cytosol Rel.Dist.***
None		13	17.8 ± 1.8		0.47	2.7 ± 0.4		0.53
DOC	10^{-7}	2	18.0 ± 1.7	n.	0.54	1.8 ± 0.1	n.	0.46
	10^{-6}	2	14.6 ± 1.6	n.	0.49	1.8 ± 0.5	n.	0.51
HC	10^{-7}	3	14.9 ± 4.3	n.	0.38	3.2 ± 0.7	n.	0.62
	10^{-6}	2	11.9 ± 2.8	n.	0.79	0.35 ± 0.11	s.	0.21
HC****	10^{-7}	3	13.9 ± 3.1	n.	0.32	3.7 ± 0.9	n.	0.68
	10^{-6}	2	8.9 ± 0.9	n.	0.21	3.9 ± 0.9	n.	0.79

DOC = Deoxycorticosterone acetate; HC = hydrocortisone; n. = not significantly different from the control; s. = significantly different from the control.

*N = number of experiments

**Sp. Act. (specific activity) refers to the micromoles of carbon dioxide fixed per hour per g of protein in the test system. The standard error of the mean is recorded after each value.

***Rel. Dist. (relative distribution) refers to the fraction of the cellular complement of the enzyme that is found in the mitochondria or cytosol.

****The cells were exposed to hydrocortisone and 2.78 mM glucose (1/10 of that normally present) in the medium.

to 10^{-6} M HC was only 13 per cent as great as that of the control. This specific activity of 0.35 ± 0.11 was significantly different than the control value of 2.7 ± 0.4 at $p < .05$. Thus, the probability is less than five in 100 that the actual difference between the two means might have arisen by chance. Deoxycorticosterone at either 10^{-7} or 10^{-6} M did not cause any significant change in PEP-CK activity in the mitochondria or cytosol (Table 13). Thus, as mentioned earlier, neither HC nor DOC produced any significant variations in mitochondrial PEP-CK activity. But in contrast to the results of other studies, 10^{-6} M HC effected a drop instead of a rise in the PEP-CK activity of the cytosol. As a result of the cortisol-induced decrease in PEP-CK activity of the cytosol only 21 per cent of the total cellular enzyme was found in the cytosol as compared to the normal 53 per cent.

The pyruvate carboxylase activity of mitochondria and cytosol derived from cells that were exposed to 10^{-7} or 10^{-6} M HC or DOC was not significantly different from that of the control (Table 14). Under these conditions the fraction of the total cellular pyruvate carboxylase that was found to be located in the cytosol remained at 99 per cent. As reported earlier, some studies have shown increases in pyruvate carboxylase activity due to treatment with HC, and other studies have shown none. Perhaps in L-cells pyruvate carboxylase is not a regulatory enzyme in gluconeogenesis, i.e. it is not present in limiting amounts.

The activity of the malate enzyme of either the mitochondria or the cytosol did not change significantly when the cells were exposed to 10^{-7} or 10^{-6} M HC or DOC (Table 15). The specific activity of the malate enzyme in the cytosol from cells that were exposed to 10^{-6} M HC was 19.9

TABLE 14

EFFECTS OF HYDROCORTISONE AND DEOXYCORTICOSTERONE ON PYRUVATE CARBOXYLASE ACTIVITY IN THE MITOCHONDRIA AND CYTOSOL OF L-CELLS

Media Additions	(M)	N*	Mitochondria Sp. Act. **	Rel. Dist. ***	Cytosol Sp. Act. **		Rel. Dist***
None		15	0.71 ± 0.10	0.01	9.3 ± 0.6		0.99
DOC	10^{-7}	3	0.59 ± 0.09	n. 0.01	9.5 ± 2.2	n.	0.99
	10^{-6}	3	0.47 ± 0.03	n. 0.01	9.9 ± 2.1	n.	0.99
HC	10^{-7}	2	0.68 ± 0.25	n. 0.01	8.0 ± 0.8	n.	0.99
	10^{-6}	3	0.80 ± 0.13	n. 0.01	9.1 ± 2.8	n.	0.99
HC****	10^{-7}	3	0.88 ± 0.24	n. 0.02	5.5 ± 1.0	s.	0.98
	10^{-6}	4	0.70 ± 0.12	n. 0.02	7.9 ± 2.4	n.	0.98

DOC = Deoxycorticosterone acetate; HC= hydrocortisone; n.= not significantly different from the control; s.= significantly different from the control.

*N= number of experiments

**Sp. Act. (specific activity) refers to the micromoles of carbon dioxide fixed per hour per g of protein in the test system. The standard error of the mean is recorded after each value.

***Rel. Dist. (relative distribution) refers to the fraction of the cellular complement of the enzyme that is found in the mitochondria or cytosol.

****The cells were exposed to hydrocortisone and 2.78 mM glucose (1/10 of that normally present) in the medium.

TABLE 15

EFFECTS OF HORMONES ON MALATE ENZYME ACTIVITY IN CELLULAR CONSTITUENTS

Media Additions	(M)	N*	Mitochondria Sp. Act.**	Mitochondria Rel. Dist.***	Cytosol Sp.Act.**	Cytosol Rel.Dist.***
None		5	2.7 ± 0.3	0.02	27.6 ± 2.2	0.98
DOC	10^{-7}	1	2.5	n. 0.01	28.3	n. 0.99
	10^{-6}	1	1.7	n. 0.01	25.4	n. 0.99
HC	10^{-7}	2	2.9 ± 0.1	n. 0.02	26.7 ± 0.5	n. 0.98
	10^{-6}	3	2.3 ± 0.6	n. 0.02	19.9 ± 3.3	n. 0.98

DOC = Deoxycorticosterone acetate; HC = hydrocortisone; n.= not significantly different from the control.

*N= number of experiments

**Sp. Act. (specific activity) refers to the micromoles of carbon dioxide fixed per hour per g of protein in the test system. The standard error of the mean is recorded after each value.

***Rel. Dist. (relative distribution) refers to the fraction of the cellular complement of the enzyme that is found in the mitochondria or cytosol.

as compared to 27.6 micromoles of carbon dioxide fixed per hour per g of protein for the control, but the test for significance showed $.05 < p < .10$. Since the probability must be less than .05 in order to be statistically significant, these two values are probably not significantly different. Nearly all of the malate enzyme activity was still located in the cytosol following the various treatments with the hormones. That the malate enzyme activity did not change in L-cells exposed to HC or DOC was not unexpected because adrenalectomy or the administration of glucocorticoids to rats had little effect on the liver content of the enzyme (141, 143, 144, 223). Whether the malic enzyme plays an important role in gluconeogenesis can not be determined from these results. The enzyme might be present in high enough concentrations to handle the cells' needs so that on a teleological basis its synthesis would not be expected to be influenced by glucocorticoids.

Effects of HC and DOC on Glucose Utilization and Lactate Production

Glucose utilization by L-cells in 10^{-7} or 10^{-6} M HC- or DOC-containing media may be slightly less than in the normal medium, but none of the values are statistically significant (Table 16). However, lactate production by L-cells in 10^{-7} or 10^{-6} M HC is reduced to only 1/10 that of cells in normal medium (Table 16). Lactate production was not decreased significantly by 10^{-7} M DOC. At 10^{-6}M DOC a significant drop to 25 per cent of normal lactate production was noted.

The percentage of glucose that was converted to lactate under each condition is also listed in Table 16. Cells in normal medium converted 30 per cent of the utilized glucose to lactate, while cells in DOC media converted a smaller percentage of glucose to lactate. Only

TABLE 16

GLUCOSE UTILIZATION AND LACTATE PRODUCTION
IN L-CELLS UNDER VARIOUS CONDITIONS

Media Additions	(M)	N*	Glucose Util.** moles·10^{13}/cell		Lactate Prod.** moles·10^{13}/cell		Lactate Yield from Glu.(%)
None		11	32.1 ± 2.3		19.5 ± 3.6		30
DOC	10^{-7}	3	29.3 ± 2.4	n.	13.5 ± 4.3	n.	23
	10^{-6}	3	23.7 ± 2.0	n.	5.0 ± 1.7	s.	11
HC	10^{-7}	4	29.2 ± 2.1	n.	1.7 ± 0.8	s.	3
	10^{-6}	4	26.9 ± 3.1	n.	2.1 ± 0.8	s.	4
TES		4	55.5 ± 8.3	s.	44.2 ± 9.6	s.	40

DOC = Deoxycorticosterone acetate; HC = hydrocortisone; n.= not significantly different from the control; s.= significantly different from the control.

TES= N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid. Bicarbonate buffer was replaced with TES buffer, and a KOH trap for carbon dioxide was also used.

*N= number of experiments and each experiment was run in duplicate.

**The glucose utilized was the difference between the initial glucose on feeding on day four and that found in the medium on day seven. The lactate produced was that found in the medium on the seventh day. The standard error of the mean is recorded after each value.

three to four per cent of the glucose was converted to lactate in cells in HC media. In other studies on L-cells that were grown in normal media, 19 (129) and 24 (52) per cent of the consumed glucose was shown to be converted to lactate.

The amount of lactate that was found in the medium of the seven-day-old cultures was used to calculate the extent to which lactate is produced from glucose by the cells. Lactate converted to pyruvate is probably counterbalanced by lactate that is formed from components other than glucose. The interconversion of glucose and glycogen would not be expected to influence strongly the uptake of glucose from the medium by the cells because the glycogen concentration per cell after seven days of incubation was found to be only $\frac{1}{2}$ to 1 per cent of the glucose utilized per cell over the three day period after refeeding.

Cells that had been growing in the normal medium for seven days were found to contain 2.9×10^{-12} g of glycogen per cell. Cells that were exposed for the last three days of the incubation period to 10^{-6} M HC contained almost twice as much glycogen (5.6×10^{-12} g of glycogen per cell) as cells in the normal medium. This result is similar to that seen in intact animals in which cortisol was reported to cause an increase in glycogen levels in normal rats by 60 to 75 per cent (69) and in starved rats by 350 to 500 per cent (69, 114).

Since L-cells that were grown in 10^{-6} M HC produced only 1/10 of the lactate that cells in normal medium did, it was thought that HC might have lowered lactate dehydrogenase (LDH) activity or affected the isozymes of LDH. But no differences were found in LDH activity or iso-

zymes from cells in 10^{-6} M HC or normal medium. The specific activity of LDH from L-cells in normal medium was 0.50 ± 0.10 mmoles of NADH formed per min per g of protein. The specific activity of L-cells exposed to 10^{-6} M HC was 0.51 ± 0.05 mmoles of NADH formed per min per g of protein. Regardless of whether it was derived from normal or HC treated cells the enzyme exhibited only a single LDH isozyme band by sepraphore electrophoresis. Glinos et al. (82b) reported that L-cells contain a single LDH isozyme.

L-cells did not multiply as fast in HC or DOC media as in normal media. The number of cells that were found after three days exposure to 10^{-7} M or 10^{-6} M DOC media was only 87 or 74 per cent respectively of the number of cells found in non-hormonal medium. The number of cells that were found after three days exposure to 10^{-7} M or 10^{-6} M HC media was only 64 or 60 per cent respectively of the number of cells found in normal medium.

Effects of Low Concentrations of Exogenous Glucose on L-Cells

Glucose is normally present at 27.8 mM in the nutrient medium and is in excess of the cells' needs for it. As was mentioned in Chapter II, the presence of glucose is known to cause a decrease in the PEP-CK and pyruvate carboxylase activities of certain tissues, and in others to have no effect. The effects of varying amounts of glucose in the medium on the PEP-CK activity of L-cells are shown in Table 17. Except in one experimental situation, mitochondrial PEP-CK activity of L-cells was not lowered significantly by low levels of glucose. The activity of mitochondrial PEP-CK from L-cells exposed to 0.56 mM glucose (1/50 the normal amount of glucose) for three days was only 31 per cent of the control.

TABLE 17

EFFECTS OF LOW CONCENTRATIONS OF EXOGENOUS GLUCOSE ON PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY IN CELLULAR CONSTITUENTS

Glucose Present in Media (mM)	Exposure Time in Hrs.	N*	Mitochondria Sp.Act.**		Rel.Dist.***	Cytosol Sp.Act.**		Rel. Dist.***
27.8	72	13	17.8±1.8		0.47	2.7±0.4		0.53
2.78	72	4	16.7±2.3	n.	0.31	5.4±0.8	s.	0.69
0.56	72	2	5.6±3.4	s.	0.11	6.5±2.8	s.	0.89
None	12	2	18.8±0.9	n.	0.34	4.1±0.5	n.	0.66
None	24	2	16.7±0.4	n.	0.28	4.9±1.3	s.	0.72
None	46	2	13.7±2.8	n.	0.29	4.3±2.0	n.	0.71
27.8 + TES	72	3	17.2±2.2	n.	0.30	5.9±0.6	s.	0.70

TES = N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid. Bicarbonate buffer was replaced with TES buffer, and a KOH trap for carbon dioxide was also used; n.= not significantly different from the control; s.= significantly different from the control.

*N= number of experiments

**Sp. Act. (specific activity) refers to the micromoles of carbon dioxide fixed per hour per g of protein in the test system. The standard error of the mean is recorded after each value.

***Rel. Dist. (relative distribution) refers to the fraction of the cellular complement of the enzyme that is found in the mitochondria or cytosol.

But it was questionable whether the cells were still viable after this extended exposure to 0.56 mM glucose. All the cells had become small and round and a great number had come off the culture flask surface into the medium. Besides these abnormal characteristics, the number of cells present in 0.56 mM glucose was only 53 per cent of the number present in 27.8 mM glucose at 72 hours. The number of cells present in 2.78 mM glucose at 72 hours was 82 per cent of the number present in 27.8 mM glucose.

PEP-CK of the cytosol was increased 100 per cent by exposure of the cells for 72 hours to 1/10 of the normal amount of glucose, and 141 per cent by exposure to 1/50 of the normal amount of glucose (Table 17). Exposure of L-cells to no glucose for periods of 12 to 46 hours caused a 50 to 80 per cent increase in the PEP-CK of the cytosol (Table 17). The effects of decreased amounts of glucose on the PEP-CK from L-cells are similar to those from starved rats. In the guinea pig, fasting enhanced the soluble but not the mitochondrial PEP-CK (144, 178). In ciliated protozoa the PEP-CK of the cytosol, which is involved in gluconeogenesis in these organisms, was found to decrease in response to the presence of exogenous glucose (218). In intact or adrenalectomized rats starvation caused a doubling of the soluble PEP-CK activity but had no effect on mitochondrial PEP-CK (69, 177, 223). It appears that PEP-CK from the cytosol of L-cells may be a rate-limiting enzyme in glucose synthesis.

Mitochondrial pyruvate carboxylase activity from cells that had been deprived on glucose for 24 or 46 hours was lower than that from cells which had been growing in media containing normal amounts of glucose (Table 18). But the pyruvate carboxylase activity is quite low in mitochondria from cells on normal medium, constituting only about one per cent of

the total pyruvic carboxylase activity of the cells.

Pyruvate carboxylase activity of the cytosol of cells exposed to 1/10 of the normal amount of glucose was unchanged from that of cells in the normal medium (Table 18). However, pyruvate carboxylase of the cytosol was significantly decreased in cells that were exposed to a medium containing 1/50 the normal amount of glucose for 72 hours or no glucose at all for 12, 24, or 46 hours (Table 18). In the latter three conditions the activity of the enzyme was 56, 57, and 33 per cent, respectively, of that observed for cells maintained in the normal high-glucose medium. In cells exposed to 1/50 of the normal glucose for 72 hours, no mitochondrial pyruvate carboxylase activity was detected and the enzyme activity of the cytosol was only 5 per cent of the control. But for the same reasons given in the PEP-CK section, L-cells exposed to 1/50 the normal glucose for 72 hours were probably not viable.

Since the pyruvate carboxylase activity of the cytosol from cells that had been completely deprived of glucose decreased, it appears that either pyruvate carboxylase does not play an important role in the synthesis of glucose in L-cells or that this enzyme is not involved in regulating the process.

The malate enzyme was not affected by exposure of the cells to 2.78 mM glucose (1/10 of the normal glucose concentration) for 72 hours. The specific activities of the malic enzyme of the mitochondria and cytosol from cells grown on 2.78 mM glucose were 2.2 ± 0.3 and 29.2 ± 0.8 micromoles of carbon dioxide fixed per hour per g of protein respectively. These values are close to those from cells grown on normal medium (Table 15). The activity of this enzyme in rat liver falls during fasting, and

TABLE 18

EFFECTS OF GLUCOSE DEPRIVATION ON PYRUVATE CARBOXYLASE ACTIVITY IN THE MITOCHONDRIA AND CYTOSOL OF L-CELLS

Glucose Present in Media (mM)	Exposure Time in Hrs.	N*	Mitochondria Sp.Act.**		Rel.Dist.***	Cytosol Sp.Act.**		Rel. Dist.***
27.8	72	15	0.71±0.10		0.01	9.3±0.6		0.99
2.78	72	4	1.1 ±0.5	n.	0.02	9.5±1.5	n.	0.98
0.56	72	2	0.00	s.	0.00	0.45±0.32	s.	1.00
None	12	2	1.5±1.1	n.	0.03	5.2±2.0	s.	0.97
None	24	2	0.40±0.00	n.	0.01	5.3±1.1	s.	0.99
None	46	2	0.07±0.02	s.	0.00	3.1±1.2	s.	1.00
27.8 + TES	72	3	1.2±0.2	n.	0.02	7.7±0.3	n.	0.98

TES= N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid. Bicarbonate buffer was replaced with TES buffer, and a KOH trap for carbon dioxide was also used; n.= not significantly different from the control; s.= significantly different from the control.

*N= number of experiments

**Sp. Act. (specific activity) refers to the micromoles of carbon dioxide fixed per hour per g of protein in the test system. The standard error of the mean is recorded after each value.

***Rel. Dist. (relative distribution) refers to the fraction of the cellular complement of the enzyme that is found in the mitochondria or cytosol.

rises, together with lipogenesis upon refeeding the animal with a high carbohydrate diet (9, 141, 143, 144, 223, 278, 284). In L-cells the malic enzyme might be present in any contingency at high enough concentrations to handle the cells' demands for lipogenesis or gluconeogenesis.

EFFECTS OF LOW BICARBONATE ON L-CELLS

L-cells were grown in TES medium which contained no added bicarbonate. The TES medium was not entirely free of carbon dioxide because this gas was picked up from the air as the medium was being made-up and was produced by the cells as a product of metabolism. A KOH trap was used to absorb most of the carbon dioxide. The activities of mitochondrial PEP-CK from cells exposed to TES medium or normal medium for 72 hours were the same (Table 17). However, the activity of the PEP-CK of the cytosol from cells exposed to TES medium was higher than that from cells in the normal medium by 119 per cent (Table 17). The activities of pyruvate carboxylase of the mitochondria or cytosol of cells that were grown in TES and normal media were about equal (Table 18).

Glucose utilization and lactate production per cell in TES medium were 73 and 126 per cent greater, respectively, than in normal medium (Table 16). Forty per cent of the used glucose was converted to lactate by cells in the TES medium compared to 30 per cent by cells in the normal medium.

It was mentioned earlier that the number of cells present after 48 to 72 hours in a culture containing TES medium and a KOH trap was only 63 to 38 per cent that of cells in the normal medium. Thus, in the absence of carbon dioxide the cells fare poorly.

As mentioned in Chapter II, reported Km values of bicarbonate for PEP-CK (2 to 25 mM) are higher than those for pyruvate carboxylase (0.62 to 2.7 mM). Thus, because of the low bicarbonate concentration in TES medium PEP-CK would not be expected to operate at maximum efficiency. Therefore, the cell might have to synthesize more enzyme if the activity of PEP-CK fell below the cells' need for it. An increase in PEP-CK activity of the cytosol of cells exposed to TES media was seen.

It is puzzling why a cell in TES medium would utilize almost twice as much glucose and produce more than twice as much lactate as a cell in normal medium. The cells in TES medium are multiplying at $\frac{1}{2}$ the rate of cells in normal medium. Perhaps the decreased carbon dioxide fixation by L-cells in TES medium is compensated for by increased emphasis on pathways that utilize glucose. It seems that cells in TES medium carry on glycolysis at a higher rate than cells in normal medium. The reduced rate of carbon dioxide fixation by cells in TES medium could lead to a lowered production of intermediates for the Krebs cycle and a rate of gluconeogenesis lower than is usually found in cells that are growing in normal medium. For these reasons, cells in TES medium might be expected to utilize more glucose and have a high rate of glycolysis.

TABLE 19

SPECIFIC ACTIVITIES OF CARBON DIOXIDE FIXING ENZYMES
FOUND IN THE CRUDE EXTRACTS OF L-CELLS
UNDER NORMAL CONDITIONS

Enzymes	Mitochondria Specific Activity	Cytosol Specific Activity
PEP-carboxykinase	17.8	2.7
Pyruvate carboxylase	0.71	9.3
Malate enzyme	2.7	27.6
Carbamyl phosphate synthetase	*	0.83
Isocitrate dehydrogenase	*	27.8
Phosphogluconate dehydrogenase	*	13.3
PEP-carboxylase	*	0.0
PEP-carboxytransphosphorylase	*	0.0
Ribulosediphosphate carboxylase	*	0.0

* These enzymes were not assayed for in the mitochondrial fraction. The procedure for each enzymatic assay is described in Chapter II. The specific activity refers to the micromoles of carbon dioxide fixed per hour per g of protein in the test system.

CHAPTER V

SUMMARY

Mouse L-cells (strain 929) that were grown in a synthetic medium fix carbon dioxide into lactate, glutamate, alpha-ketoglutarate, proline, asparagine, aspartate, citrate, and small amounts of malate and succinate. Carbon dioxide was incorporated into the combined protein and DNA, the RNA, and the lipid fractions in approximately equal amounts. Aspartate was the only newly-formed amino acid that did not exchange rapidly with its extra-cellular counterpart.

About the same percentages of products were formed from carbon dioxide in normal and bicarbonate-free media. With only two exceptions significant changes in radioactive products formed from cells exposed to C-14 bicarbonate for 48 to 96 hours were not seen. Low concentrations of radioactive alpha-ketoglutarate were found at 48 hours and higher concentrations on lengthened exposure. Radioactive lactate, which was found in high concentrations at 48 hours, decreased with time.

All of the radioactivity in glutamate was located in carbon one. In lactate ninety per cent of the radioactivity was found in carbon one. From the distribution of the label in glutamate and lactate the fixation products, oxaloacetate and/or malate, would have had to randomize, probably through fumarate. From the labeling pattern in glutamate alpha-ketoglutarate synthetase was ruled out as operating in L-cells.

The rate of carbon dioxide fixation by the L-cells during the exponential growth phase (day 4 to day 5 after transferring) was 12 x 10^{-15} moles per cell per hour. This rate was 5 to 10 times as great as that of the older, slower growing cells (day 5 to day 7). Cells that were grown in bicarbonate-free media multiplied at about ½ the rate of cells that were grown in the normal medium. After two weeks on bicarbonate-free medium L-cells appeared abnormal. Normal growth and appearance of the cells was rapidly restored by adding bicarbonate to the medium. Therefore, bicarbonate appears to be essential for the normal growth of L-cells. Cells in bicarbonate-buffered medium incorporated carbon dioxide 12 to 20 times as fast as those in bicarbonate-free medium. Glucose utilization and lactate production per cell in bicarbonate-free medium were 73 and 126 per cent greater, respectively, than in normal medium. It appears that the decreased carbon dioxide fixation by L-cells under low bicarbonate conditions is compensated for by the cell placing greater than usual emphasis on glycolysis.

Pyruvate carboxylase was found almost exclusively (99 per cent) in the cytosol fraction of L-cells. Pyruvate, ATP, and Mg^{+2} are essential for the enzyme's activity. Under the conditions of this study pyruvate carboxylase had a pH optimum of 6.9. Acetyl-CoA did not appear to be a necessary cofactor, and in fact, at 0.2 mM it caused 50 per cent inhibition. No significant changes were caused in pyruvate carboxylase activity in either mitochondria or cytosol by exposure for three days of L-cells to either HC or DOC at either 10^{-7} or 10^{-6} M. Pyruvate carboxylase of the cytosol, but not the mitochondria, was significantly decreased in cells that were deprived of glucose for 12, 24, or 46 hours to 56, 57

and 33 per cent respectively of that observed for cells maintained in normal glucose medium. It appears that either pyruvate carboxylase does not play an important role in the synthesis of glucose in L-cells or that this enzyme does not have a regulatory function in the process. The activities of pyruvate carboxylase in the mitochondria or cytosol of cells that were grown in bicarbonate-free media were unchanged from those of cells grown in normal medium.

Phosphoenolpyruvate-carboxykinase (PEP-CK) is distributed almost evenly between the mitochondria and cytosol of L-cells. A nucleotide diphosphate is essential for the PEP-CK activity in the carboxylation direction. IDP, GDP, or ADP are used equally well in the reaction. The enzyme requires a divalent metal ion (Mn^{+2} or Mg^{+2}) and PEP. The carboxylation reaction with PEP-CK was completely inhibited by 10 mM ATP. DOC at either 10^{-7} or 10^{-6} M did not cause any significant change in PEP-CK activity in the mitochondria or cytosol. The specific activity of mitochondrial PEP-CK was lowered only slightly by 10^{-7} and 10^{-6} M HC. However, PEP-CK from the cytosol of cells exposed to 10^{-6} M HC was only 13 per cent of that of the control. PEP-CK of the cytosol was increased 100 and 141 per cent by exposure of the cells for 72 hours to 1/10 and 1/50 respectively of the normal amount of glucose. No glucose for 12 to 46 hours caused a 50 to 80 per cent increase in activity respectively. It appears that PEP-CK from the cytosol of L-cells may be a rate-limiting enzyme in glucose synthesis. However, the presence of cortisol in the growth medium causes the level of the enzyme to decrease. PEP-CK of the cytosol, but not of the mitochondria, from cells exposed to bicarbonate-free media for 72 hours was 110 per cent higher than that from cells in

the normal medium. Thus, the absence of ample carbon dioxide brings about an increase in the amount of this carbon dioxide fixing enzyme.

The malate enzyme is located almost entirely (98 per cent) in the cytosol of L-cells, only 2 per cent being found in the mitochondria. Pyruvate, NADPH, and Mn^{+2} are necessary for malic enzyme activity in the direction of carboxylation. Under the conditions of these studies the pH optimum for the reaction was found to be 6.5. The activity of the malate enzyme of either the mitochondria or the cytosol did not change significantly when the cells were exposed to 10^{-7} or 10^{-6} M HC or DOC. The enzyme was not affected by exposure of cells to 1/10 the normal glucose concentration for 72 hours. The malate enzyme is probably present in concentrations that are high enough to handle the cells' demands for lipogenesis or gluconeogenesis.

Although 10^{-7} and 10^{-6} M HC did not cause any significant changes in glucose utilization by the cells, they did reduce the production of lactate to only 1/10 that of the hormone-free controls. Only three to four per cent of the glucose was converted to lactate under hormone-supplemented conditions compared to 30 per cent under normal conditions. No changes were caused in either the specific activity or the single isozyme band of lactate dehydrogenase by exposure of L-cells to 10^{-6} M HC for three days. Cells exposed to 10^{-6} M HC contained almost twice as much glycogen (5.6×10^{-12} g of glycogen per cell) as cells in the normal medium. However, the glycogen concentration per cell after seven days was only ½ to one per cent of the glucose utilized per cell over the three-day period after refeeding. It appears that, when exposed to HC, the cells metabolize glucose by pathways other than glycolysis.

These additional carbon dioxide fixing enzymes were found in the cytosol of the crude cell homogenate of L-cells: carbamyl phosphate synthetase, isocitrate dehydrogenase, and phosphogluconate dehydrogenase. Phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxytransphosphorylase, and ribulosediphosphate carboxylase were not found in the cytosol of the crude cell homogenate of L-cells. Isocitrate, NADPH, and Mn^{+2} are necessary for isocitrate dehydrogenase activity in the direction of carboxylation.

Carbon dioxide was seen to play an important role in the metabolic activities of L-cells because: 1) the absence of bicarbonate in the medium resulted in decreased cell growth and abnormal cell morphology; 2) the label from C-14 bicarbonate was found in many products; and 3) many enzymes were shown to fix carbon dioxide.

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APPENDIX

ABBREVIATIONS

acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
C	curie
cm	centimeter
CoA	coenzyme A
CO_2	carbon dioxide
CO_3^{-2}	carbonate
cpm	counts per minute
CTP	cytosine triphosphate
DNA	deoxyribonucleic acid
DOC	deoxycorticosterone acetate
ΔF^o	change in standard free energy
g	gram
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HC	hydrocortisone, cortisol
HCl	hydrochloric acid
HCO_3^-	bicarbonate
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
IDP	inosine diphosphate
ITP	inosine triphosphate
K	equilibrium constant
K_m	Michaelis constant

L-cells	established line of mouse cells NCTC clone 929 (strain L, Earle)
MDH	malate dehydrogenase
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
NAD	DPN, nicotinamide adenine dinucleotide
NADH	DPNH, reduced NAD
NADP	TPN, nicotinamide adenine dinucleotide phosphate
NADPH	TPNH, reduced NADP
NaOH	sodium hydroxide
OAA	oxaloacetate
OAS	oxalosuccinate
pCO_2	partial pressure of unhydrated gas, CO_2 physically dissolved in water
PEP	phosphoenolpyruvate
PEP-CK	phosphoenolpyruvate carboxykinase
pH	-log of hydrogen ion concentration
pK_a	-log of K_a (ionization or dissociation constant of an acid)
RNA	ribonucleic acid
rpm	revolutions per minute
S	Svedberg unit (1×10^{-13} second)
$s^{\circ}_{20,w}$	sedimentation coefficient in water at 20 degrees

ABBREVIATIONS (cont'd.)

TCA	trichloroacetic acid
TES	N-tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid
Tris	Tris (hydroxymethyl) aminomethane
TTP	thymidine triphosphate