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The Metabolic Role of Carnitine in
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THE METABOLIC ROLE OF CARNITINE IN THE YEAST, TORULOPSIS BOVINA

By

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ABSTRACT

THE METABOLIC ROLE OF CARNITINE IN THE YEAST, TORULOPSIS BOVINA

By

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This study shows that carnitine participates in the biosynthesis of amino acids in the carnitine-requiring yeast, Torulopsis bovina ATCC 26014, a finding which is both new and novel and provides a basis of the metabolic role of carnitine in this yeast.

When 0.5-5 μM L-carnitine is added to the media, the growth rate of T. bovina is doubled for both aerobic and anaerobic cultures. The stimulation occurs about 18 minutes after carnitine is added. High concentrations of glutamate stimulate growth without altering the carnitine content of the cells. Cells grown without added carnitine contain 0.4 nmol/g wet weight of carnitine while cells grown with 5 μM carnitine accumulate 1400 nmol/g wet weight by the end of exponential growth.

Very high levels of carnitine acetyltransferase (CAT) activity are present in this yeast, the levels being unaffected by anaerobiosis or chloramphenicol but decreasing nearly 50% in yeast grown with carnitine. The substrate specificity and kinetic parameters of the enzyme in cell-free extracts were determined. The transferase is most active with acetyl-, propionyl-, and isobutyrylCoA. When provided with carnitine, acetylcarnitine is the only acylcarnitine formed in vivo. Adding

[1-^{14}C]acetylcarnitine to cultures of *T. bovina* doubles the growth rate with much of the radioactivity becoming cell associated. The majority of the ^{14}C is incorporated into cell protein although some ^{14}C is recovered in the fatty acid fraction of saponified cells. Analysis of the amino acids derived from radiolabeled protein revealed that acetylcarnitine contributes carbons to the synthesis of glutamate, arginine, proline, leucine, and lysine. In contrast, [1-^{14}C]acetate only labels leucine and lysine. The labeling pattern with [^{14}C]acetate plus carnitine is the same as that with [^{14}C]acetylcarnitine.

Isopycnic density gradient analysis of lysed spheroplast preparations revealed that CAT is mostly associated with mitochondria while acetylCoA synthetase is in the cytosol. Catalase was not detected indicating the absence of peroxisomes. Less than 15% of the CAT activity is soluble in yeast preparations in which the mitochondria are ruptured. Thus the subcellular localization of CAT is consistent with carnitine facilitating the transfer of acetyl groups from the cytosol into the mitochondria where 2-oxoglutarate is synthesized.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CAT	Carnitine acetyltransferase (EC 2.3.1.7)
CPM	Counts per minute
acylCoA	Acylcoenzyme A
Ci	Curies
CoASH	Coenzyme A
DNA	Deoxyribonucleic acid
DPM	Disintegrations per minute
DTNB	5,5-'dithiobis-(2-nitrobenzoic acid)
D_4^{20}	Density measured at 20°C and corrected to 4°C, in g/cm^3
EDTA	(Ethylenedinitrolo)-tetraacetic acid
G6PDH	Glucose-6-phosphate dehydrogenase
HPLC	High pressure liquid chromatography
K_i	Inhibition constant
K_m	Michaelis constant
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
N_2/CO_2	5% CO_2 in nitrogen
pI	isoelectric pH
RNA	Ribonucleic acid
TCA	Tricarboxylic acid (citric acid) cycle
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl) aminomethane
V_{max}	Maximum velocity
[ ]	Concentration of

INTRODUCTION

THESIS STATEMENT

The growth of the yeast, Torulopsis bovina ATCC 26014, is stimulated by carnitine (1-4). One known function of carnitine is to shuttle long-chain fatty acids into mitochondria for β oxidation (5-8). However, a preliminary study showed that T. bovina lacked fatty acid oxidase (9). Thus carnitine had an unknown function in this yeast. The purpose of this thesis was to determine the metabolic role of carnitine in T. bovina.

LITERATURE REVIEW

BIOCHEMISTRY OF CARNITINE, ACETYLCARNITINE, AND CARNITINE ACETYLTRANSFERASE (CAT)

Carnitine participates in the transport of long-chain acylCoA derivatives across the acylCoA impermeable inner membrane of mitochondria and thereby facilitates oxidation of fatty acids (6-8,15,16). However, other roles for carnitine must exist since carnitine acyltransferase is associated with peroxisomes and microsomes as well as mitochondria (70), and since all mammalian tissues contain significant quantities of short-chain acylcarnitines (28,130,224). Furthermore, carnitine affects branched-chain amino acid metabolism in mammals (44,45), and pyruvate oxidation in the fatty acid oxidase-deficient flight muscle of the

blowfly, Phormia regina (79). Some possible roles for carnitine in intermediary metabolism have recently been summarized (225). For an understanding of carnitine metabolism in T. bovina, the possible functions of acetylcarnitine and CAT are most relevant.

Carnitine, acetylcarnitine and CAT are present in numerous animal tissues, some plant tissues and some microorganisms although few analyses have been made in the latter two categories (10-14). Fraenkel observed that since carnitine was not present to the same extent in all tissues and species, it may be involved "in two different processes, one of which requires it in minute quantities and the other in large quantities" (17). T. bovina and Tenebrio molitor are two organisms that require small quantities of carnitine.

CARNITINE AS A GROWTH FACTOR

Tenebrio

Carnitine, Vitamin B_T, was first recognized as a growth factor for larvae of the mealworm, Tenebrio molitor (18), although the B_T factor was not identified as carnitine until 1951 (19). Other beetle larvae belonging to the family Tenebrionidae require carnitine (20-22). Optimal survival of T. molitor was obtained with L-carnitine concentrations of 0.35 µg/g dry weight of diet (inner salt, 19) while 1.5 µg L-carnitine/g dry weight of diet was required for fully successful growth (23). Under certain culture conditions the beetles ceased to show a carnitine requirement but the deficient state could be induced by feeding the larvae 4-N-trimethylaminobutyrate. This provided the first clue that the β-hydroxyl group was important to carnitine metabolism. The

4-N-trimethylaminobutyrate induced deficient state showed symptoms similar to carnitine deficiency with disturbances in the casting of the skin during molting and also bulges in the midgut (22).

The function of carnitine in Tenebrio is unknown. Fraenkel and Chang found that in starved larvae the fat content decreased from 43% to 12% while the water content increased from 58.5% to 71%. In contrast, in carnitine deficient larvae the fat content only decreased to 24% and the water content fell to 42% (24). McFarlane found no decrease in fat content in carnitine deficient larvae (25). These results suggest that carnitine deficiency blocks lipid catabolism. No histological deficiency symptoms were observed in muscle or nervous tissue (26) although it is uncertain that lipid droplets would have been observed around the muscle mitochondria as has been described in recent reports of muscle carnitine deficiency in humans (27). Carnitine concentration must be greater than 10 μM in order to stimulate fatty acid or fatty acylCoA oxidation by mitochondria (26). This represents a tissue concentration of at least 10 nmol/g wet weight or 50 nmol/g dry weight assuming 80% water content. When fed a diet containing carnitine, Tenebrio contained about 142 nmol L-carnitine/g dry bodies (23) or nearly three times the minimum amount necessary to stimulate fatty acid oxidation. However, McFarlane found no difference in the rate of oxygen consumption or in the respiratory quotient of carnitine deficient larvae compared to either normal young larvae or normal older larvae (25). It seems doubtful, therefore, that carnitine-stimulated fatty acid oxidation is the immediate cause of death. The carnitine deficient larvae appear to die because of water loss due to improper hardening of the cuticle following molting (24). It would be interesting to determine if the insect contains CAT or carnitine

palmityltransferase and what carnitine derivatives form *in vivo*. An intriguing possibility is that acetylcarnitine participates in acetylation reactions necessary for proper cuticle hardening. Support for the existence of CAT in *Tenebrio* is provided by the data of Bhattacharyya *et al*. (29) indicating that acetylcarnitine substitutes for carnitine in the nutrition of this insect.

Torulopsis

The structural similarity of carnitine and choline prompted some investigators to search for lipid bound carnitine but instead of finding phosphatidylcarnitine, long-chain acylcarnitines were isolated and identified (16,30,31). Phosphatidyl-β-methylcholine was found when carnitine replaced choline in the diets of some insects (32) although carnitine does not generally have a choline-sparing function (16). For example, carnitine did not support the growth of choline-less mutants of *Neurospora crassa* (33). However, Travassos *et al*. (1) were able to isolate from a choline-requiring yeast a carnitine-requiring strain of *Torulopsis bovina*. Their isolate, *T. bovina* ATCC 26014, was sensitive to 0.1 µg% DL-carnitine or 2.5 pmol/ml L-carnitine with optimum growth requiring >1.5 µg% DL-carnitine (37.5 pmol/ml in L-carnitine).

Travassos and his colleagues studied the growth requirements of *T. bovina* ATCC 26014 in detail paying particular attention to those compounds that might serve as precursors of carnitine, as carnitine was thought to be synthesized in 1961, and to the possible methyl denoting capacity of carnitine's quaternary ammonium group. The yeast's growth was stimulated by 5 mg% L-glutamic acid but none of the other intermediates in the biosynthesis of carnitine proposed by Guggenheim in

1951 (glutamic acid⟶4-aminobutyrate⟶4-N-trimethylaminobutyrate⟶crotonobetaine⟶carnitine) stimulated growth (42). Maximum growth stimulation required both carnitine and choline although methionine substituted for choline. The combination of leucine plus methionine stimulated growth in the absence of choline and carnitine.

Somewhat later, Miranda et al. (2) discovered that carnitine accumulated in the growth media and concluded that carnitine biosynthesis was not impaired in this yeast but that it must "leak" carnitine and thus grow more poorly than the parent strain where a "retention mechanism" operates. However, their study did not actually measure the carnitine content of the yeast or the media. Nor did their study find any lipid bound carnitine in the lipid extract of cells grown 48 h with DL-[methyl-^{14}C]carnitine.

Except for a preliminary study by Bieber et al. (9), the reports on T. bovina ATCC 26014 have all been by members of the laboratory from which the original report issued. They did not perform any biochemical analyses of the yeast although they observed that acetylcarnitine substituted for carnitine suggesting, as demonstrated in the preliminary report from this laboratory, the existence of CAT in T. bovina ATCC 26014. Phosphorylcarnitine also stimulated yeast growth (3) leading one to imagine a high energy storage function for carnitine although the intervention of a non-specific phosphatase might be explanation enough. Phosphorylcarnitine is hydrolyzed by crude phosphatase preparations from sperm (3).

Other Organisms

The initial description of carnitine as a "growth factor" for Tenebrio caused many investigators to test the growth promoting

characteristics of carnitine on a variety of systems. For example, Liebecq-Hutter, who worked with Tenebrio also claimed an effect of carnitine on the development of embryonic chick bone but the effect was later attributed to crotonobetaine (149). Sakuguchi (150) reported that 10 μg/ml DL-carnitine permitted growth of the soy sauce lactic acid bacterium, Pediococcus soyae, but no additional reports followed. Strack and Rotzsch tested carnitine on a variety of animals (151,152): tadpoles grew faster in water containing carnitine; carnitine induced weight gain in young rats presumably by stimulating appetite; and carnitine antagonized the effects of thyroxine on tadpoles and rats. Reynier (135) found that carnitine lowered the ratio of nitrogen catabolism and increased survival time in starving rabbits. Many other examples could be cited but too little is known about the biochemistry of these effects to make a listing worthwhile.

Discovery of CAT

During their work with Tenebrio, Friedman and Fraenkel (34) discovered that DL-carnitine inhibited the acetylation by acetylCoA of p-aminobenzoate catalyzed by preparations from pigeon liver acetone powders. DL-carnitine was inhibitory at a concentration of 10 μM and appeared to be specific for carnitine with acetylcarnitine formed in the absence of p-aminobenzoate. Furthermore, acetylcarnitine itself acetylated p-aminobenzoate thereby demonstrating the equivalent bond energy of acetylCoA and acetylcarnitine which was not expected of a secondary o-acetyl ester group (35). In fact, acetylcarnitine formed acetylCoA when the liver extracts were incubated with CoASH and the following reversible enzymatic reaction was proposed:

$$\text{O-acetyl-L-carnitine} + \text{CoASH} \underset{\text{Reverse}}{\overset{\text{Forward}}{\rightleftharpoons}} \text{acetylCoA} + \text{L-carnitine} \quad \text{(eqn. 1)}$$

At the time (1955) acetylcarnitine had not been found in animals although it had not really been looked for. Acetylcarnitine was known to replace carnitine in the diet of Tenebrio (29) and it seems peculiar that Friedman and Fraenkel did not look for either CAT or acetylcarnitine in this insect.

CAT Involvement in Fatty Acid Oxidation

The purification of CAT was not reported until almost a decade after the above report. In the meantime, a number of studies implicated carnitine having a role in lipid metabolism. Fritz (52,53,36) found that DL-carnitine stimulated rat liver slices and homogenates to oxidize palmitate to CO_2 and ketone bodies. Carnitine augmented fatty acid oxidation in liver particulate fractions but not in soluble systems, a finding which first suggested that carnitine had some transport function. Similar results were obtained using skeletal and cardiac muscle with carnitine stimulating fatty acid oxidation of isolated mitochondria (55,56). Indeed, mitochondria very actively oxidized long-chain acylcarnitines (15,16) which were formed by mitochondrial preparations (40,16) and also identified in the lipid extracts of various tissues (30,31). Carnitine did not act by stimulating the long-chain acylCoA synthetase reaction (36).

In these early preparations, carnitine did not stimulate palmitylCoA oxidation but further research showed that carnitine did in fact stimulate mitochondrial long-chain acylCoA oxidation (16). From these data came the proposal that fatty acid oxidation was compartmentalized

inside the mitochondria behind a CoASH impermeable barrier that was permeable to acylcarnitines (6). The inner mitochondrial membrane is impermeable to CoASH (57) although carnitine is restricted to the same mitochondrial space as CoASH (57-60). Thus, there was a physiological need for a reversible long-chain carnitine acyltransferase catalyzed reaction analagous to CAT. Some evidence for a long-chain carnitine acyltransferase had been obtained that suggested the enzyme was different from CAT (61). Shortly thereafter, carnitine palmityltransferase was purified from calf liver (62). Thus it was established that carnitine stimulated long-chain acylCoA oxidation via acylcarnitine formation and transport into the mitochondrial matrix with subsequent transfer of the acyl group back to CoASH at the site of the fatty acid oxidase system.

The physiological need for a short-chain carnitine acyltransferase such as CAT was not apparent from the discovery of the role of carnitine in fatty acid oxidation. Carnitine did not stimulate octanoate or butyrate oxidation in rat liver slices or particulate fractions (36) and has not been found to be obligatory for the oxidation of medium- or short-chain fatty acids. However, the oxygen consumption of rat kidney, heart, brain, and testis mitochondria was strongly stimulated by acetyl-DL-carnitine and in particular by acetyl-L-carnitine (37). Other studies also showed that short- and medium-chain acylcarnitines are oxidized by mitochondria (15,16,38). Bremer observed that when presented with an excess of pyruvate, isolated mitochondria convert carnitine to acetylcarnitine but acetylcarnitine oxidation is inhibited when pyruvate is added (37). These results suggested that the reversible enzymatic acetylation of carnitine was responsible for the stimulation by carnitine of fatty acid oxidation. More importantly, however, Bremer pointed out

that acetylcarnitine formed during pyruvate oxidation might be a "loophole" in the compartmentation of the cell allowing "active acetate" derived from carbohydrate breakdown to leave the mitochondria and thus be available for extramitochondrial biosyntheses, e.g., fatty acids and cholesterol. Citrate was not recognized as an acetyl carrier for fatty acid synthesis at the time and Bremer's proposal sparked a flurry of research (see below).

After Fritz and Yue discovered that carnitine stimulated palmitylCoA oxidation (16), they again investigated the oxidation of acetate by heart muscle mitochondria and found that indeed unlike acetate, acetylCoA oxidation was carnitine dependent (63). Similar results were obtained with butyrylcoA and hexanoylCoA and the lack of stimulation by carnitine of short-chain fatty acid oxidation was attributed to the intracellular localization of the different chain-length acyl thiokinases. Carnitine presumably stimulated acetylCoA oxidation by facilitating its transfer into mitochondria. However, acetylCoA is ordinarily generated inside the mitochondria by the operation of the fatty acid and pyruvate oxidase systems. Thus it made sense to reason that under physiological conditions, carnitine facilitated the transfer out of the mitochondria of the acetyl groups in the intramitochondrial acetylCoA pool. This view is indirectly supported by the fact that (+)-acetyl-O-carnitine, a potent inhibitor of CAT (see below) has no effect on carnitine stimulated palmitate oxidation by rat heart mitochondria (64) but this result should be interpreted with caution because (+)-acetyl-O-carnitine exchanges very poorly with intramitochondrial L-carnitine (41). Strictly speaking, therefore, carnitine-stimulated palmitate oxidation does not require any CAT activity external to the inner mitochondrial membrane.

CAT Purification and Properties

CAT was partially purified from pig heart by Fritz et al. (35) developing four different assay procedures in the process for both the forward and reverse direction (see eqn. 1). The enzyme remained particulate in homogenate prepared in 0.25 M sucrose but was easily solubilized using 0.1 M K_2HPO_4. The enzyme preparation had slight or no fatty acylCoA hydrolase or acetylcarnitine esterase activity and did not react with choline or its derivatives. The apparent equilibrium constant at pH 7 was 0.6 in the direction of acetylCoA formation and the apparent K_m value for acetyl-DL-carnitine was 6.2×10^{-4}M. The high group potential of the O-acetylester bound ($\Delta F^{\circ\prime} = -7.9$ kcal) was unexpected but emphasized the "active" acetate nature of acetylcarnitine.

High specific activities of CAT were found associated with the isolated mitochondria from rat, pigeon, and locust muscle with much lower activities in rat liver and kidney mitochondria and no detectable activity in bee flight muscle (46). As in pig heart, the CAT activity in the other tissues remained particulate when the mitochondria were prepared in isotonic sucrose but was easily solubilized by homogenization in 0.1 M phosphate buffer, pH 7.2. Under the conditions of the assay, butyryl-, decanoyl-, and palmitylcarnitine inhibited the CAT reaction from which it was erroneously concluded that the same enzyme transferred acyl groups from short-, medium-, and long-chain acylcarnitines to CoASH. The presence of CAT in locust flight muscle which utilizes fatty acids for energy and its absence from a carbohydrate utilizing tissue, bee flight muscle, caused the authors to stress that CAT was only important in fatty acid oxidation.

The above study showed that pigeon breast muscle was a much better source of CAT than pig hearts and Chase et al. (47) quickly reported the preparation of crystalline CAT from pigeon breast muscle. The molecular weight of the enzyme determined by Sephadex chromatography was 55,000. Further characterization of the enzyme's kinetic behavior (48), its dependence on pH (49) and its substrate specificity (50) were quickly reported. The enzyme is strictly a short-chain carnitine acyltransferase with the interesting property that the Michaelis constants for acylCoA substrates from acetyl- to octanylCoA are experimentally identical (38 ± 6 μM), whereas V_{max} for the catalyzed reaction decreases about 10-fold over this range of substrates. The equilibrium constant for the reaction measured in the direction of acetylCoA formation is 0.6 (65), identical to the value obtained for pig heart CAT (35).

Long-chain acylCoA's reversibly inhibit the pigeon breast enzyme, mostly by decreasing CAT's affinity for L-carnitine (50). The Ki for palmitylCoA is 0.43 μM, a value which is probably physiologically significant. Palmitylcarnitine (100 μM) did not inhibit the enzyme but bromo-acetylCoA was a potent irreversible inhibitor at nearly a 1:1 ratio of enzyme to inhibitor (66).

Fritz and Schultz (51) found that acetyl-D-carnitine is a potent inhibitor of pig heart CAT with a Ki of 2.5×10^{-4}M when assayed in the direction of acetylcarnitine formation. The enzyme is inhibited competitively by D-carnitine with an apparent K_i of 2.1×10^{-3}. The K_m for DL-carnitine is 6.2×10^{-4}M. They reported that comparison of reciprocal plots for DL-carnitine with those for L-carnitine showed an increased K_m for L-carnitine and an unaltered V_{max} for the racemic mixture. On the other hand, Chase and Tubbs

(48) found that a fixed ratio of D- to L-carnitine produces a parellel decrease in V_{max} and K_m in the pigeon breast muscle enzyme and that the K_m's for the L-forms of carnitine and acetylcarnitine are approximately equal to the K_i's for the D-forms. In addition acetyl-L-carnitine was a competitive inhibitor of L-carnitine in pigeon breast CAT whereas it was a non-competitive inhibitor for pig heart CAT. These differences might have been due to Fritz and Schultz measuring the rate of thiol release from acetylCoA while Chase and Tubbs measured the reverse reaction by coupling acetylCoA formation to citrate formation.

CAT was recently purified from beef heart mitochondria as part of a larger study to isolate carnitine octanyltransferase activity as a separate enzyme (67). The CAT activity was purified over 400-fold and was greater than 95% pure. The enzyme had similar molecular weights determined by Sephadex G-200 chromatography (60,500) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis (62,600). Isoelectric focusing produced a single peak at pH 8.2. Studies of the forward and reverse reaction kinetics revealed a very significant difference in the relative activities measured with increasing acyl chain lengths. In the direction of acylcarnitine formation, the activity with octanyl- and decanoylCoA is 27 and 5% respectively of the rate with acetylCoA whereas in the direction of acylCoA formation the corresponding relative activities are 54 and 26%, respectively.

Mittal and Kurup noted that treating animals with hypolipidemic drugs causes major increases in CAT and proceeded to purify CAT from hepatic mitochondria of rats fed clofibrate (68). Although gel filtration indicated the molecular weight of the enzymes was 56,000, two non-identical subunits were obtained using SDS gel electrophoresis.

Solberg was unable to purify calf liver CAT because the carnitine acetyl-, octanyl- and palmitoyl-transferases all eluted as a single peak with molecular weight about 68,000 (69). Thus, it is not known if the two subunit native of the clofibrate induced hepatic CAT is the result of proteolysis, a reflection of a difference between muscle CAT and hepatic CAT, or the result of clofibrate treatment. In all other respects, the clofibrate induced hepatic CAT was very similar to the partially purified CAT from pig heart (35,51).

Mitochondria contain 60-70% of the cellular CAT (see below) but significant levels of extramitochondrial CAT exist (70-76) and the microsomal and peroxisomal activities have been partially purified by Markwell and Bieber (74). The microsomal enzyme activity was very labile in sucrose or dilute phosphate buffer solutions but stable in the presence of 0.4 M KCl. Gel filtration of the enzymes on Sephadex G-100 resulted in the recovery of only 10% of the applied CAT activity which eluted at an apparent molecular weight of 59,000. Both the peroxisomal and microsomal enzymes had similar pI values of 8.3 with the microsomal preparation exhibiting a minor peak at pI 5.3 under some conditions. Both enzyme preparations had the same apparent K_m for acetylCoA (69 μM) and L-carnitine (146 μM) with nearly identical substrate specificities. However, the reported relative activities with hexanoylCoA or octanoylCoA are much lower than the corresponding activities of beef heart and pigeon breast muscle CAT preparations (67). The peroxisomal and microsomal enzymes were also reported by Markwell and Bieber (74) to function with malonylCoA and acetoacetylCoA at 50 and 75% respectively of the rate with acetylCoA. Scholte's study of liver malonylCoA decarboxylase concluded that malonylCoA is not a substrate for CAT but, although not specified,

this result was probably obtained using the commerical preparation of CAT from pigeon breast muscle (77). Bressler and Katz (78), in their study of fatty acid synthesis in guinea pig liver, reported that CAT is 23% as active with acetoacetylCoA as it is with acetylCoA but they too used a CAT enzyme derived from muscle tissue.

Distribution of CAT

The preceeding sections demonstrate the presence of CAT in pig heart (35), pigeon breast muscle (47), rat liver (74), and liver of clofibrate-treated rats (68), pigeon and sheep liver (34), calf liver (69) and beef heart (67). CAT was present in locust flight muscle but absent from bee flight muscle (46), a result confirmed by Childress et al. (79).

The tissue distribution of CAT was indirectly determined by Beenakkers and Klingenberg (46) in their survey of mitochondrial preparations. Actual study of the CAT distribution in various tissues of rat was done by Marquis and Fritz (80). Highest activity was present in heart and testis, somewhat lower activity in brown adipose tissue and relatively low levels in skeletal muscle, kidney and brain. CAT was extracted most successfully from the tissues using a buffer (pH 8.0) containing 0.1 M K_2HPO_4, which seems to be a universal finding. Skeletal muscle CAT activity was lower but the specific activity in mitochondria isolated from skeletal muscle (410 nmol/min/mg protein) was essentially equal to the specific activity in heart muscle mitochondria (440 nmol/min/mg protein). These values are higher but qualitatively similar to those of Beenakkers and Klingenberg.

It is noteworthy in the data of Marquis and Fritz that of all the tissues only testis contains a much higher ratio of CAT to carnitine in an equivalent weight of tissue. A closer study of male rat reproductive tissues revealed that epididymal sperm contain the highest CAT activity of any tissue so far examined (276 μmol/min/g dry weight). Ram and human sperm (88) and presumably bovine sperm (100) are similarly rich in CAT. In rat relatively high levels of CAT were also associated with testis, caput epididymis, caudal epididymis, and vas deferens with no activity in cell-free epididymal fluid (81).

A more extensive tissue distribution study was performed by McCaman et al. (82) using a unique assay system yielding results in good agreement with Marquis and Fritz even though the two groups assayed the reaction in reverse directions. Sciatic nerve, lung, spleen, small intestine, thymus, and liver were the least active tissues while heart and testis were the most active. Kidney, skeletal muscle, brain, and adrenal gave intermediate values. In contrast, choline acetyltransferase was absent from kidney, liver, thymus, intestine, and lung with activities in heart, testis, brain, and skeletal muscle 1×10^{-2} to 5×10^{-5} times the CAT activities in these tissues. There was little variation in the CAT levels present in 9 different regions of rabbit brain with no difference between white and gray matter. Four day old rabbit brain cortex only contained 10% of the adult level of CAT.

As part of a study to determine if mitochondrial CAT was accessible to externally added substrates, Barker et al. (83) compared the relative rates of the CAT catalyzed reaction in both the forward (acetylcarnitine formation) and reverse direction using three different assays and thereby demonstrated the variability of the results obtained by the different

methods. CAT was present in the mitochondria of sheep liver and the mitochondria of liver and mammary gland of guinea pig, goat, and rat. CAT values in rat liver mitochondria were 20- to 40-fold lower than in the other animals' livers. Barker et al. claim their rat liver value is much lower than that found by Marquis and Fritz (80, see above) but they must have confused the units because the measured values were 5.1 and 4.2 nmol/min/mg protein respectively in the two reports.

The levels of CAT as well as the isobutyryl-, isovaleryl- and octanylcarnitine acyltransferase activities in tissues of fed and fasted rats were also recently examined by Choi et al. (84). The carnitine acyltransferases in liver increased after 8 days of fasting while the levels in heart, skeletal muscle, kidney, and testes remained unchanged. Variations in CAT activity in tissues of rat exposed to fasting and cold were studied by Kerner et al. (85). Significant increases of CAT activity were observed in brown fat and liver of cold adapted rats compared to controls. In another study by the Pecs group, CAT was found in gastrocnemius muscle of the frog (86).

CAT is present in both normal and dystrophic murine skeletal muscle (94) and in mouse liver and cardiac tissue (95).

A good correlation exists between CAT activity and acetylcarnitine levels in developing embryonic tissues as first demonstrated by Casillas and Newburgh (96) using embryonic chick brain, heart, and liver. CAT was also present in the yolk sac. CAT is absent in immature rat ovary but the enzyme appears during development induced by pregnant mare gonadotropin and remains active during steroidogenesis (97). Fetal and adult monkey skeletal muscle, heart, liver, and brain fat all contain CAT (98). CAT activity is relatively high in liver, soleus, tibialis, fetal

liver, and fetal heart of female guinea pigs and rabbits (99) and, so too in brown adipose tissue (89).

Human arterial and venous tissue from various locations all contain low levels of CAT (101) as do human platelets (87). Relative to adults, lower activities of CAT are present in human fetal heart, liver, brain, and skeletal muscle (102). CAT is present in human placenta (102) and also in the placenta of mouse, marmoset and tamarin (103). Human kidney and fibroblasts also contain CAT and in one case a general tissue deficiency of this enzyme was implicated as the cause of fetal ataxic encephalopathy (104).

There is an overwhelming interest in the CAT levels of mammalian tissues. CAT has been reported in yeast (9,90,91) and, with the recent interest in peroxisomal metabolism, CAT activity has been reported in goldfish intestine (92) and carp liver (93). However, CAT is reported absent from a number of plant tissues (70) including spinach leaf peroxisomes (76). African trypanosomes do not oxidize fatty acids. Yet the blood stream form of Trypanosoma lewisi has an extremely high level of CAT activity (105) and other trypanosomes contain CAT (106).

Intracellular localization of CAT

Differential centrifugation of a large number of different tissues has repeatedly confirmed that the majority of cellular CAT activity sediments with the mitochondrial fractions (37,80,82,83,107). Given the prevailing view that CAT facilitates mitochondrial export and import of acetylCoA, it is not surprising to find CAT localized predominantly in mitochondria. However, Markwell and coworkers noted that CAT was significantly skewed to higher densities than was the mitochondrial

marker enzyme in a rat liver homogenate fractionated by isopycnic centrifugation in a very steep sucrose gradient (109). Careful investigation of similarly fractionated homogenates of rat and guinea pig kidney and liver revealed that CAT was indeed a mitochondrial enzyme in kidney but in liver substantial (nearly 50%) activity was also present in the peroxisomes and microsomes (70). Since then other investigators have looked for extramitochondrial CAT. The enzyme is only present in the mitochondria of guinea pig intestine (108) while in rat heart a small but definite percentage of the total CAT resides in the microsomes (75). Peroxisomal CAT has been demonstrated in mouse liver (110), goldfish intestinal mucosa (92), carp liver (93), brown adipose tissue (111), and alkane-grown yeast (91).

Rat liver peroxisomal CAT is a soluble matrix enzyme (74) while the microsomal activity is membrane associated. Part of the microsomal activity faces the cytosol and part is exposed on the lumenal surface (112). The picture concerning the intramitochondrial localization of CAT is confusing.

Pearson and Tubbs argue, and many agree, that the role of acetylcarnitine is to provide an "acetyl sink" for mitochondrial matrix acetylCoA (10), a role that requires CAT only on the matrix side of the mitochondrial inner membrane. Although histochemical data obtained from rat heart (113) and skeletal muscle (114) indicate CAT occupies the space between the outer and inner mitochondrial membrane, most other reports conclude there is very little CAT in the outer mitochondrial compartment, most of the activity being present on the matrix side of the inner mitochondrial membrane.

In one study, Tubbs and Chase (115) obtained preparations of rat heart mitochondria that oxidized acetylcarnitine but would not oxidize acetylCoA without the addition of carnitine. Based on the pattern of inhibition of oxidation of these substrates by bromoacetylCoA and bromoacetylcarnitine, suicide inhibitors of CAT (116), these workers concluded that there were two pools of CAT in mitochondria, one accessible to acetylCoA (outer compartment) and one accessible to acetylcarnitine (matrix compartment). However, in a later study (117) liver mitochondria oxidized acetylCoA + carnitine at an insignificant rate and they concluded the carnitine dependent oxidation observed for rat heart was probably due to stimulation of endogenous fatty acid oxidation.

Two separate studies indicate the rate of oxygen consumption does not adequately reflect the activity of external CAT. In the first study, Fritz and Yue (63) showed that even though the rate of oxygen consumption increased only 1.6 times, carnitine stimulated the degradation of [1-^{14}C]acetylCoA by rat heart mitochondria 50-fold. The second study deals with the oxidation of isobutyrylCoA which is also a substrate for CAT. In their study, Choi _et al_. (39) report that beef and rat liver mitochondria oxidize isobutyryl CoA + carnitine at a negligible rate due to inhibition of isobutyrylcarnitine translocation rather than lack of formation of isobutyrylcarnitine. By one method these workers showed that nearly 50% of mitochondrial CAT was external to the inner membrane while a second method indicated 10-20% was external.

The formation of acetylcarnitine from pyruvate by mitochondria of rat heart (41); rat heart, kidney, and liver (37); and bovine sperm (100) demonstrates that CAT is accessible to acetylCoA generated in the matrix

compartment. At least 90% of mitochondrial CAT is reported to be latent and therefore presumably in the matrix compartment in sheep liver, kidney cortex, heart, and skeletal muscle (107). Similarly, 10% of the CAT activity of disrupted mitochondria was measurable in intact mitochondria of goat and guinea pig liver and mammary glands, sheep liver, and rat mammary gland (83). A somewhat higher percentage (25%) of mitochondrial CAT was assigned to the outer compartment based on subfractionation studies of rat liver and pig kidney mitochondria with digitonin (118). These workers reported that the rate of oxidation of acetylCoA + carnitine by pig kidney mitochondria decreased 3-fold when the outer compartment CAT was removed by digitonin treatment but that oxidation capacity could be restored if purified CAT was added back to the depleted mitochondria. Solberg also used digitonin fractionation and found little or no outer compartment CAT in rat and mouse liver mitochondria (119). Likewise in blowfly flight muscle mitochondria, separate investigators (120,79) reported the total absence of outer CAT because, whereas acetylcarnitine was rapidly oxidized, acetylCoA + carnitine was not. External CAT may depend upon developmental age. Mitochondria from fetal bovine heart do not oxidize acetylCoA + carnitine whereas the mixture is oxidized by calf heart mitochondria. Both tissues oxidized acetylcarnitine (121).

CAT isozymes

In general, the above studies suggest two non-interchangeable pools of CAT in mitochondria. Also, the peroxisomal CAT activities might represent different enzymes and tissue specific isozymes of CAT might explain the proposed differences in the function of CAT in different

tissues. However, all the evidence obtained to date indicate a single type of CAT under all these conditions.

Rat liver peroxisomal CAT is a soluble matrix enzyme and microsomal CAT is a membrane bound enzyme, but both enzymes have similar molecular weights, similar isoelectric points, similar chromatographic properties, and similar kinetic constants (74). The similarities between pigeon breast muscle CAT (47,48,50) and pig heart (35) CAT have already been discussed and the somewhat different molecular weight and subunit composition of clofibrate induced rat liver CAT (68) has been noted.

Although species differences in the isoelectric points of partially purified CAT from rabbit, human, and pigeon tissues were reported by White and Wu (122), essentially identical isoelectric focusing patterns were observed for CAT from different tissues of the same species. Edwards et al. (117) found that CAT from ox heart, ox liver, sheep liver, and pigeon breast muscle were separarted similarly by isoelectric focusing, ion-exchange chromatography and centrifugation. They observed two interconvertible forms of the enzyme, one attributed to its being membrane associated. Clarke and Bieber (67) and Markwell et al. (74) have confirmed these findings in beef heart and rat liver respectively. Thus, only a single type of CAT appears to exist within a given tissue.

It is noteworthy that, whereas White and Wu (122) found commercially prepared pigeon breast muscle CAT to contain three different enzyme peaks after electrofusing, several other investigators report only a single peak near pH 8 for this same enzyme preparation (67,74,117). Thus it is uncertain that species differences in CAT actually exist.

Acetylcarnitine metabolism

Marquis and Fritz (11) were the first to observe that tissues containing a high level of CAT usually contain a high concentration of carnitine and acylcarnitine. Assuming that CAT operates near equilibrium, changes in the ratio [acetylcarnitine]/[carnitine] are expected to reflect identical changes in the [acetylCoA]/[CoASH] ratio. However, it is not proven that in different metabolic states CAT operates at equilibrium in each compartment of the cell in all cell types. These data are really only available for perfused rat heart, locust flight muscle, and for several tissues of sheep.

Perfusing rat hearts with appropriate substrates causes very large changes in acetylCoA levels but the corresponding changes in acetylcarnitine are compensatory with the ratio approaching the theoretical value of 0.6 under most conditions (10). Very similar results were obtained by Whitmer et al. (123) using control, ischemic and hypoxic perfusion conditions with either glucose or glucose + palmitate as substrates. Direct measurements of metabolite compartmentation shows approximately 95% of the cellular CoA but only 10% of cellular carnitine is in the mitochondrial compartment in rat heart (124). This data is supported by the fact that the plot of the mass action ratio of CAT by Pearson and Tubbs (10) passed through the origin. Thus not only does CAT appear to operate near equilibrium in perfused rat heart, but the whole of the acetylCoA pool in this tissue appears to be in equilibrium with the acetylcarnitine pool. These results caused Pearson and Tubbs to propose that acetylcarnitine buffers the acetylCoA/CoA ratio allowing a larger input of substrate into the cell without depleting the free CoASH

which would destroy the ability of the mitochondria to function especially under conditions of high energy requirement.

Worm et al. (125) applied this same reasoning to a study of the metabolite changes occurring during flight in the flight muscle of Locusta migratoria. Plots of the mass action ratio for CAT versus flight time indicated the reactants were not in equilibrium in resting insects or during the first minute of flight although equilibrium was attained immediately thereafter. The data in this study also suggested that there were two distinct pools of acetylCoA one of which was not in equilibrium with acetylcarnitine.

Snoswell and Kaundakjian (107) performed a similar study of normal and diabetic sheep tissue. Not finding any other obvious function for CAT in sheep, these authors concluded that the enzyme buffered the acetylCoA/CoA ratio. The apparent equilibrium constants for the CAT reaction calculated for both normal and diabetic sheep heart and skeletal muscle were somewhat higher (range 1.3-4.2) then the expected value (0.6). The calculated intracellular equilibrium constant for CAT in sheep liver and kidney cortex was nearly 30-fold higher (direction of acetylCoA formation) than under in vitro additions. Liver and kidney perform different functions than the heart and carnitine could have other roles in the liver than buffering the acetylCoA/CoA ratio. Liver cells maintain a fairly equal distribution of their cellular CoASH between the extra- and intra-mitochondrial compartments (126). The carnitine content of liver is one-half to one-quarter the carnitine content of heart muscle and would not be expected to represent the same buffering capacity as exists in heart. Thus it is not surprising that the situation in sheep liver appears more complex.

Pearson and Tubbs measured the levels of free carnitine and acetylcarnitine in a large member of tissues including liver and kidney taken from rats in various nutritional and metabolic states. Whereas the total acid soluble carnitine content of each tissue remained relatively constant, major changes in the acetylcarnitine/carnitine ratio (from <.04 to 6) were observed for the various tissues in different metabolic states (10).

Still assuming that CAT operates near equilibrium, Bohmer and coworkers (127,128) measured significant changes in the acetylcarnitine/carnitine ratio in the liver, kidney, heart, and adipose tissues of rat in various nutritional and metabolic states. Ciman et al. (129) performed a similar analysis of resting and exercised rat muscle.

Under normal conditions acetylcarnitine is the predominant short-chain acylcarnitine in rat tissues (130), however, it is more precise to be concerned with the total acylcarnitine composition of a tissue and whether or not it is in equilibrium with the cellular acylCoA pool. For CAT it is approximately the C_2 to C_6 short-chain acylcarnitine/carnitine ratio that is relevant and this ratio does change with nutritional status (10,12,131) although over a smaller range than the acetylcarnitine/carnitine ratio. For example, the ratio of acetylcarnitine to carnitine in rat liver drops from 0.24 in the fed animal to less than 0.05 in the starved, carbohydrate re-fed rat while the acid soluble acylcarnitine/carnitine ratio remains constant at 0.7 (10). Determining the acylcarnitine composition would more precisely reflect the metabolic state of these cells. In a study of anoxia in rat heart and pig heart, fairly minor changes in the short-chain

acylcarnitine/carnitine ratio were recorded while major changes in the tissue levels of some specific acylcarnitines occurred (133).

One other aspect of acylcarnitine metabolism explored by Brass and Hoppel (132) using rat liver is the extent to which the cellular acylcarnitine pools turnover and mix with the acylCoA pool. In isolated rat liver mitochondria oxidizing palmitylcarnitine, exogenous carnitine has no effect on the mitochondrial content of acetylCoA, acid-soluble CoA or acid-insoluble CoA, or no effect on oxygen consumption or citrate formation even though acetyl units are shunted to acetylcarnitine. Approximately 5% of the acetyl groups generated from palmitylcarnitine by β-oxidation appeared in acetylcarnitine without affecting the acylCoA/CoA ratio. From these data plus data obtained in vivo (131) the authors concluded that carbon flux through the short-chain acylcarnitine pool is sluggish compared to the flux through the CoA pool. However, Brass and Hoppel made a very interesting calculation; the rate of acetylcarnitine formation by mitochondria in state 3 oxidizing palmitylcarnitine is about 1 nmol/min/mg mitochondrial protein very nearly equal to the specific activity of CAT in rat liver mitochondria. Thus, this study indicates that CAT can shuttle acetylcarnitine out of the mitochondrion at a rate nearly equal to its specific activity without affecting the intramitochondrial acetylCoA/CoA ratio. In other words, it appears as if CAT couples the cytosolic acetylcarnitine/carnitine ratio directly to the intramitochondrial ratio of acetylCoA/CoA. Metabolism of acetylcarnitine at a rate equal to its rate of delivery to the cytosol would make this system behave like an acetyl pump exporting "active acetate" from the mitochondrion.

Involvement of acetylcarnitine in fatty acid synthesis and biological acetylations

Carnitine is thought to play a central role in the control of fatty acid utilization and fatty acid synthesis (134,143). Reynier (135) reported that carnitine is antiketogenic in fasting rabbits as has also been seen to occur in other animals (137,139,142). On the other hand, some investigators report that carnitine stimulates acetoacetate production (138,140,36). These contradictory results may be caused by carnitine having opposite effects in fed and fasting animals (136). Moreover, the carnitine concentration determines if ketogenesis is stimulated or depressed. (137,138).

Fritz found that carnitine stimulated fatty acid oxidation in rat liver homogenates and reported that acetoacetate production was stimulated simultaneously (36). Carnitine-stimulated acetoacetate production was studied by Bressler and Katz using fasted, pregnant guinea pig liver homogenates (78). They attributed the increased acetoacetate production to a faster rate of delivery of long-chain fatty acids to the site of β-oxidation, a result readily understood in terms of the known role of carnitine in fatty acid oxidation. Bressler and Katz also suggested that acetoacetylCoA transport out of the mitochondria was carnitine dependent but this scheme has not been substantiated and it is accepted that acetoacetate is produced in the mitochondrion via the HMG-CoA cycle (141).

During their study of acetoacetate production, Bressler and Katz noted that exogenous carnitine stimulated the conversion of [2-^{14}C]pyruvate to fatty acids by liver homogenates of fed guinea pigs. Bremer had already proposed that activated acetyl groups exported

out of mitochondria as acetylcarnitine could be available for fatty acid or cholesterol biosynthesis (37). In a subsequent study, this time using fed male guinea pig liver homogenates, Bressler and Katz (145) showed that carnitine stimulates the conversion of pyruvate to acetylcarnitine and to long-chain fatty acids and that in vivo carnitine stimulates the conversion of glucose, pyruvate, and acetate to fatty acids in liver and adipose tissue. Carnitine had no effect on the conversion of citrate to fatty acids. It should be pointed out that although DL-carnitine caused a 2- to 3-fold increase in the amount of pyruvate carbon incorporated into fatty acids, the absolute rate of incorporation was only of the order of 0.002 nmol/min/mg protein. Under in vivo conditions the rate of incorporation was even lower, 0.0005 nmol/min/mg protein.

A year later Bressler and Brendel (144) reported a similar investigation of fatty acid synthesis in pigeon liver but this time the rate of incorporation of added substrates into fatty acids were between 0.025 to 0.05 nmol/min/mg protein. The purified fatty acid synthesizing system of pigeon liver incorporates acetylCoA into fatty acids at the rate 6.5 nmol/min/mg protein (146). In this second study, Bressler and Brendel concluded that in the absence of added carnitine, 75% of the pyruvate converted to fatty acids probably went via the indirect citrate pathway but that this decreased to 60% when L-carnitine was added. These values were calculated using assumptions which if not true would have overestimated the percentage of pyruvate converted to fatty acid via the citrate pathway. Thus in the presence of exogenous carnitine, at least 40% of fatty acid carbons are derived from pyruvate by a non-citrate pathway, presumably as acetylcarnitine. Furthermore, the acetylation of

sulphanilamide at a rate of about 0.5 nmol/min/mg protein by pigeon liver derived nearly 50% of its acetyl groups from pyruvate by a non-citrate pathway. The role of carnitine in biological acetylations is discussed further below.

Lowenstein (147,148) absolutely refutes any role for acetylcarnitine in fatty acid synthesis. However, he used progressively purer preparations of the fatty acid synthesizing system in his studies which by his own data lack any detectable CAT activity. It is not surprising, therefore, that acetylcarnitine emerges as a very poor substrate for fatty acid synthesis in his studies. Marquis et al. (143) demonstrate that carnitine and palmitylcarnitine have complex effects on the rate of fatty acid synthesis in partially purified systems unrelated to the function of CAT.

Lowenstein claims (147) that CAT is not active enough to account for the cell's rate of fatty acid synthesis. The maximum rate of acetylCoA incorporation into fatty acids by high speed supernatant fractions of livers is about 1 or 2 nmol/min/mg protein. But even in rat liver where the level of CAT is low compared to other animals, Brass and Hoppel showed (see previous section) that acetylcarnitine is produced and pumped out of the mitochondrion at a rate nearly equal to the specific activity of CAT in the mitochondria, 1/nmol/min/mg protein.

These arguments do not prove that acetylcarnitine is formed and transferred out of the mitochondrion and then used for fatty acid synthesis. They do indicate that some of the older literature should be re-evaluated on this point. A related issue that has not received much attention is the role of cytosolic acetylcarnitine in biological acetylations. Biological acetylations are a heterogenous group of

reactions some of which appear to have important functions in the control of cellular growth, development and metabolism. The most obvious example is the acetylation of histone and non-histone chromosomal proteins which reactions require extramitochondrial acetylCoA that may even have to be transported into the nucleus as acetylcarnitine. As a class the sialyl residues on secretory proteins are O-acetylated and there are many more specific examples of O-acetylated or N-acetylated proteins. It is very tempting to speculate that microsomal CAT may play a direct role in these acetylation reactions. The mitochondrial CAT may simply deliver active acetate to the cytosol but whereas cytosolic citrate may be committed to fatty acid synthesis, cytosolic acetylcarnitine may be available for other purposes.

It is amazing that so much could be known about an enzyme and yet its importance in metabolism be so poorly understood.

Yeast metabolism

Typically, glucose fermenting yeast are studied because of their economic value. However, glucose has a very particular effect on the metabolic state of yeast. The classical glucose effect was described many years ago by Epps and Gale (153) and J. Monod (154). As the biochemistry of the glucose effect came to be better understood, Magasanik coined the term "catabolite repression" (155). In its simplest terms, glucose or one of its "catabolites" causes the repression of other catabolic enzymes not related to glucose fermentation. In modern terms "catabolite inactivation" is defined as "the loss *in vivo* of catalytic activity of certain enzymes subsequent to the addition of glucose or related sugars to cells adapted to a non-sugar carbon source or to

starved cells" (156). Some of the enzymes inactivated include fructose-1,6-bisphosphatase, phosphoenolpyruvate carboxykinase, cytoplasmic malate dehydrogenase, α-glucoside-(maltose) permease, the galactose uptake system, uridine nucleosidase, and aminopeptidase I. The glyoxalate pathway is also suppressed and although cytochromes may be synthesized they may not be assembled into a functional electron transport system. In this thesis it is catabolite repressed yeast that are studied.

It is quite certain that most yeast growing on glucose would, if fatty acids were added, still use only glucose for their energy metabolism. Under these conditions, most of the added fatty acid would be incorporated into the yeast lipids (157). I. bovina ATCC 26014 is no exception, [1-^{14}C]palmitate, when added to the growth media with glucose, is recovered almost completely in the cell lipids and protein/cell wall residue (9). The ability of Saccharomyces species to use long-chain fatty acids as sole carbon sources has not been investigated. There is a group of alkane utilizing yeast of which Candida tropicalis is a well-studied example. This yeast contains peroxisomes (158) and extremely high levels of CAT (91), probably even higher than reported because the enzyme was assayed with subsaturating concentrations of acetylCoA (assuming its K_m for acetylCoA is similar to that reported for CAT purified from other sources, see above). CAT is present in both the peroxisomes and mitochondria of C. tropicalis and Kawamoto et al. (91) propose that CAT participates in shuttling "activated acetate" from the peroxisome where it is formed exclusively to the mitochondria where it is a substrate for the TCA cycle.

At the other extreme are the oleaginous yeast, a group of yeast that accumulate lipids and are so far the only yeast found to contain ATP:citrate lyase (159). Non-oleaginous yeast grown on glucose must synthesize fatty acids to grow. Kohlhaw and Tan-Wilson (90) found high levels of CAT in a commercial preparation of Baker's yeast and suggested that acetylcarnitine shuttles acetyl groups out of the mitochondria for use in fatty acid synthesis when yeast are grown on non-fermentable substrates. Presumably in yeast grown on glucose, pyruvate is decarboxylated and dismutated to acetate and ethanol, the acetate being activated to acetylCoA in the cytosol and used for fatty acid synthesis. Direct evidence for either of these hypotheses is lacking.

Severe biosynthetic requirements are placed on yeast grown on glucose. As a result a number of synthetic pathways for various compounds depend on a condensation reaction between acetylCoA and an α-keto acid. Lysine is biosynthesized via the α-aminoadipate pathway and the first intermediate homocitrate, is formed by the condensation of acetylCoA with α-ketoglutarate catalyzed by homocitrate synthase (160). AcetylcoA is also required to condense with oxaloacetate forming citrate in order to produce α-ketoglutarate. The condensation of α-ketoisovaleric acid with acetylCoA catalyzed by α-isopropylmalate synthase is the first reaction in leucine biosynthesis (161). O-Acetylhomoserine is an intermediate in methionine biosynthesis in Neurospora (162) and N-acetylglutamate is a key intermediate of arginine biosynthesis in a number of organisms including Saccharomyces (163).

In 1957 Van Uden and Do Carma-Sousa (164) described a yeast isolated from the caecum of a cow and named it Candida bovina CBS 2760 (it should be noted that Candida bovina has been transferred to the genus

Torulopsis). The yeast does not form spores, ferments and assimilates only glucose, does not split arbutin, and does not assimilate ethanol or nitrate. C. bovina was very similar to Torulopsis pintolopesii and Kreger-vanRij took C. bovina to be the imperfect stage of Saccharomyces tellustris (165). Thus it is not unusual to compare the metabolism of T. bovina to that of Saccharomyces. These three yeast are part of a group of thermophilic yeast that are regarded as obligate saprophytes of warm blooded animals growing best at around 40°C although they do grow well at 30°C but poorly at 20°C or lower (165). Cury et al. (166) studied the nutritional requirements of this group of yeast and discovered that some of them including T. bovina needed choline to grow. The isolation of a carnitine-requiring strain of T. bovina has already been described (see above).

Watson and coworkers have studied the lipid and cytochrome content and composition of the wild type T. bovina ATCC 22987 with the following results. The yeast is a facultative anaerobe (167) but it is competent of respiration and its cytochrome content is significantly greater than that of other thermophilic yeast (168). The major phospholipids in T. bovina are phosphatidylcholine, cardiolipin, and phosphatidylethanolamine in nearly equal amounts. Palmitoleic acid is the predominant fatty acid component followed by oleic acid (169).

The above data are presented for comparisons sake; there are no data to indicate that the carnitine-requiring strain has the same properties as the wild type yeast. In fact, Watson's data suggests that T. bovina ATCC 22987 assimilates ethanol whereas the type description stated that ethanol was not assimilated. Thus Watson's data were collected with

yeast grown on ethanol and would not be expected to represent the biochemistry of glucose grown cells.

Restatement of the problem

With this introduction then, the problem is to determine the role of carnitine in a yeast grown on glucose, thus not oxidizing fatty acids nor presumably requiring any shuttle mechanism for acetylCoA in order to synthesize fatty acids. Because the electron transport chain is suppressed by glucose, carnitine is not expected to act as a high energy reservoir of acetyl units for consumption in the TCA cycle; the TCA cycle is used to supply growing yeast cells with biosynthetic intermediates, not to oxidize acetyl groups. Determining the metabolic role of carnitine in T. bovina ATCC 26014 may provide the first unequivocal demonstration of a function for CAT and its possible importance in the metabolism of other organisms.

EXPERIMENTAL METHODS AND RESULTS

MATERIALS AND METHODS

Materials

Sodium [1-^{14}C]acetate (58.3 Ci/mol) and standard [^{14}C]toluene were purchased from New England Nuclear (Boston, MA). The [^{14}C]acetate was used for the synthesis of [1-^{14}C]acetylcarnitine as recently described (170). Phthaldialdehyde and ethanethiol were obtained from Pierce Chem. Co. (Rockford, IL). Avicel coated uniplates were purchased from Analtech, Inc. (Newark, DE) and most of the solvents were purchased from Mallinckrodt (St. Louis, MO) except the acetonitrile used for HPLC analysis which was glass distilled, Omnisolv from MCB Manuf. Chemists, Inc. (Cincinnati, OH). Glass beads (0.45 mm) were purchased from Thomas Scientific Co. (Philadelphia, PA. Nephelometer flasks were purchased from Bellco Glass, Inc. (Vineland, NJ). L-carnitine·HCl was generously provided by Otsuka Pharmaceutical Co., Japan. All acylCoA derivatives were purchased from PL Biochemicals Inc. (Milwaukee, WI) but [1-^{14}C]acetylCoA as well as DL-[methyl-^{3}H]carnitine was purchased from Amersham (Arlington Heights, IL). Type H-2 β-glucuronidase from Helix pomatia was purchased from Sigma Chemical Co. (St. Louis, MO). Prepurified nitrogen and 5% CO_2 in nitrogen were purchased from Airco, Inc. (Montvale, NJ). All other chemicals were of reagent grade from commercial sources.

Organism

Torulopsis bovina ATCC 26014 was used throughout this study. It is the carnitine-requiring mutant of the parent *T. bovina* ATCC 22987 isolated as described by Travassos *et al.* (1).

Growth media

Yeast were grown on a modified Wickerham's yeast carbon base medium (171). Four stock solutions (A,B,C, and D) were prepared. Their composition is indicated in Table 1. Solution C was used in the preparation of Solution D. A stock solution of ten-times concentrated media was prepared consisting of 5 parts solution D supplemented with 2% (w/v) L-asparagine and 3 parts distilled water. This was sterilized by autoclaving 20 minutes at 15 psi and then combined with 1 part A and 1 part B. Growth media was prepared by dissolving the carbon source in 5 parts 0.1 M potassium phthalate buffer, pH 4.5, adding 4 parts water, autoclaving to sterilize, and then diluting the sterile concentrated media ten-fold into this solution.

The carbon source was glucose at 1% (w/v) final concentration. When present, L-carnitine was added to the media with the carbon source to a final concentration of 1 μg/ml or to the final concentrations indicated in the specific figures and tables. Basal media lacks carnitine.

Maintenance and growth conditions

T. bovina was grown at either 37°C or 30°C with L-carnitine included in all maintenance cultures. For most experiments, cells were grown in 7 ml of synthetic media in 18 x 150 mm test tubes or in 100 ml of media in 500 ml nepholometer flasks with an 18 mm diameter side arm. Cultures

TABLE 1. COMPOSITION OF STOCK SOLUTIONS FOR *T. BOVINA* MEDIA PREPARATION

A[a]

Ingredient	g/200ml
L-Phenylalanine	1.60
L-Tryptophan	0.50
L-Histidine	0.20
L-Methionine	0.20

B[b]

Ingredient	g/500ml
Choline-Cl	0.050
Biotin	0.005
Ca-Pantothenate	0.100
Thiamin	0.100
Pyridoxine	0.100
Nicotinic Acid	0.100
Inositol	0.500
Cytosine	0.500
Adenine	0.500

C[c]

Ingredient	mg/L
H_3BO_3	500
$CuSO_4$ $5H_2O$	40
$FeSO_4$ $7H_2O$	40
Na_2MoO_4	200
$ZnSO_4$	400
$CoCl_2$ $6H_2O$	40
$MnSO_4$ H_2O	400

D[d]

Ingredient	g/L
KH_2PO_4	20.0
$MgSO_4$ $7H_2O$	10.0
NaCl	2.0
KI	.002
$CaCl_2$ $2H_2O$	1.0
Trace Elements Solution C	20 ml

[a]100x Concentrated Amino Acid Solution: dissolve in the smallest volume of 1 N HCl, dilute to 180 ml, adjust the pH to 4.5, make the volume to 200 ml and autoclave to sterilize.
[b]100x Concentrated Vitamin Solution: dissolve the nucleosides in 1 N HCl and add to the vitamins in 400 ml water. Adjust the pH to 4.5 make the volume to 500 ml and autoclave to sterilize.
[c]1000x Concentrated Trace Elements Solution: add trace elements in order to 800 ml water, make the volume to 1 Liter and store in a brown glass bottle.
[d]20x Concentrated Salts Solution: dissolve the salts in order in 750 ml water. Predissolve the $CaCl_2 \cdot 2H_2O$ in water + 4-5 drops 1 N HCl. Adjust the pH to 4.5, make the volume to 1 Liter and add 4 ml $CHCl_3$ as preservative.

were shaken continously on a New Brunswick gyratory shaker and growth was monitored by measuring the absorbance of the cell suspension at 600 nm in a Coleman Jr. II spectrophotometer. Growth rates were calculated as the slope of the least squares line fitted to a plot of the logrithm of the absorbance ($Log_{10}A$) versus incubation time in hours. In Figure 1 the equation for this line was used to calculate relative incubation time by taking as time zero that point at which the cells reached a density equivalent to 0.01 absorbance. Exponential phase cells were used for the inoculation of all test media. Cells were harvested by centrifugation and washed three times with phthalate buffered saline (potassium phthalate, 10 mM; KH_2PO_4, 7.4 mM; NaCl, 137 mM; KCl, 2.7 mM; pH 4.5). The cells were suspended in phthalate buffered saline to an absorbance of 0.1, the cell density being about 3×10^6 cells/ml. This suspension was diluted 100 fold and a 0.1 ml sample was used to inoculate a 7 ml culture and a 1.0 ml sample was used to inoculate a 100 ml culture.

Anaerobic cultures were obtained by purging flasks containing media with gas filtered first through sterile cotton then through sterile water to saturate it with water vapor and finally through a glass tube plugged with sterile cotton. The flasks were sparged with gas for 1-2 hours, inoculated with cells, and then gassed another 30 minutes before sealing them with a bunsen valve. A fish tank air pump hooked to a similar gas train was used to continuously aerate aerobic cultures.

Uptake of radioactive carnitine

Cells were grown in 100 ml of the synthetic media without added carnitine. They were harvested by centrifugation and suspended in 5 ml of ice cold uptake buffer (KH_2PO_4, 10 mM; $MgSO_4 \cdot 7H_2O$, 1 mM;

NaCl, 50 mM; KCl, 2.7 mM; pH 4.5). A 0.5 ml sample was removed for dry weight determination. Carnitine uptake was determined by adding 0.2 ml of DL-[methyl-^{3}H]carnitine solution (1.18 μCi at .25 Ci/mmol diluted with 55.6 nmol unlabeled L-carnitine·HCl) to 5 ml of uptake buffer warmed to 37°C and mixing this solution with 4.5 ml of cold cell suspension at time zero. The cells were incubated in 25 ml Erlenmeyer flasks at 37°C in a Dubnoff water bath. At the times indicated (the first time point was 10 sec) 1 ml samples were removed, collected on glass fiber filters using a millipore apparatus, dried at 60°C for 8 hours, weighed and then placed into scintillation vials. The filters were wetted with 1 ml water, 10 ml of Triton X-100 based scintillation cocktail was added (173) and the cell associated tritium counted with 15% efficiency.

Carnitine analysis

Carnitine analysis was a modification of the methods of Cederblad and Lindstedt (174) and Parvin and Pande (175). The reaction was buffered at pH 7.6 and contained in 0.2 ml:288 pmol [1-^{14}C]acetylcoenzyme A (0.0167 μCi), 50 μg N-ethylmaleimide (prepared fresh on day of use), 20 μmol potassium phosphate, and the sample (in 100 μl) to be analyzed. This was used to assay 1-20 pmol L-carnitine. Unlabeled acetylCoA (333 pmol) was added to assay 10-200 pmol of L-carnitine. The reaction was initiated by the addition of 25 μℓ of CAT (0.425 units/assay) and was terminated by putting a 200 μℓ sample into a column (5 x 25 mm) of Dowex 1-x8 (100-200 mesh) in chloride form, washing it through with 1 ml of water and collecting the total eluate in a scintillation vial for detection of ^{14}C. The enzymatic blanks were very low and equal to the non-enzymatic blanks when phosphate buffer was used.

Duplicate analyses were performed on each sample with and without a known amount of L-carnitine added and the sample values were corrected to 100% recovery of the added L-carnitine.

Carnitine was generally extracted from cells by boiling for 3 min. The data in Table 2 shows that boiling carnitine and/or acetylcarnitine solutions made up in basal media did not interfere with their analysis and that essentially 100% of the added carnitine was recovered after saponification of the sample. Total carnitine was determined in all samples after saponification of the extract in 0.2 N KOH for at least 30 min at 50°C after which a solution of potassium phosphate, pH 7.6, was added to 0.1 M final concentration to aid neutralization to pH 7.6 with HCl prior to analysis.

The endogenous carnitine content of T. bovina was determined as follows. Cells were grown for 36 h in basal media (no carnitine present), harvested, washed, and then inoculated into three flasks each containing 1 L of basal media. The cells were harvested in the late exponential phase of growth (0.5-0.6 absorbance), collected by centrifugation and extracted by boiling 5 min with 2 volumes of distilled water. Cell debris was removed by centrifugation and the pellet washed twice with 2 volumes of water. The hot water extracts were lyophilized and then made to a known volume with water. This solution was assayed directly for carnitine and total carnitine was assayed after saponification of a measured sample of the extract.

Carnitine uptake and its esterification by whole cells was measured as follows. Cells were grown at 30°C in media supplemented with 5 μM L-carnitine. Samples were taken at various times after inoculation and the cells collected by filtration on Whatman GF/C glass fiber filters

TABLE 2. CARNITINE RECOVERIES IN BOILED MEDIA SOLUTIONS

Sample	Carnitine Found nmol/100 ml culture
1. Stock L-carnitine solution (5 μM)	470
2. 5 μM L-carnitine basal media	468
3. Basal media	<15
4. 5 μM acetylcarnitine	<15
5. 5 μM carnitine + 5 μM acetylcarnitine in basal media	502
6. Same as 5 plus boil 3 min	519
7. Same as 6 plus saponification	1006

using a Millipore apparatus. The filtered media was assayed directly for free carnitine while the cells and filters were immersed in 2 ml water in a capped culture tube and placed into a boiling water bath for 3 min. The filter and cell debris were removed by centrifugation and the hot water extract analyzed for free carnitine. Total carnitine was determined in saponified samples of the media and cell extracts and esterified carnitine was calculated from the difference between the total and free carnitine values.

The amount of acetylcarnitine synthesized by the yeast was determined in cells grown with 5 μM L-carnitine in 1 L of media in 2.8 L Fernbach flasks stoppered with cotton. Cells were harvested by centrifugation in the late exponential phase of growth and extracted by shaking 1 g of wet packed cells 4 min with 10 g of 0.45 mm glass beads in 4 ml of 6% (w/w) $HClO_4$. The extracts were filtered on fritted glass Buchner funnels, the filtrates neutralized to pH 6.5 with KOH and centrifuged to remove salt. Acetylcarnitine was assayed in the acid extracts enzymatically by coupling to the formation of citrate as described previously (176).

Fermentation analysis

Cells were grown on 1% glucose at 30°C with or without L-carnitine. Samples of the cultures were taken during the measurable stage of exponential growth and the fermentation fluid separated from the cells by centrifugation for 10 min at 10000 x g. The supernatant fluid was analyzed in duplicate for glucose, ethanol, acetate, and lactate. The assay procedures for glucose, ethanol, and lactate were taken from Bergmeyer (177-179). Acetate was assayed by the method of Guynn and

Veech (180). Standard curves were generated for each compound in its respective assay and all samples were listed in duplicate for recovery of added standard. In a different experiment, 100 ml cultures were grown without phthalate, one with and one without carnitine added, and the spent media (88 ml) was lyophilized and redissolved in 10 ml of water. Samples (1 ml) were then acidified, saturated with NaCl and extracted with ether. The ether extracts were analyzed by gas chromatography for volatile fatty acids (181).

RADIOACTIVE TRACER STUDIES

Growing cells were incubated for various times with either [1-^{14}]acetate or [1-^{14}C]acetylcarnitine (approximately 5 μM with the exact concentrations given in the individual experiments). The cells were then fractionated into various components and analyzed for ^{14}C content as described in the following sections.

Lipid extraction

Cells were grown in 100-200 ml of media to an absorbance of 0.2 at which time [^{14}C]acetate or [^{14}C]acetylcarnitine was added and the cells allowed to grow for 2 h more. The cells were then collected by centrifugation, washed with cold distilled water, and pelleted in 50 ml, thick-walled glass centrifuge tubes. The cells were treated with 0.5 ml of saturated KOH solution plus 15 ml of 95% ethanol and a couple boileezer glass chips added. The tube was heated, uncovered, in a glycerol bath at 80°C. When the volume was reduced to 0.5-1 ml, the tube was cooled and the neutral lipids extracted with 2 or 3, 10 ml volumes of

petroleum ether or until no further ^{14}C was extracted. The residual solution was treated with one drop of cresol red indicator solution (0.1 g in 26.2 ml of 0.01 N NaOH plus 223.8 ml water) and acidified with H_2SO_4 (concentrated acid diluted 1 to 2 with water). After the second color change to red, the acidified lipids were extracted as described above with petroleum ether. Warm water (25-50 ml) was then added to the residual cell material most of which dissolved. This solution was centrifuged and decanted to obtain the soluble non-lipid fraction and the insoluble residue. The ^{14}C in the neutral lipids, acidic lipids, soluble non-lipids and insoluble residue was determined by scintillation counting as described below.

Phenol extraction

Cells were harvested by centrifugation at 2000 x g for 10 min and ruptured by shaking 1 g of cells (wet weight) with 20 g glass beads and 10 volumes water until >90% of the cells were broken as judged by microscopic observation. The glass beads were removed by filtration on a coarse fritted glass buchner funnel and the homogenate repeatedly centrifuged at 860 x g for 2 min until no further pellet was formed. All the pellets were combined and washed with water by similar repeated centrifugation until negligible radioactivity could be washed from the pellet. This low speed pellet constituted the cell wall fraction. The pellet washes were combined with the original 860 x g supernatant fluid and acidifed with concentrated $HClO_4$ to a final concentration of 6% (w/v). Denatured protein was collected by centrifugation at 10,000 x g for 10-15 min. The supernatant fluid was decanted, neutralized to pH 7 with KOH, removed of salt by centrifugation and assayed for acid soluble

radioactivity. For protein isolation, the denatured protein pellet was dissolved directly in phenol. The protein pellet was first treated with chloroform-methanol (1:1) to extract lipids. The lipid extraction was repeated 3 or 4 times with 2 ml solvent, the pellet being dispersed each time by sonication and re-collected by centrifugation. In some instances, the lipid extracted protein pellet was hydrolyzed 18 h at 37°C in 0.3 N KOH (182) to isolate ribonucleotides which were analyzed as described elsewhere (183). Otherwise, the lipid extracted protein pellet was dissolved in ammoniacal phenol and acetone precipitated essentially as described previously (184).

Protein hydrolysis

About 4 mg of methanol washed, dry protein isolated as described above, was hydrolyzed in constant boiling HCl containing 2% thioglycollate (185) and a crystal of phenol as described by Moore and Stein (186). Hydrolysis was performed for 72-96 h, the hydrolysate centrifuged and transferred to a small boiling flask with 1 ml of 1 N HCl and evaporated under reduced pressure to dryness. The condensing flask of the rotary evaporator contained 25 ml of 1 N KOH solution cooled in ice to trap any [^{14}C]acetate. The amino acids were dissolved in 0.5 ml of water and the pH adjusted to 7-8 with KOH. Samples were removed for determination of ^{14}C and amino nitrogen. Amino nitrogen was assayed with ninhydrin using leucine as standard (187).

Amino acid analysis

The amino acids in the protein hydrolysate were isolated by reverse phase HPLC chromatography of the phthaldialdehyde derivatives essentially

as described by Schubert and Coker (188) using a Beckman HPLC system connected to an Aminco Fluoro-colorimeter interfaced to a Hewlett-Packard integrator. The eluate from each run was collected in 0.5 min fractions directly in mini vials and combined with 4 ml of Triton X-100 based scintillation cocktail and enough 67% ethanol to obtain a homogenous solution for detection of ^{14}C.

Heyns and Walter (189) described a paper chromatographic procedure in separating isomers of leucine and isoleucine which we combined with solvent system II of vonArx and Neher (190) to effect the specific separation of lysine, leucine, isoleucine, histidine, and proline. The amino acids were separated on cellulose TLC plates by development in the first direction for 3 h with isopropanol formic acid (88%), water (160:9:39) and in the second direction, twice, for 3 h with pyridine, amyl alcohol, water (35:35:30). Amino acids were visualized with ninhydrin spray reagent (191). Radioactivity associated with the amino acids was detected by running two identical plates, spraying one with ninhydrin and using it as a template for the second plate. The second plate was still lightly sprayed with ninhydrin to be sure that leucine and isoleucine were properly separated. The spots were scraped from the second plate, placed into scintillation vials with 1 ml water and 10 ml Triton based cocktail and counted for ^{14}C.

Radioactivity measurements

Radioactivity in all aqueous and particulate fractions was measured by scintillation counting in a Triton X-100 based cocktail (173) and all lipid fractions were counted in an identical cocktail in which toluene replaced the Triton. Lipid fractions in chloroform were evaporated under

a stream of N_2 and redissolved in absolute ethanol prior to assaying ^{14}C. DPM were determined by the channels ratio method of quench correction or by inclusion of an internal standard ([^{14}C]toluene). All samples were counted on a Packard Tri-Carb spectrometer.

CAT STUDIES

Preparation of mechanically disrupted cell-free extracts

Cells were collected by centrifugation and washed once with ice cold 50 mM phosphate buffer, pH 6.5 in either a tared or graduated centrifuge tube. The cells were packed by centrifugation at 2000 x g for 10 min and the wet weight or packed cell volume recorded. For disruption, the cell pellet was suspended in 20 volumes of cold buffer and a 4 ml sample combined with 8 g glass beads (0.45 mm) in an 18 x 150 mm capped culture tube. Higher cell densities, i.e., only 5-10 volumes cold buffer per g wet weight of cells, were used when the extract was to be applied to a sorbitol gradient. The mixture was agitated at full speed with a Vortex-Genie for 2 min unless stated otherwise. The homogenate was siphoned off with a Pasteur pipet and the glass beads washed with 4 ml buffer. The homogenate and washes were combined and "crude extracts" prepared by centrifuging the solution for 10 minutes at 500 x g to sediment whole cells and cell wall debris. Crude extracts were used immediately or frozen at -80°C. Enzyme levels were measured in homogenates prepared in 50 mM phosphate buffer, pH 6.5 and differential centrifugation was performed on crude extracts prepared in sorbitol buffer (sorbitol, 0.45 M; Tris·HCl, 10 mM; KH_2PO_4, 5 mM; K_2HPO_4, 5 mM; NaCl, 50 mM; pH to 6.5).

Samples (about 5-7 mg protein) of cell-free homogenates prepared in 0.25 M sorbitol, 50 mM phosphate buffer, pH 6.5 were layered on top of 32 ml linear gradients of 10-40% (w/w) sorbitol in 50 mM phosphate buffer, pH 6.5, containing 2 ml cushions of 55% (w/w) sorbitol in the same buffer. Centrifugation was performed in a Beckman SW 27 rotor in a Beckman L2 Ultracentrifuge run at 22,000 rpm for a period of 16-18 hr at 4°C. Subcellular particles were collected by puncturing the tube from the bottom and collecting 1.5 ml fractions. The fractions were either kept at -80°C or they were made to 0.1% (w/v) in sodium azide and kept at 4°C.

Preparation of spheroplasts and isolation of mitochondria

Spheroplasts were prepared and lysed as described by Linnane and Lukins (192) except 1 ml of β-glucuronidase was added to 5 ml of cell suspension and 0.5 ml β-mercaptoethanol was added to 100 ml of cell suspension. Also, spheroplasts were lysed by dilution without further treatment in the French Press. Spheroplast formation was followed by measuring the decrease in turbity and was about 90% effective in 40 min as judged by microscopic observation. Mitochondria were isolated by differential centrifugation or purified by isopycnic density centrifugation in linear gradients of 15-66% (w/w) sorbitol in 1 mM Tris·carbonate buffer, pH 7.0 and 1 mM EDTA. Centrifugation was performed in a Beckman SW 27 rotor at 22,000 rpm for 3 h at 4°C. Subcellular organelles were collected by puncturing tubes from the bottom and collecting 1.3 ml fractions.

Enzyme assays

CAT was assayed in the presence of 0.4 mM acetylCoA in either Buffer I (0.1 M Tris, pH 8.0, 1.1 mM EDTA, 1.25 mM L-carnitine, 0.15 mM DTNB, 0.1% (v/v) Triton X-100) or Buffer II (50 mM glycylglycine, pH 8.2, 1.25 mM L-carnitine, 25 mM $MgCl_2$, 0.15 mM DTNB) as previously described (70). Rates were recorded continuously at 25°C using a Gilford 250 or Zeiss PM6 spectrophotometer. Citrate synthase was assayed exactly as CAT using Buffer II except 10 mM cis-oxalacetic acid was substituted for L-carnitine. Fumarase was assayed as described by Hill and Bradshaw (193). Catalase was assayed as described in Bergmeyer (194). Isocitrate dehydrogenase was assayed as described by Cook and Sanwall (195) using either NAD^+ or $NADP^+$. Glucose-6-phosphate dehydrogenase was assayed in a 0.2 ml reaction mixture containing 4 mM glucose-6-phosphate, 0.26 mM $NADP^+$, 50 mM Tris buffer, pH 7.3, and 0.1 ml of enzyme solution. NADH (and NADPH) dehydrogenase was assayed according to Mackler (196). Malate dehydrogenase was assayed by following the reduction of oxalacetate according to Kitto (197). α-Oxoglutarate dehydrogenase was assayed by following the reduction of NAD^+ according to Reed and Mukherjee (198). The assay for α-glucosidase (maltase) was by the method of Halvorson (199). NADPH-cytochrome c reductase was assayed as described by Masters et al. (200) assuming an E_{550} of 19.7×10^6 $mole^{-1}cm^2$. AcetylCoA synthetase was assayed spectrophotometrically by coupling citrate synthase and malate dehydrogenase to the reduction of NAD^+ as modified by Pearson (201). The assay contained 100 mM Tris·HCl and 15 mM L-malic acid adjusted to pH 7.8, 2.4 mM dipotassium ATP, 3.0 mM $MgCl_2$, 0.25 mM NAD^+, 0.1 mM NADH, 1.60 mM CoASH (free acid), 1.8 mM potassium acetate, 1 unit malate dehydrogenase, 0.4 units citrate

synthase, and enough enzyme solution and water to bring the final volume to 0.25 ml. The blank lacked acetate. ATP-citrate lyase was assayed by both the hydroxamate method and the spectrophotometric method described by Takedo et al. (202) except glutathione was substituted for mercaptoethanol. Citrate lyase was assayed as described by Dagley (203).

Protein determinations

Protein was determined using Coomassie Blue G250 (204). Protein, 0-50 mg, in a volume of 0.1 ml was mixed with 5 ml of the dye reagent and its absorbance measured at 605 nm. The dye reagent was prepared by dissolving 300 mg Coomassie Blue G250 (Brilliant Blue G, Lot No. 18C-0132, Sigma Chemical Co., St. Louis, MO) in 50 ml absolute ethanol, adding 70 ml of 60% (w/v) $HClO_4$, and making the volume to 2 liters. The reagent was filtered and the absorbance at 605 nm adjusted to 0.2 by dilution with 2.1% $HClO_4$. The dye dissolves readily in absolute ethanol but only with difficulty in 95% ethanol. The 2.1% $HClO_4$ produced the best linear response with BSA as standard, but other batches of dye may require a different concentration of $HClO_4$.

RESULTS

GROWTH STUDIES

Carnitine stimulation of growth

Very low concentrations of carnitine promote the growth of the yeast, Torulopsis bovina ATCC 26014 (1) but the published data do not show whether carnitine affects cell size, final cell density, lag time or growth rate. The data in Figure 1 show that cells grown in media supplemented with 1 µg/ml L-carnitine grow at nearly twice the rate of control cells grown without carnitine. Similar results were obtained with cells grown at 30°C or 37°C; see Table 3. Cultures grown on 1% glucose with or without carnitine entered the stationary phase at approximately the same cell density (0.6 absorbance) indicating that carnitine did not noticeably affect cell yield.

One attempt was made to count cells. Cell numbers varied linearly with the absorbance of the culture, see Figure 2, but the ratio of cell number to absorbance (the slopes of the lines in Figure 2) was different when carnitine was present than in the control. Microscopic examination of the cultures revealed that the carnitine supplemented culture contained mostly quadruplet strings of cells whereas the control culture contained mostly doublet and single cells. Thus the accelerated growth rate induced by carnitine caused the morphological appearance of the yeast to change. The data in Table 4 show that the dry weights of cells

Figure 1. Effect of carnitine on the growth rate of T. bovina. Cells from an exponentially growing culture containing 1 µg/ml L-carnitine were diluted to 400 cells/ml and incubated at 30°C. Δ--Δ; no added carnitine; O—O, 1 µg/ml L-carnitine added.

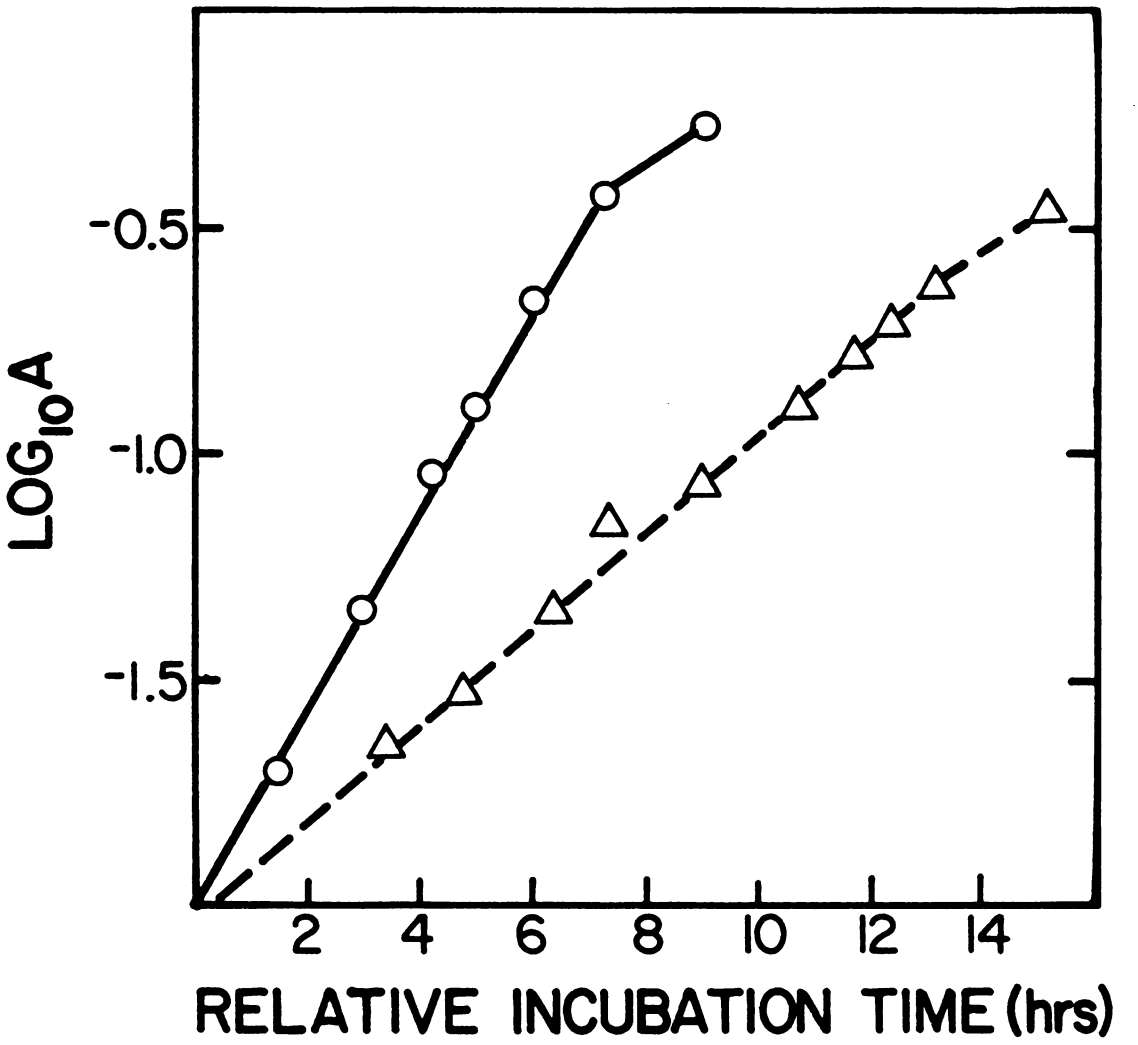


Figure 1

Figure 2. Covariance of cell number and culture absorbance. Cells were grown with 6 mM glucose in 100 ml of media at 30°C. Samples were removed at the indicated absorbance values and cells counted using an haemocytometer. The data represent the results obtained from duplicate cultures. ●—●, cells grown in basal media. ▲—▲, cells grown in basal media plus 5 μM carnitine.

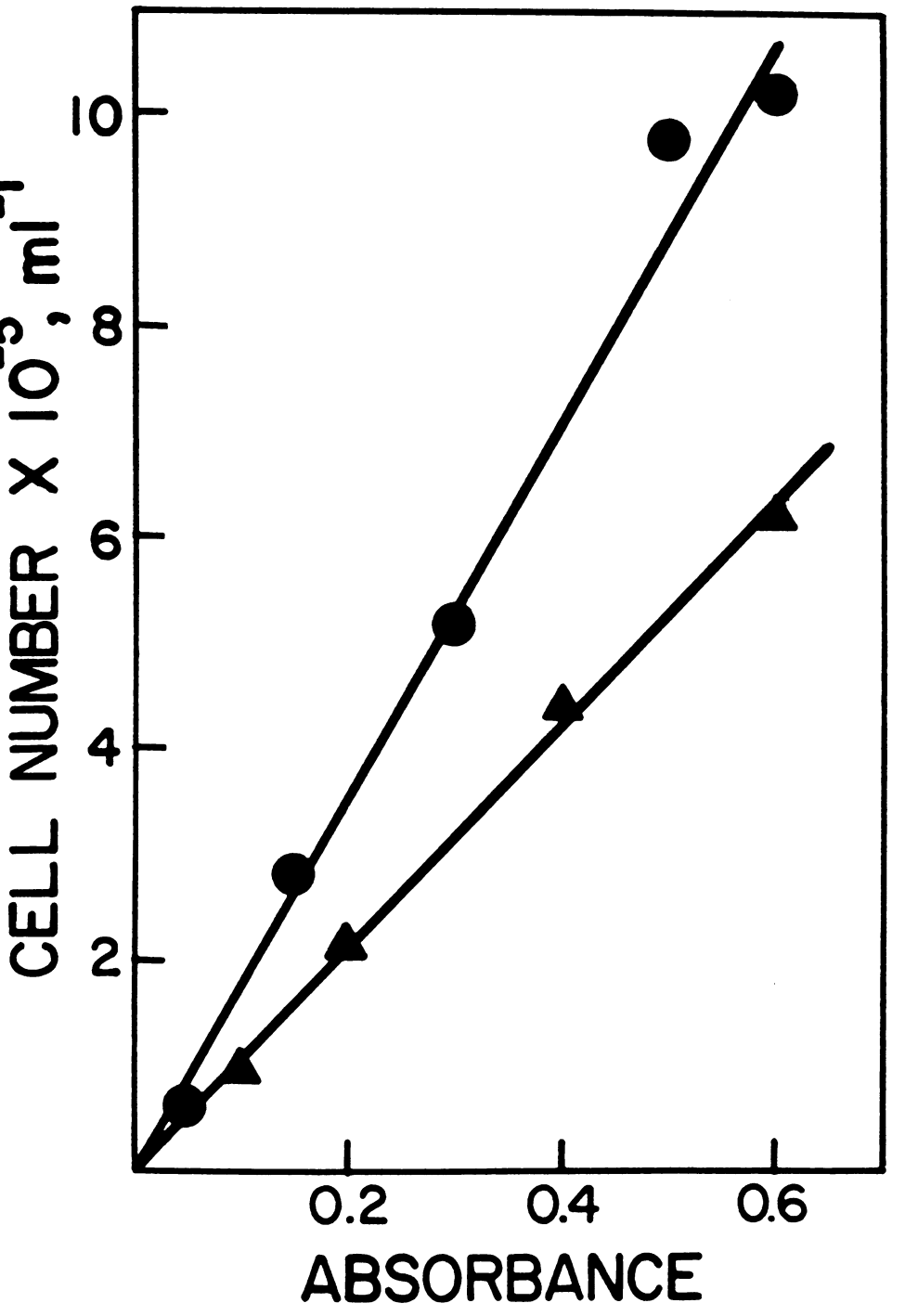


Figure 2

TABLE 3. THE EFFECT OF L-CARNITINE ON YEAST GROWTH RATE

Growth Temperature	Growth Rate (H^{-1}) L-carnitine	Control
37°C	.286 ± .045 (n=23)	.176 ± .039 (n=18)
30°C	.180 ± .010 (n=13)	.111 ± .013 (n=7)

For each experiment the same inoculum cells were added to a group of tubes containing either control media (no carnitine added) or media containing 1 μg/ml L-carnitine and the cultures incubated at the indicated temperature. The total number of cultures analyzed in all the experiments is indicated in parenthesis. The L-carnitine rate differs from the control at a significance level of $p<0.01$ by the Wilcoxon signed rank test (172).

TABLE 4. EFFECT OF CARNITINE ON CELL YIELD

Experiment	mg dry weight per unit absorbance[a]	
	+ Carnitine	- Carnitine
1	1.28	1.17
2	1.33	1.20
3	1.35	1.33
Average	1.32 ± .04	1.23 ± .08

Yeast were grown with or without 5 μM carnitine as shown and harvested in the late exponential phase of growth. A sample of each cell suspension was then filtered and the cells dried 24 h at 60°C before weighing.

[a]Values were normalized by calculating the ratio of the cell dry weight (in mg/ml of culture) to the culture absorbance at the time of harvest.

from cultures grown with or without carnitine are the same (7% difference between the average values) and that the absorbance is proportional to the total cell mass.

If carnitine affected glycolytic metabolism, this might be reflected in the fermentation balance. The data in Figure 3 demonstrate that I. bovina produces ethanol in approximately equal quantity to the amount of glucose consumed. Carnitine did not have a major effect on the fermentation balance. However, in other experiments, it was shown that the pH of the media decreased from 4.5 to 3 in the absence of carnitine but remained at 4.5 when carnitine was added. Addition of 0.05 M phthalate maintained the pH at 4.5 under all growth conditions without altering the growth response. Thus the slower growth rate in the absence of carnitine was not due to a decrease in pH. Analysis of the fermentation liquor indicated that lactic acid was not produced in measurable quantity nor were any standard volatile fatty acids produced. If acetic acid was produced, it was present at less than 40 μmol/L. When phthalate was left out of the media, the amount of titratable acid produced in cultures without added carnitine was 2 meq/L.

Effect of air and anaerobiosis on carnitine stimulated growth

The effect of carnitine on growth could be associated with this yeast's aerobic or anaerobic metabolism. To explore this possibility, growth tests were conducted in test tubes containing synthetic media to a height of 15 cm, one series with and one without added carnitine. The yeast grew equally well in both except in the top 1-2 cm of the tubes without carnitine where growth was retarded during the first 24 hours of incubation. This suggested that growth was inhibited by air and

Figure 3. The amount of glucose fermented to ethanol during exponential growth. Cells were grown with 6 mM glucose in 100 ml of media at 30°C. Samples of the cultures were taken at the indicated cell densities and the media analyzed for glucose and ethanol as described in the Materials and Methods. The data represent the results obtained from duplicate cultures. A, cells grown in basal media plus 5 μM carnitine. B, cells grown in basal media. ● ●, glucose. ▲ ▲, ethanol.

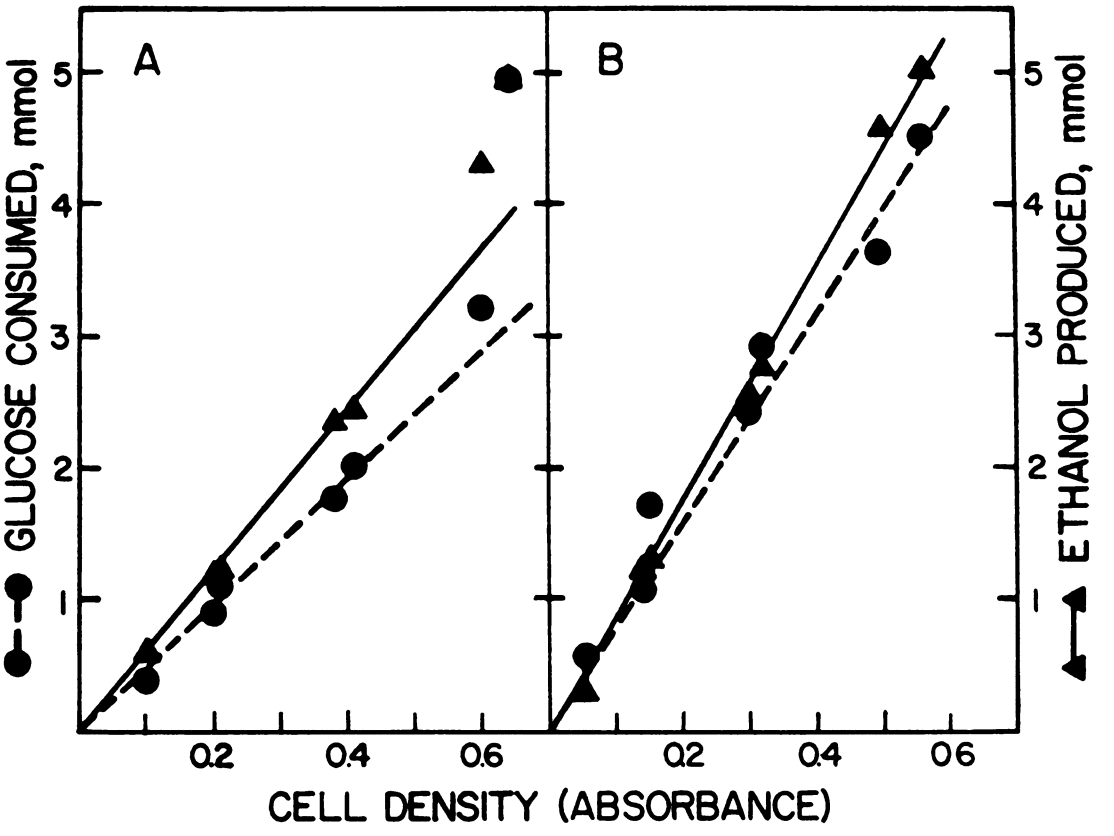

A
B
GLUCOSE CONSUMED, mmol
ETHANOL PRODUCED, mmol
1
2
3
4
5
0.2
0.4
0.6
CELL DENSITY (ABSORBANCE)

Figure 3

carnitine was somehow able to counteract the inhibition. We tested this directly by purging cultures with (95:5) N_2/CO_2, a purified gas mixture which contained less than 15 ppm total contaminants. Figure 4 shows growth in this gas mixture and the growth curves measured under air or pre-purified nitrogen. The growth rates in the absence of added carnitine were .17, .20, and .13 h^{-1} and in its presence .32, .31, and .33 h^{-1} under N_2/CO_2, nitrogen or air, respectively. Carnitine stimulated the growth rate to the greatest extent in aerated cultures, but it had an almost equal stimulatory effect in anaerobic cultures. Air presumably did not leak into the flasks during the period of incubation as indicated by the finding in Figure 4 that the gas phase had a marked and characteristic effect on the relative lag time of the cultures. In this series of experiments, all cultures were started at 0 time. Two other cultures were grown anaerobically under N_2/CO_2 gas with 1 mg/ml chloramphenicol to prevent mitochondrial development. In these latter cultures, the growth rate in the absence of carnitine was 0.17 h^{-1} and 0.31 h^{-1} in its presence.

Figure 4 also shows that the lag times of the cultures are affected by the composition of the gas phase. The pH of the medium (4.5) was not altered by purging it with N_2/CO_2 gas nor was the lag time of aerobic cultures reduced by adding 10 mM sodium bicarbonate to media buffered at pH 6.5. The reversed lag times for the plus and minus carnitine cultures grown under nitrogen were noted but not investigated.

The data in Figure 4 indicate that carnitine affects this yeast's anaerobic as well as its aerobic fermentative metabolism. All attempts to culture the yeast on a carbon source other than glucose were unsuccessful; see Table 5. Acetate was tested at both 30°C and 37°C to

Figure 4. Effect of aerobic and anerobic culture conditions on growth. Cells were inoculated at time zero into 100 ml of media in flasks purged with the indicated gas. N_2/CO_2 is 5% CO_2 in 95% N_2. ± CARN indicates the presence or absence of 5 μM carnitine in the media. All cultures were incubated at 37°C.

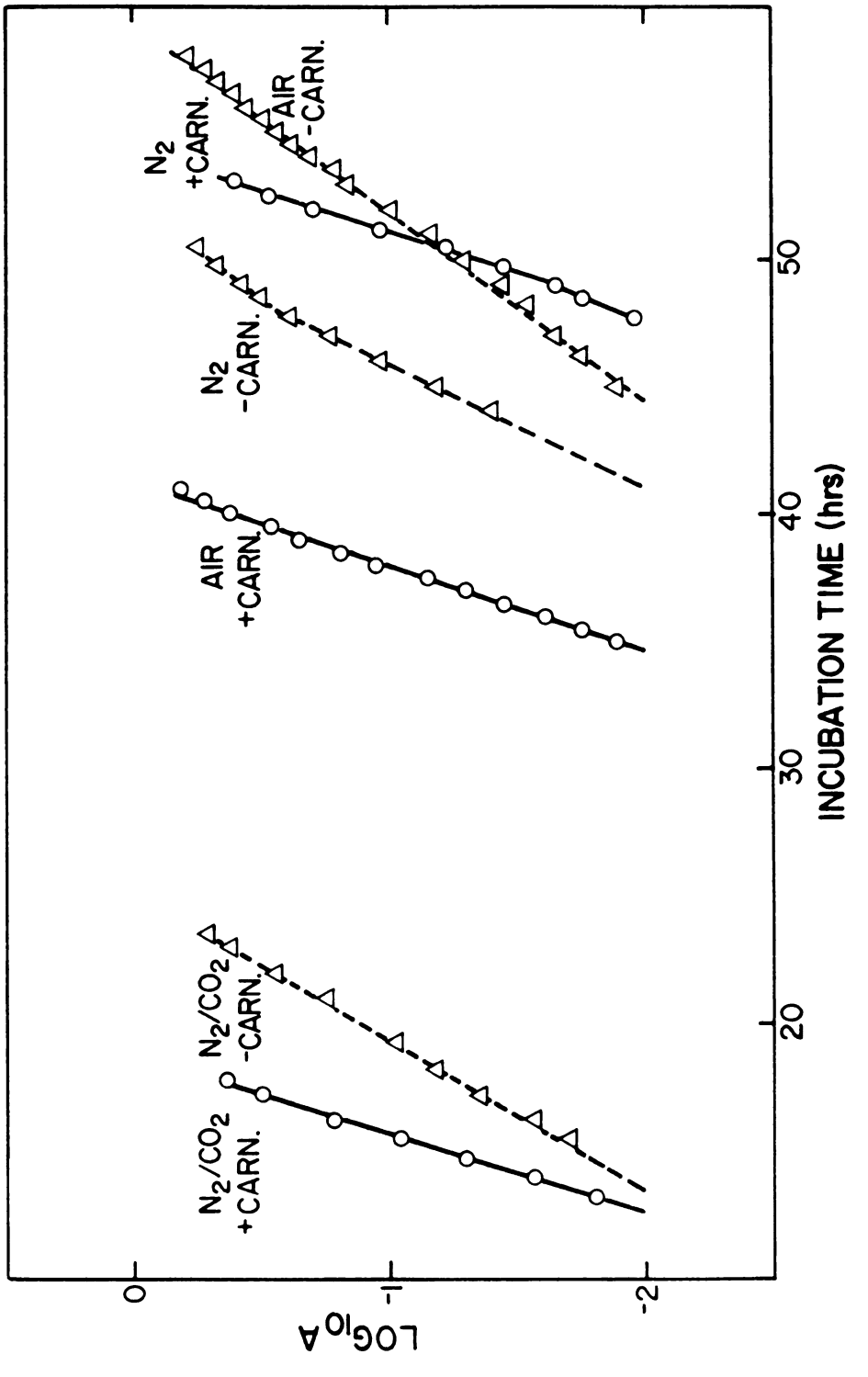

LOG10 A
0
-1
-2
20
30
40
50
INCUBATION TIME (hrs)
N2/CO2 +CARN.
N2/CO2 -CARN.
AIR +CARN.
N2 -CARN.
N2 +CARN.
AIR -CARN.

Figure 4

TABLE 5. EFFECT OF DIFFERENT CARBON SOURCES ON GROWTH

Growth Characteristics	Compounds Tested	
Rapid, Dense Growth	Glucose	
Poor Growth in Complex Media[a]	acetate glycerol	malate + acetate succinate malate
NO GROWTH	propionate n-butyrate isobutyrate n-valerate isovalerate caproate caprylate citrate valine + isoleucine	carnitine choline ethanol galactose lactate glutamate valine isoleucine

Tests were conducted at 37°C in tubes with loosely fitting caps. All compounds were tested at a final concentration of 1%(w/v) except ethanol which was added to 2% (v/v). Acids were neutralized with NaOH before addition to the media.

[a]Carbon source 1%, yeast extract 0.5%, peptone 1%.

be sure that the elevated temperature (37°C) did not prevent the yeast from growing on non-fermentable substrates but the cells still failed to grow. During studies utilizing isolated mitochondria, a difference spectrum was run on a sample of mitochondrial suspension and, as shown in Figure 5, cytochromes c, b, and aa_3 are present in this yeast.

Response time of cells to carnitine addition/depletion

In order to determine if the cell's response to carnitine required a period of induction, a series of flasks were inoculated with 400 cells/ml and purged with N_2/CO_2. Carnitine was added successively to each flask at 2 hour intervals over a ten hour period. One flask did not receive carnitine. The time courses for the growth of these cultures were essentially the same as the N_2/CO_2 labeled growth curves depicted in Figure 4. Each culture with carnitine had a greater growth rate than the one without, see Table 6, suggesting that not more than 3 hours exposure to carnitine was required before the growth rate was stimulated. The inoculum in this experiment was a 28,000-fold dilution from carnitine containing media and, if all the carnitine had been cell associated, only 1.25 pmol of carnitine was carried over in the inoculum. Apparently, this small amount of carnitine does not support maximum growth rates.

Figure 6 demonstrates what happens to the growth rates when the inoculum cells are diluted 37,800-fold in two steps (data obtained at 30°C). In step one, panel A, the cells were diluted 135-fold from carnitine containing media (5 μM) into carnitine free media. No change in growth rate was observed, but when this culture was further diluted 280-fold, the growth rate declined, as seen in panel b. The cells in

Figure 5. Difference spectrum of *T. bovina* mitochondrial cytochromes. Mitochondria were isolated as described in Methods and clarified with lauryl maltoside (3.3% final concentration). Cytochromes were reduced and oxidized by adding a few crystals of sodium dithionite to one cuvette and potassium ferricyanide to the other and recording the difference spectrum on a Perkin-Elmer UV/visible spectrometer, model 559. Scan speed 60 nm/min; 2 nm slit width.

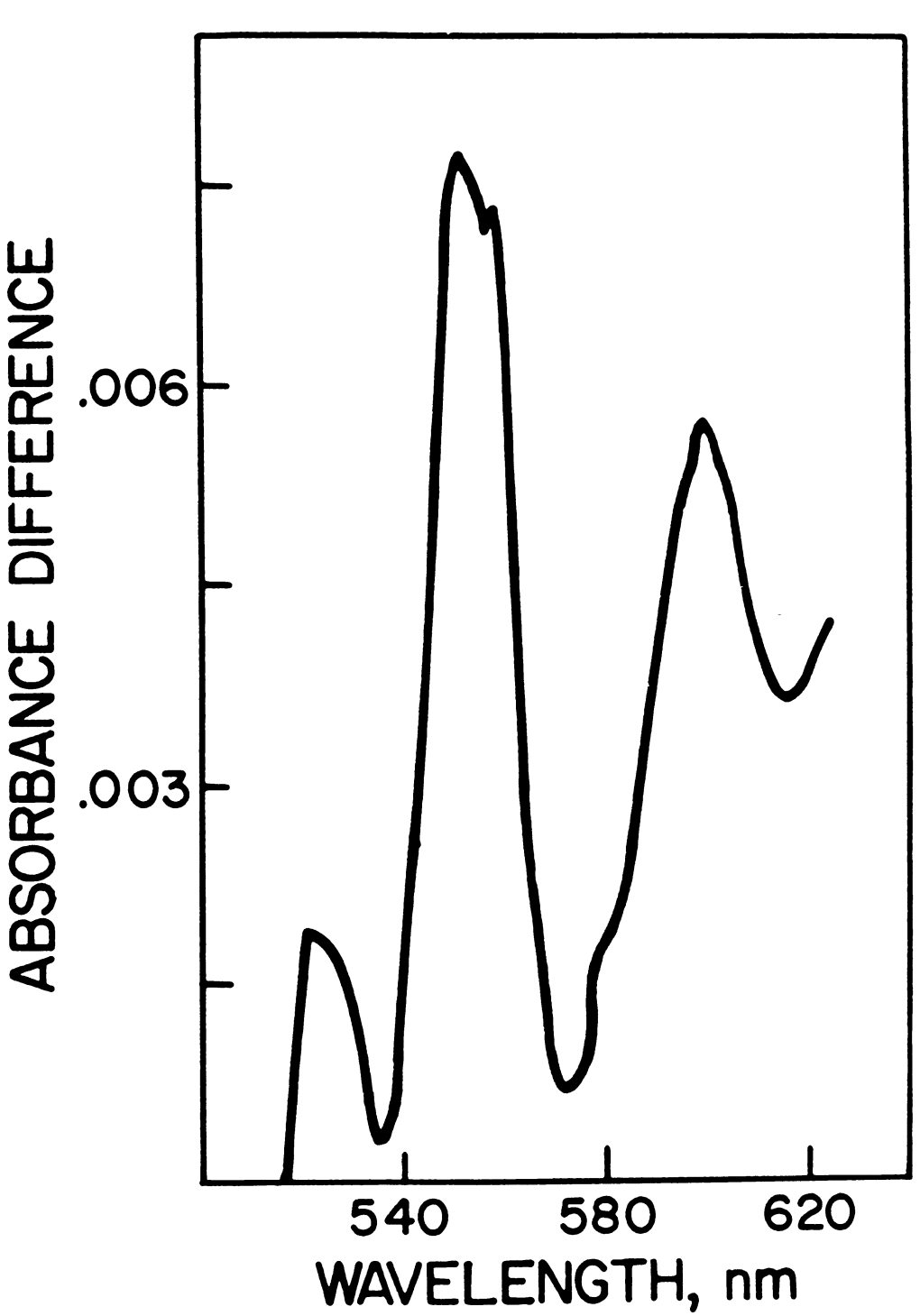


Figure 5

TABLE 6. GROWTH RATES WITH CARNITINE ADDED AT VARIOUS TIMES AFTER INCUBATION

Time of Carnitine Addition, h	0	2	4	6	8	10	No Addition
Growth Rate, h^{-1}	.33	.35	.32	.34	.33	.34	.17

Yeast were grown at 37°C in 100 ml of media purged with N_2/CO_2. Each flask was inoculated with 200 cells/ml at time zero and carnitine added at the indicated times after inoculation. Each culture was regassed for 15 min after adding carnitine.

Figure 6. Effect of dilution of cell-associated carnitine on growth rate. Inoculum cells were grown on 1 μg/ml L-carnitine and then diluted 135-fold into carnitine free media (A). These cells were then harvested and diluted again 280-fold in (B) into carnitine free media. Cells from B were harvested and diluted 330-fold into plus and minus carnitine containing media (C). Cells were grown at 30°C and the growth curves of carnitine containing control cultures are included in (A) and (B). ●—●, 1 μg/ml L-carnitine present; △–△, carnitine absent.

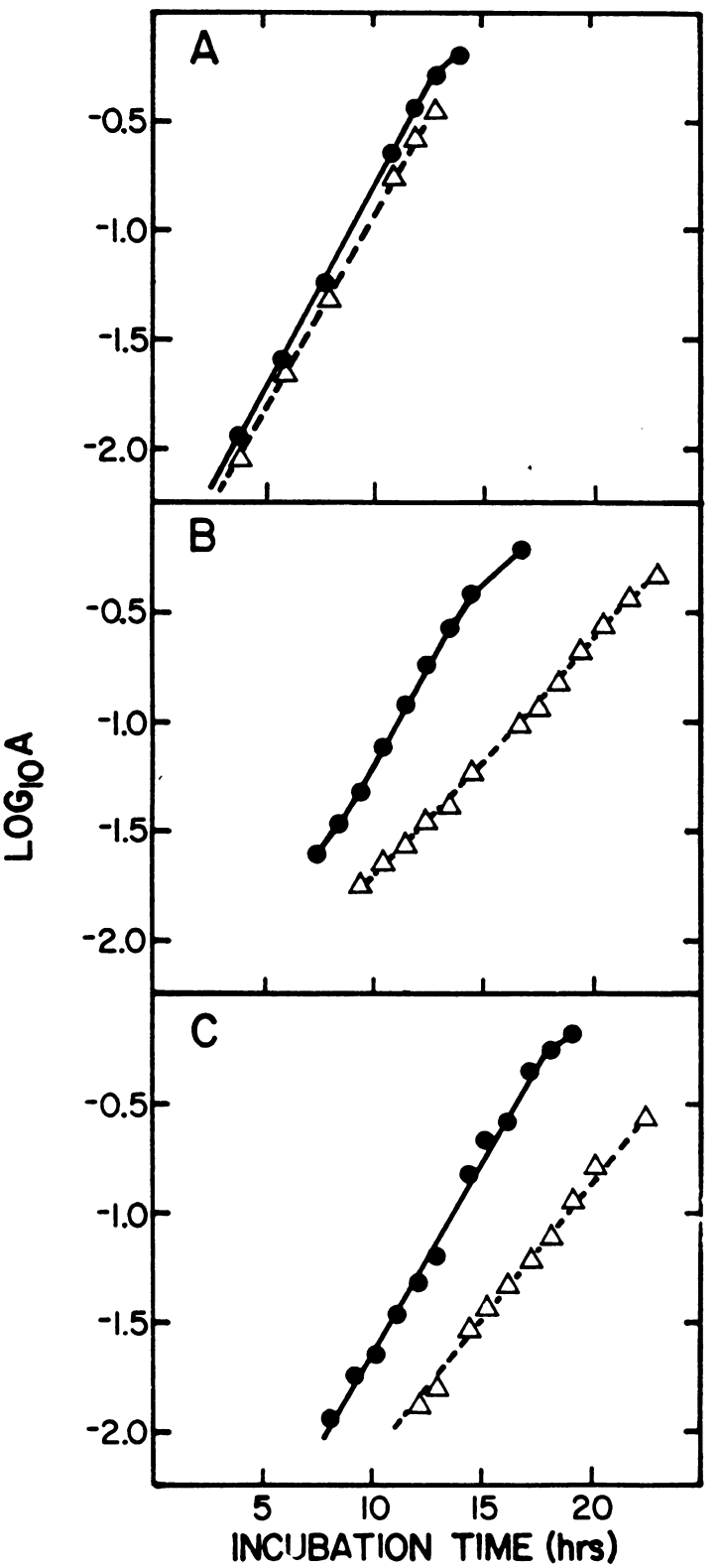


Figure 6

panel B were then diluted 330-fold, panel C, and the higher growth rate in carnitine supplemented media was again observed with a calculated lag time of 3.1 hours. During the same experiment another group of cells were transferred from carnitine containing media into carnitine containing media for which the calculated lag time of the culture was 2.9 hours. The difference of 0.3 hours or 18 minutes between the two groups of cells indicates that carnitine stimulates growth almost immediately. In retrospect then, diluting cells grown with carnitine somewhat more than 135-fold should decrease the amount of carnitine per cell to such an extent that the growth rate slows measurably over the growth phase. In fact, the growth rate does slow down when the cells are diluted 400-fold and become starved for carnitine as the cell numbers increase as shown in Figure 7. Since the growth rate appears to slow continuously over the growth curve the intracellular concentration of carnitine seems to determine the yeast's rate of metabolism and thus its rate of growth in between the limits shown in Table 3.

The simplest model for the carnitine effect on growth rate would be a first order dependence of growth rate on carnitine concentration. However, when a dose-response curve was generated, see Figure 8, the data could not be fit to a linear expression analogous to the Lineweaver-Burke transformation of the Michaelis-Menton equation for a first order enzyme reaction.

Uptake of carnitine by growth arrested cells

The dose-response curve in Figure 8 shows that only 100 ng L-carnitine/ml media is required for near maximum growth rate. Growth stimulation by such a low carnitine concentration implies that the yeast

Figure 7. Declining growth rate in carnitine starved cells. Two cultures, one with and one without carnitine added, were inoculated by the standard procedure except the final cell density was increased 100-fold to 2 x 10^4 cells/ml. The inoculum cells were grown in media containing 5 μM carnitine. ▲—▲, basal media plus 5 μM carnitine. ●—●, basal media only.

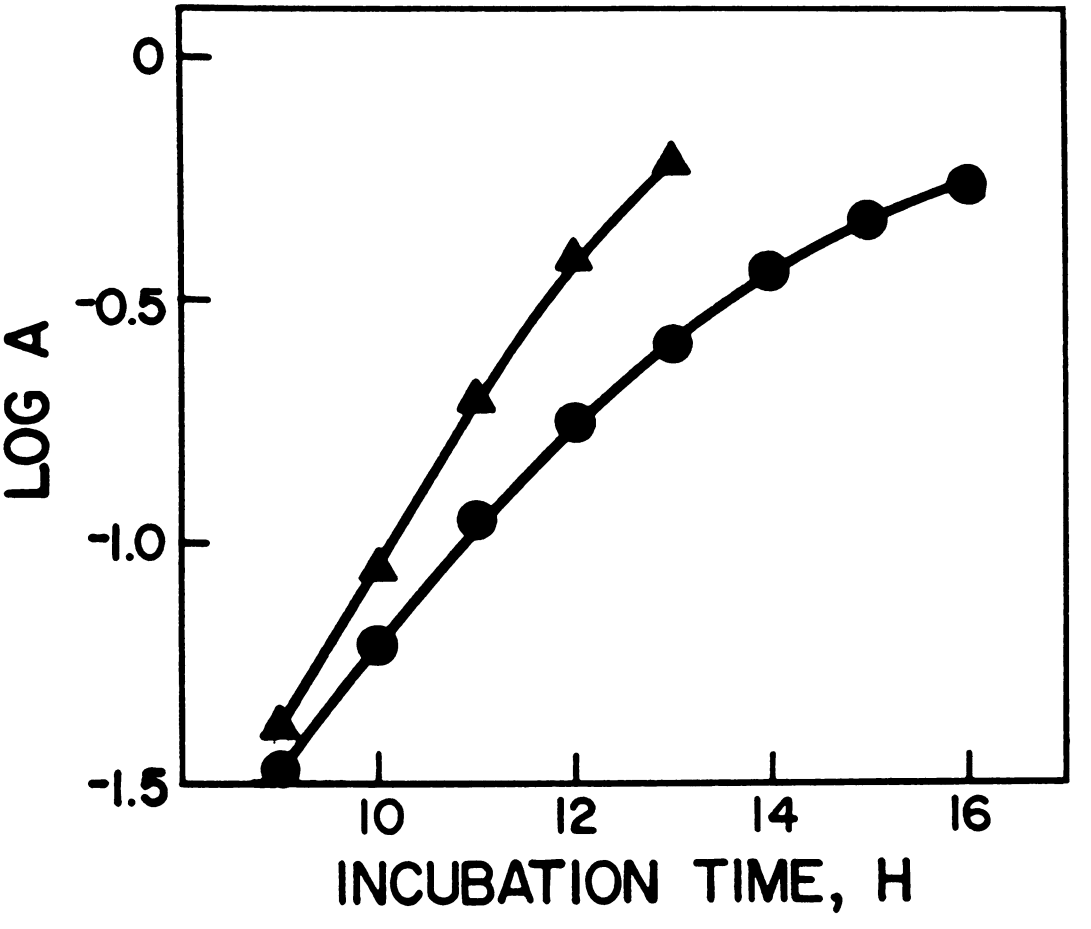


Figure 7

Figure 8. Dependence of growth rate on media carnitine concentration. Cells were grown in media lacking carnitine, harvested, and inoculated into duplicate tubes of media containing the indicated amounts of carnitine. Cultures were incubated at 30°C and their growth rates calculated. These were averaged for duplicate samples and are represented by a single point with the range indicated by the bars. The least squares line was calculated from all the points.

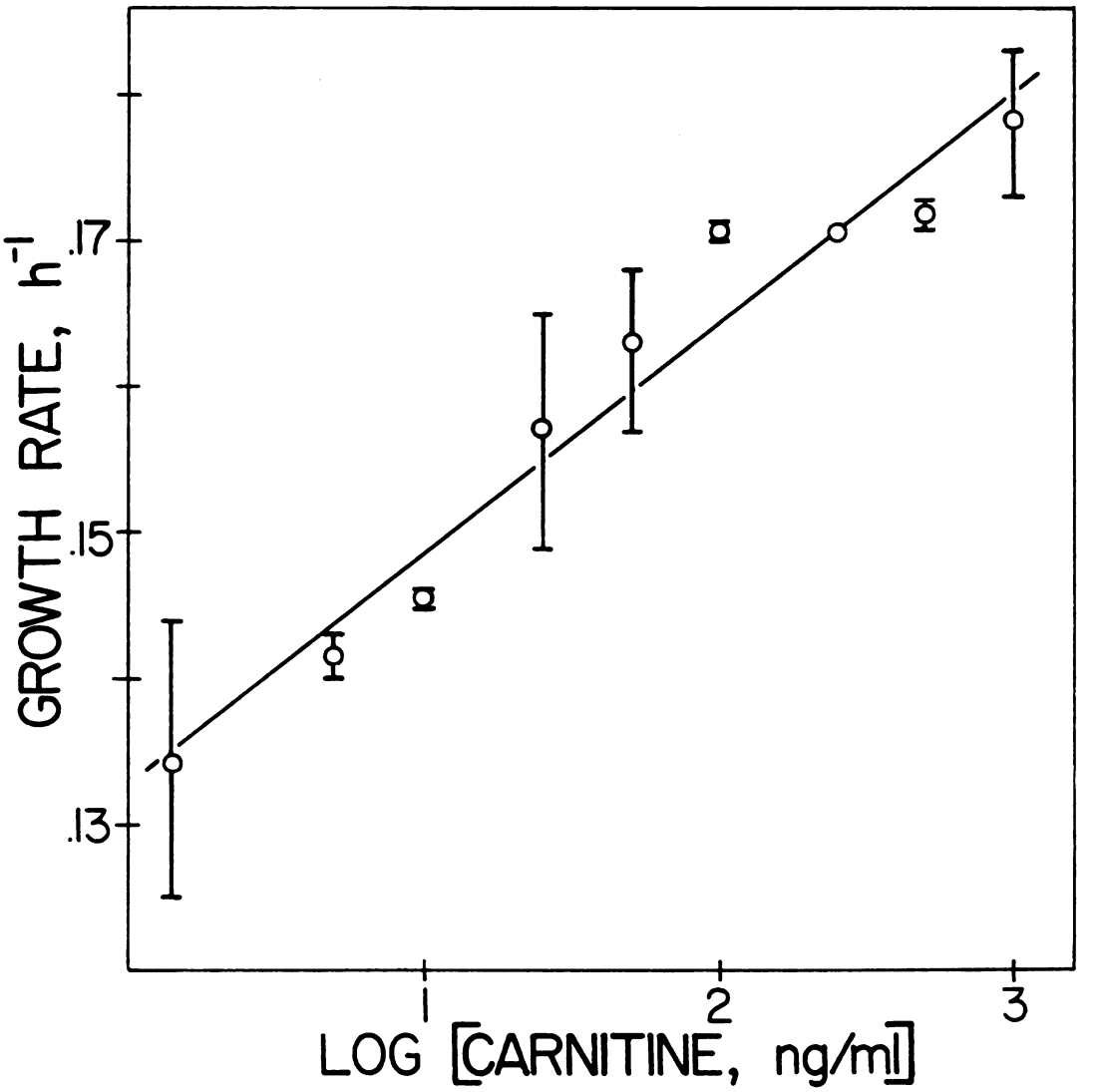


Figure 8

has a high affinity uptake system for L-carnitine. To test this, the rate and extent of DL-[^{3}H]carnitine uptake was determined as shown in Figure 9. After only 2 hours nearly 40% of the DL-[^{3}H]carnitine was cell associated. The cell mass remained relatively constant at 11.8 ± 1.4 mg dry weight/ml. Data in Table 8 show that stimulation of growth was stereospecific for the L-isomer of carnitine and makes it reasonable to assume this was the only isomer in the radioactive DL-mixture transported into the yeast. Based on this assumption, it was calculated that nearly 80% of the L-carnitine in solution (59.4 nmol) was taken up by the cells (166 mg total dry weight) during the two hour incubation period. Thus, the cells accumulated almost 0.3 μmol L-carnitine/g dry weight. In a different experiment (data not shown), early exponential phase cells (48 mg total dry weight) were incubated with 61.2 nmol L-carnitine (4.79 nmol DL-[^{3}H]carnitine, 56.41 nmol L-carnitine) and after 6.5 hours 1.8 μmol L-carnitine/g dry weight was cell associated. These levels of intracellular carnitine approach the levels found in rat liver (206). Figure 9 also shows that carnitine did not accumulate in cells at 4°C. The cell mass of this suspension stayed relatively constant at 17.6 ± 1.4 mg dry weight/ml throughout the incubation period.

Carnitine levels in T. bovina

L-carnitine was assayed in three different cultures of T. bovina grown to the early stationary phase of growth, the results of which are summarized in Table 7. The data show that T. bovina contains an extremely small amount of L-carnitine, especially when compared to mammalian tissues (207). A value of 0.4 nmol/g wet weight represents an

Figure 9. Uptake of DL-[methyl-^{3}H]carnitine by growth arrested cells of T. bovina. Experimental conditions are given in Materials and Methods. Cells were incubated in 9.7 ml of buffer with 268, 200 DPM/ml of DL-[methyl-^{3}H]carnitine containing 1.97 nmol D-carnitine and 57.6 nmol L-carnitine. ●—●, cells incubated at 37°C; ▲—▲, cells incubated at 4°C.

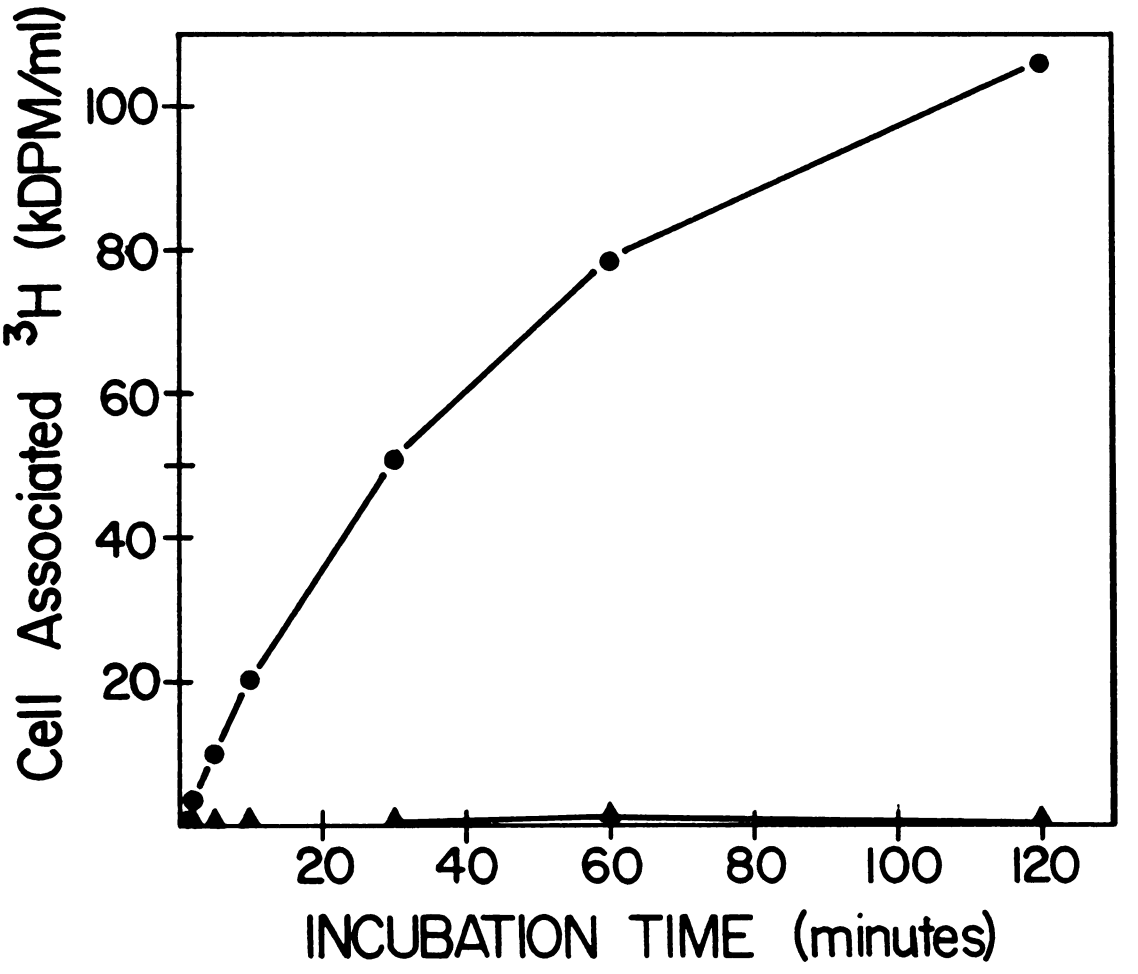

Cell Associated ^{3}H (kDPM/ml)
100
80
60
40
20
20
40
60
80
100
120
INCUBATION TIME (minutes)

Figure 9

TABLE 7. CARNITINE CONTENT OF T. bovina

pmol/g wet weight		
Total	Free	% Esterified
380 ± 206	271 ± 122	26 ± 12

Cells were grown in media lacking L-carnitine, harvested in exponential phase and inoculated into three Fernback flasks containing 1 L of media lacking carnitine. The cells were grown at 30°C to late exponential phase, collected by centrifugation and analyzed for carnitine as described in the Materials and Methods.

TABLE 8. EFFECT OF D-CARNITINE AND POSSIBLE L-CARNITINE PRECURSORS ON T. bovina GROWTH

Compound Added	Growth Rate (h^{-1})	Control Growth Rate (h^{-1})
D-carnitine 10 µg/ml	.145	.132
L-glutamate 100 µg/ml	.262	.130
4-N-trimethylaminobutyrate 1 µg/ml	.208	.216
4-aminobutyric acid 1 µg/ml	.130	.132

Cells were grown with the indicated additions in duplicate cultures at 37°C on 1% glucose exactly as described in the Materials and Methods. The controls received no additions and are tabulated on the right. The growth rate with L-carnitine added was 0.300 ± 0.16 (n=6).

intracellular concentration of about 0.57 μM assuming 70% water content. Carnitine was not detected in the spent culture media. The lower detection limit of the assay is 2 pmol per 0.1 ml sample tested or 20 nmol/L. This is nearly 30 times less than the estimated intracellular concentration. However, one liter cultures yield 5 g wet weight of early stationary phase cells containing about 2 nmol total carnitine. Thus, if the culture media was contaminated with 20 nmol/L of carnitine, the cells would only have to accumulate 10% to account for the measured intracellular level.

The data in Table 7 indicate a significant portion of the cellular carnitine is esterified and must have participated in cellular metabolism.

Other growth promoters and potential carnitine precursors

Data in Table 8 show that in the absence of added carnitine, L-glutamate also stimulates growth but at a much higher concentration than carnitine. Adding 0.5 mM glutamate plus 5 μM carnitine to the growth medium did not stimulate growth more than two-fold. Glutamate is known to participate in the synthesis of lysine in yeast. Lysine serves as a source of 4-N-trimethylaminobutyrate from which carnitine is derived in mammalian systems (208,209) and could also be a carnitine precursor in T. bovina. If so, 4-N-trimethylaminobutyrate would be expected to increase the growth rate of the yeast if it were taken up. However, the data in Table 8 show that 4-N-trimethylaminobutyrate did not stimulate growth. It is possible that L-glutamate is a carnitine precursor via its decarboxylated derivative, 4-aminobutyric acid. But as seen in Table 4, 4-aminobutyric acid also did not affect the yeast's growth rate. These

results seem to rule out the possibility that either 4-aminobutyric or 4-N-trimethylaminobutyric acid are precursors of carnitine. This conclusion is supported by the finding that when cells were grown on L-glutamate and assayed for total L-carnitine, only 0.8 nmol/g wet weight was found.

T. bovina CAT

Extraction, assay conditions, and partial purification

A previous study determined that T. bovina contains CAT without detectable levels of carnitine octanyl- or palmityl-transferase activities (9). Since CAT is probably a key enzyme in the metabolic process stimulated by carnitine, studies were initiated to characterize the CAT activity in T. bovina. Several problems were encountered, the most worrisome being the total extraction of the enzymes from the yeast. Grinding cells with sand or sonicating them with glass beads present proved to be inadequate extraction procedures. Slight adjustments in the method described by Lang et al. (210) in which cells are agitated with glass beads, produced reproducibly high yields of CAT. The enzyme was easily purified 70-fold in 2 steps; (a) precipitation of the enzyme by increasing the ammonium sulfate concentration from 1 to 2 M and (b) binding the enzyme to a Cibacron Blue column in 50 mM phosphate buffer, pH 6.5 and eluting the activity with 50 mM Tris buffer, pH 8.5, containing 1 M KCl. This preparation was stable for at least 2 weeks at room temperature with 0.02% azide added and only lost 22% of its activity after 4 months storage at 4°C dropping to 53% of its initial activity after 7 months.

During these initial studies, CAT was assayed in Buffer I (see Material and Methods) as described by Markwell et al. (70) and it was noticed that ammonium sulfate precipitation of CAT usually yielded 150- to 200% of the starting activity. Furthermore, the assay uses Tris buffer and CAT is reported to acetylate Tris (211). Therefore, a new buffer system was developed as shown in Table 9. $MgCl_2$ had an astonishing stimulatory effect while EDTA and Triton X-100 were slightly inhibitory. However, the stimulatory effect of $MgCl_2$ could be produced by NaCl, Na_2SO_4 and $MgSO_4$ and, as shown in Figure 10, these salts probably exert their effect by increasing the ionic strength. This would explain why ammonium sulfate precipitation tended to produce such high yields of enzyme. It should be noted that changes in ionic strength cause increases in carnitine palmitoyl transferase I activity in intact mitochondria from dog heart (212).

CAT production in T. bovina

Even without a satisfactory extraction procedure, it was noticed in early investigations (213) that media carnitine affected the levels of CAT and this matter was re-investigated after finding that agitating the cells with glass beads gave much higher yields of CAT. These studies were performed with crude extracts of the yeast obtained as described in the Materials and Methods. Figure 11 shows that CAT activity is linear with protein concentration whether or not the cells were grown with carnitine. Cells grown on glucose without added carnitine had very high specific activities of CAT as shown in Table 10, the activity in these crude extracts being high under both aerobic and anaerobic culture conditions. However, cells grown with carnitine had specific activities

TABLE 9. ASSAY CONDITIONS FOR *T. bovina* CAT

	Additions			
Carnitine, 1.26 mM	+	+	+	+
Glygly Buffer, 50 mM, pH 8.2	+	+	+	+
$MgCl_2$, 12.5 mM	-	+	+	+
EDTA, 1.1 mM	-	-	+	+
Triton X-100, 0.1%	-	-	-	+
	nmol/min/ml			
	1292(38)	1951(65)	1877(37)	1804(68)

Cells from two, 1 liter cultures grown on synthetic media at 30°C in 2.8 liter Fernbach flasks for 17.75 hours, were collected by centrifugation, resuspended in 10 ml of 50 mM phosphate buffer, pH 6.5, and transferred to a glass stoppered, 125 ml erlenmeyer flask containing 32 gm cold glass beads (.45 mm). The flask was shaken vigorously for 2 minutes, cooled, and shaken again for 2 minutes. The homogenate was filtered through a coarse, sintered glass funnel, washed repeatedly with buffer, and then centrifuged 15 mintues at 500 xg. The supernatant solution (58 ml) was assayed as indicated.
Besides the additions, all the assay solutions contained 0.15 mM DTNB, and 0.40 mM acetylCoA. The final reaction volume was 0.2 ml and the reaction progress was monitored at a wavelength of 412 nm. The molar extinction coefficient of DTNB was taken as 13,600.
Values are the average of three determinations with the standard deviation given in parentheses.

Figure 10. Stimulation of CAT by increasing ionic strength. CAT was partially purified by isopycnic density gradient centrifugation of a crude yeast extract (see legend to Figure 23). CAT was assayed in Buffer II with 0, 3.1, 6.2 or 12.5 mM $MgSO_4$ added or 0, 6.2, 12.5, or 25 mM $MgCl_2$ added. One assay was performed with 25 mM Na_2SO_4 added. The ionic strength of the glycylglycine buffer was taken as 0.05 M. The CAT activity with no additions (89 nmol/min/ml) was taken as 100%.

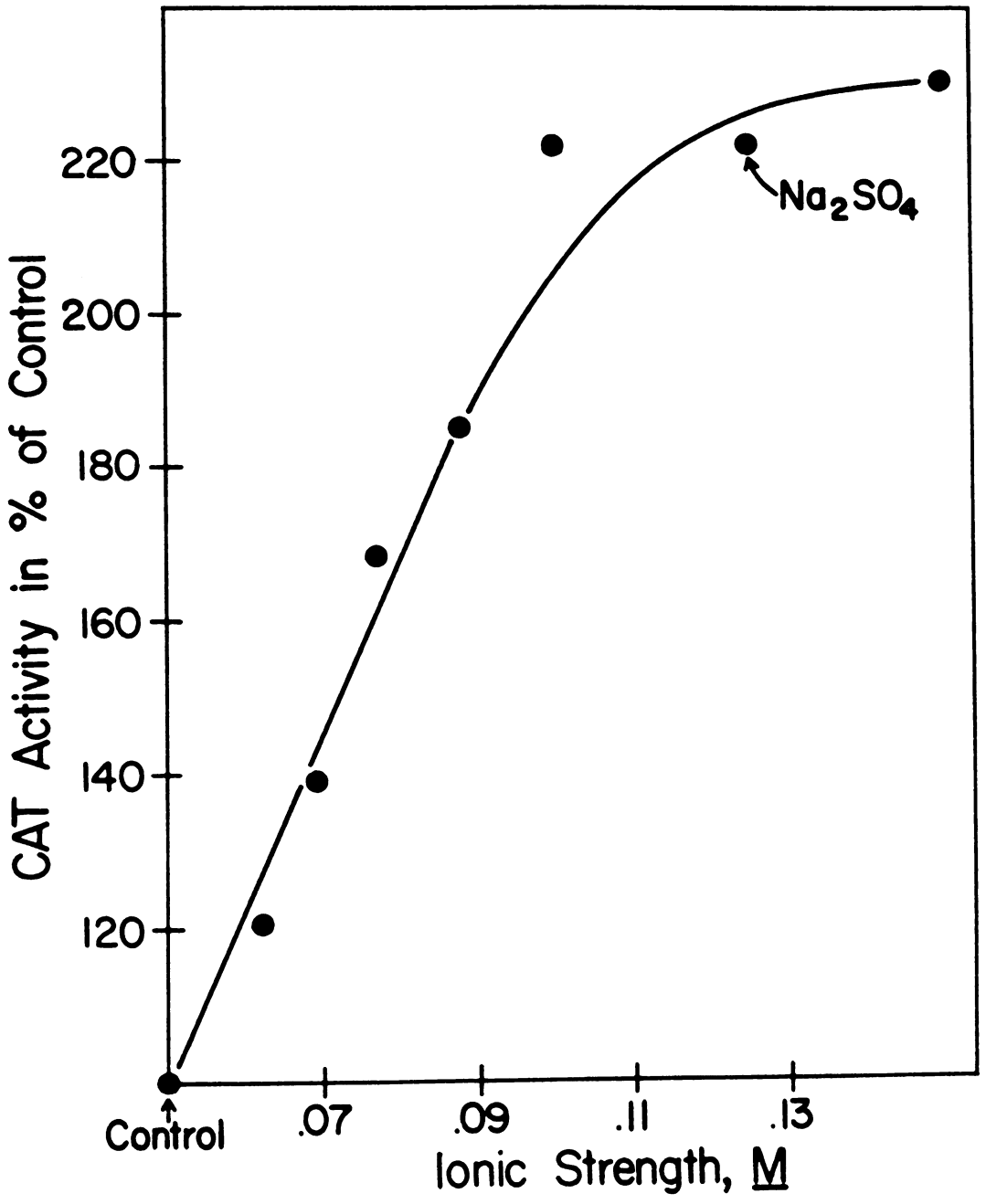

CAT Activity in % of Control
220
200
180
160
140
120
Na_2SO_4
Control
.07
.09
.11
.13
Ionic Strength, M

Figure 10

Figure 11. Linear dependence of CAT on protein concentration. Crude extracts were prepared and assayed for CAT in Buffer II as described in Materials and Methods. ●—●, cells grown without carnitine; protein concentration 0.95 mg/ml. ▲—▲, cells grown with carnitine; protein concentration 1.29 mg/ml.

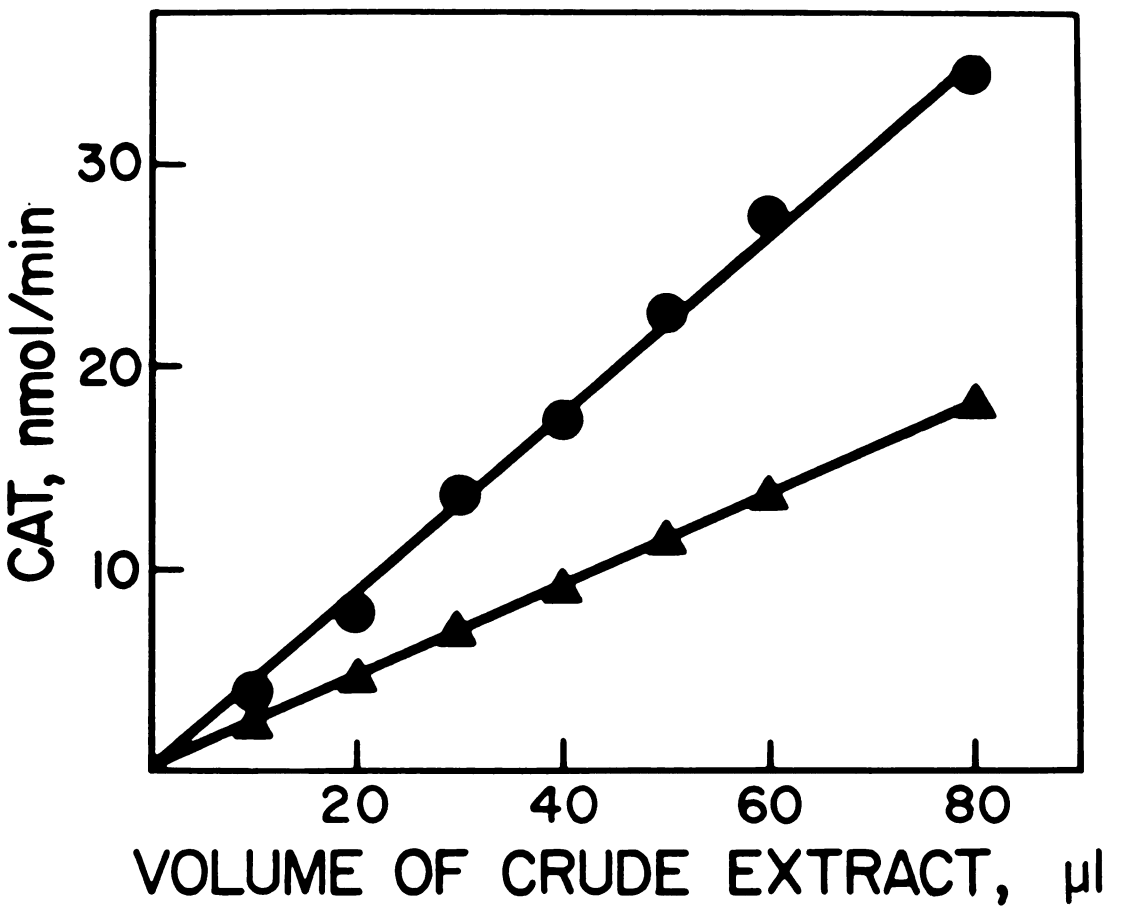


Figure 11

TABLE 10. FACTORS AFFECTING CAT PRODUCTION IN *T. bovina*

Culture Conditions	Carnitine Acetyltransferase nmol/min/mg protein	
	-carnitine	+carnitine
Anaerobic, 37°C	493 ± 70 (n=6)	258 ± 38 (n=6)
Aerobic, 37°C	492 ± 39 (n=4)	199 ± 31 (n=4)
Aerobic, 30°C	428 ± 40 (n=2)	154 ± 5 (n=2)

Cells were grown with or without 5 μM carnitine as indicated, harvested in the late exponential phase of growth, crude extracts prepared and assays performed as described in Materials and Methods. Anaerobic flasks were purged with 5% CO_2:95% N_2 gas. Aerobic cultures were grown in flasks plugged with cotton. Numbers in parentheses indicate the number of cultures analyzed.

36-57% that of cells grown without L-carnitine. In contrast under both aerobic and anaerobic conditions, glucose-6-phosphate dehydrogenase specific activity increased in carnitine supplemented cultures (data not shown). Because carnitine stimulates both aerobic and anaerobic growth, CAT is presumed to have the same function under both culture conditions. Growing the cells at 30°C instead of 37°C lowered the CAT levels slightly as indicated in Table 10.

In other experiments, cells were grown anaerobically with 1 μg/ml chloramphenicol to inhibit mitochondrial protein synthesis and in another experiment cells were grown anaerobically with the oleate ester, Tween 80 (added to reduce the need for andogenous fatty acid synthesis). CAT levels were not different than controls for either experiment.

Substrate specificity and kinetic properties

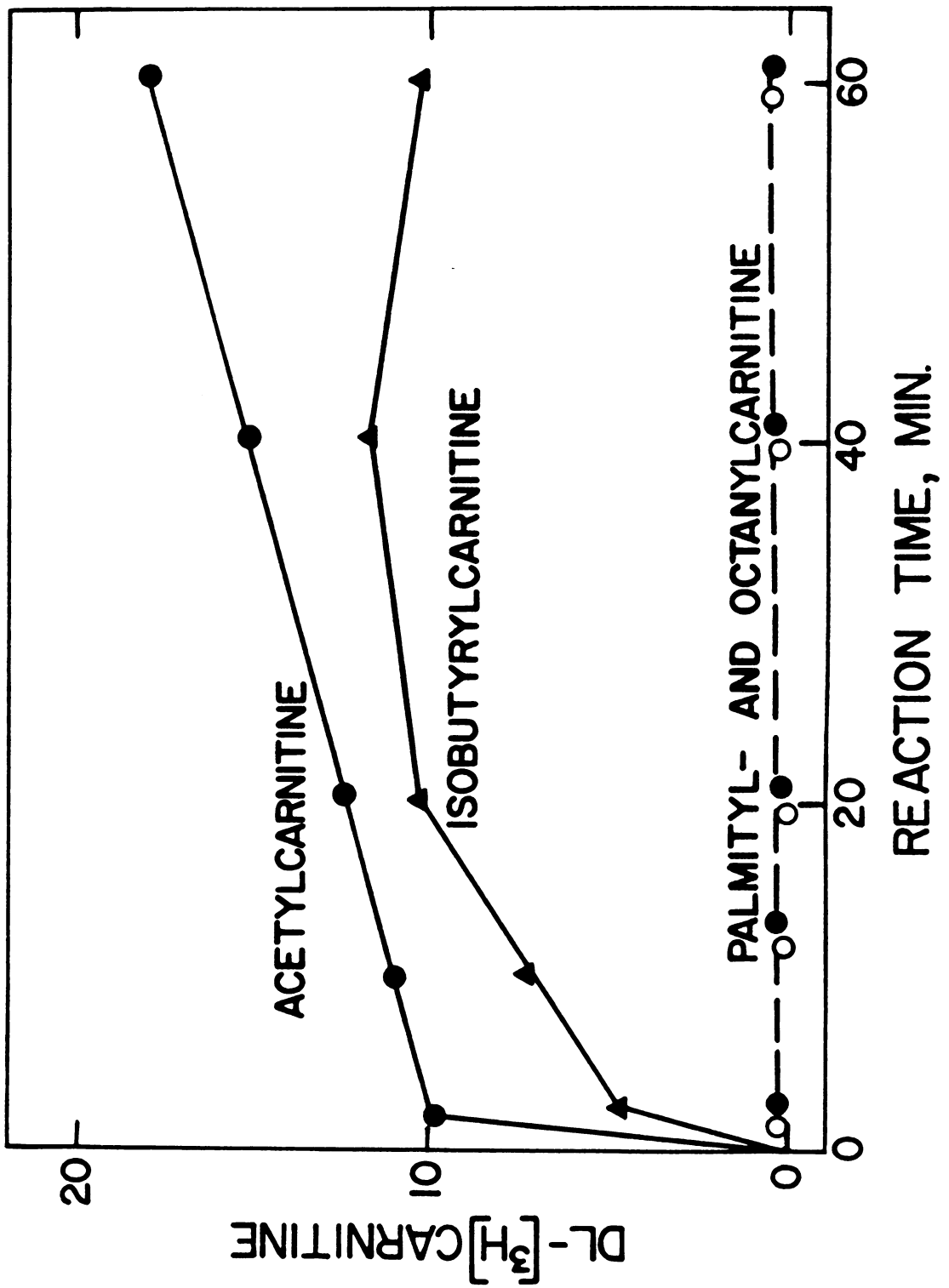
The substrate specificity of CAT was investigated and as shown in Table 11 the enzyme is a short-chain carnitine acyltransferase. Crude extracts catalyzed the transfer of acetyl-, propionyl-, and isobutyrylCoA to carnitine but were much less active with n-butyrylCoA and valerylCoA and did not have any activity with long chain acylCoA derivatives. The presence or absence of carnitine in the growth media did not alter the substrate specificity profile. The formation of acylcarnitines from the respective acylCoA derivatives by cell-free extracts of *T. bovina* was further confirmed by incubating the extracts with DL-[methyl-^{3}H]carnitine in the standard assay mixture for CAT and isolating the reaction products. Figure 12 shows that both acetyl[^{3}H]carnitine and isobutyryl[^{3}H]carnitine were formed but octanyl- and palmitylcarnitine were not.

TABLE 11. SUBSTRATE SPECIFICITY OF T. bovina CAT

AcylCoA	RELATIVE SPECIFIC ACTIVITY	
	Grown Without Carnitine	Grown With Carnitine
Acetyl-	100	100
Propionyl-	112	111
Butyryl-	12	12
Isobutyryl-	28	28
Valeryl-	<1	<1
Isovaleryl-	<1	<1

Duplicate cultures were grown aerobically at 30°C, with (5 μM) or without L-carnitine, and harvested in the late exponential phase of growth. Crude extracts were prepared and assayed in triplicate for carnitine acyltransferase activity in Buffer II with acylCoA derivatives present at a final concentration of 0.4 mM. The activity of the transferase using acetylCoA was set at 100: 428 nmol/min/mg protein for cells grown without carnitine and 154 nmol/min/mg protein for cells grown with carnitine.

Figure 12. Formation of radiolabeled acylcarnitines by cell-free extracts of T. bovina. Cell-free extracts were incubated with 0.1 mM acetyl-, isobutyryl-, or octanylCoA, or 37.5 μM palmitylCoA and DL-[^{3}H]carnitine as described previously (5) except DNTB was omitted. Acylcarnitines were isolated at the indicated times by thin layer chromatography using chloroform, methanol, ammonia (50:30:8) as solvent. Standards were run simultaneously, detected with Dragendorf spray reagent and the corresponding ^{3}H products scraped off the plates and quantitated by scintillation counting of the removed silica gel (173). The figure shows the percentage of initial [^{3}H]carnitine converted to acylcarnitine.


DL-[³H]CARNITINE
20
10
0
0
20
40
60
REACTION TIME, MIN.
ACETYLCARNITINE
ISOBUTYRYLCARNITINE
PALMITYL- AND OCTANYLCARNITINE

The apparent K_m for acetylCoA of CAT in cell-free extracts was determined from Lineweaver-Burk plots. The concentration of L-carnitine was 1.25×10^{-3} M while the concentration of acetylCoA varied from 9.5×10^{-7} M to 9.5×10^{-4} M; a K_m value of 6.3×10^{-5} was obtained for yeast grown with L-carnitine and a K_m value of 6.7×10^{-5} M was obtained for yeast grown without L-carnitine, see Figure 13. The K_m for L-carnitine was determined at an acetylCoA concentration of 4×10^{-4} M; a K_m value for L-carnitine of 3.0×10^{-4} M was obtained for yeast grown with L-carnitine and 6.7×10^{-4} M for yeast grown without L-carnitine, see Figure 14. Very similar K_m's were obtained in other determinations using partially purified enzyme.

CARNITINE AND ACETYLCARNITINE METABOLISM

Identification of acetylcarnitine as the major acylcarnitine

Figure 15 shows that growing cells take up media carnitine. The amount taken up is linear with cell density during the exponential phase of growth (culture absorbance <0.6). Nearly 80% of the media carnitine became cell associated by the time the culture reached 0.4 absorbance. From the value in Table 4, the cell dry weight of a 100 ml culture is 53 mg at 0.4 absorbance. At this absorbance, the cells contained 7.0 µmol carnitine/g dry weight or 1.4 µmol/g wet weight assuming 80% water content. This concentration is 3500 times the carnitine concentration in cells grown without carnitine (see Table 7). During the exponential phase of growth about half the cell-associated carnitine was esterified. Since all the carnitine was extracted by boiling the cells 3 minutes, it was not tightly or covalently bound to any macromolecules.

Figure 13. Determination of the K_m for acetylCoA of T. bovina CAT. CAT was assayed in crude extracts in Buffer I as described in the Methods and the K_m V_{max} calculated from the Lineweaver-Burk plots shown. A, cells grown with 5 μM carnitine; 0.79 mg protein/ml. K_m = 67 μM. B, cells grown without carnitine; 0.85 mg protein/ml. K_m = 63 μM. Each assay received 5 μl of crude extract.

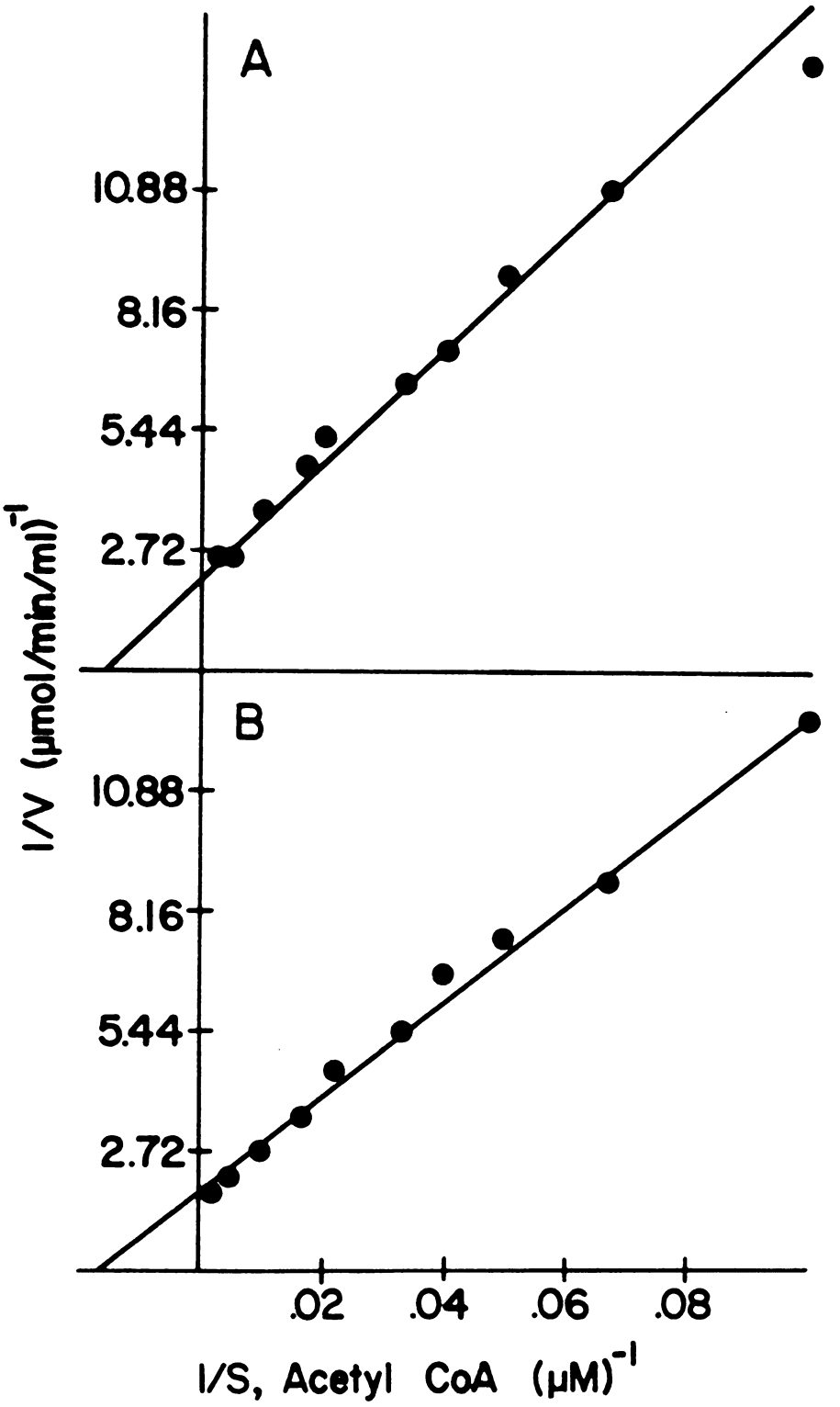


Figure 13

Figure 14. Determination of the K_m for L-carnitine. CAT was assayed in crude extracts of T. bovina in Buffer II as described in the Methods. The acetylCoA concentration was 0.4 mM while the carnitine concentration was varied as shown. A, cells grown with carnitine; each assay received 5 µl crude extract, 1.29 mg protein/ml. K_m = 0.30 mM. B, cells grown without carnitine; each assay received 10 µl crude extract, 0.63 mg protein/ml. K_m = 0.67 mM.

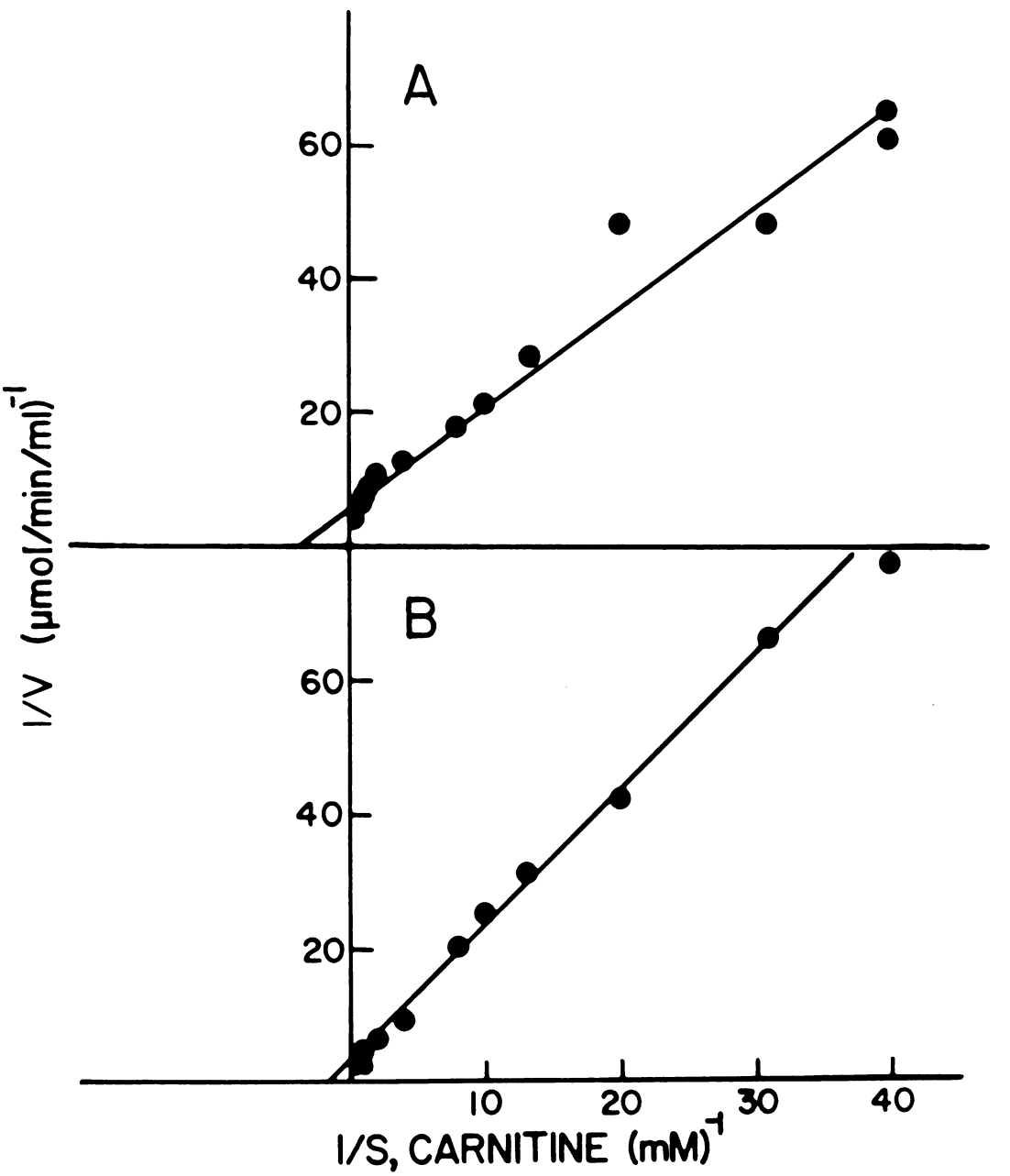


Figure 14

Figure 15. Uptake and metabolism of carnitine in T. bovina. Cells were grown in 100 ml of media with 5 mM L-carnitine and the cell associated carnitine measured at various times after inoculation as described in the Materials and Methods. The results are expressed on the basis of the amount of carnitine in the whole culture.

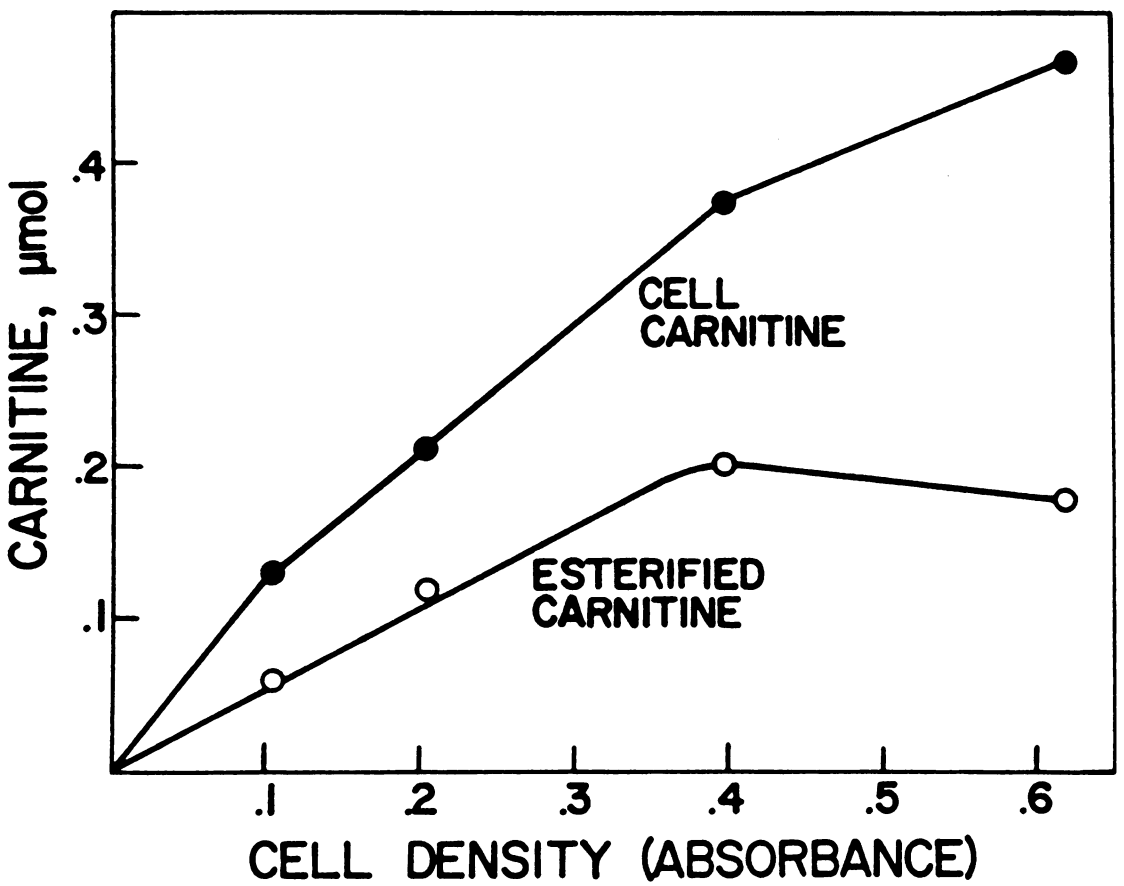

CARNITINE, µmol
.4
.3
.2
.1
CELL
CARNITINE
ESTERIFIED
CARNITINE
.1
.2
.3
.4
.5
.6
CELL DENSITY (ABSORBANCE)

Figure 15

TABLE 12. PERCENT OF ACYLCARNITINE AS ACETYLCARNITINE IN *T. bovina*.

	CARNITINE, µmol			% ESTERIFIED AS
EXPERIMENT	TOTAL	ESTERIFIED	ACETYL-	ACETYLCARNITINE
1	3.7	1.2	1.2	100
2	4.2	1.5	1.4	93

Cells were grown in 1 L cultures with 5 µM L-carnitine, harvested in the exponential phase of growth, extracted with perchloric acid and assayed for total, free and acetylcarnitine as given in the Materials and Methods section. The yeast dry weight was 521 and 530 mg in experiments 1 and 2 respectively.

Due to the substrate specificity of CAT acetyl-, propionyl-, and/or isobutyrylcarnitine were the only likely acylcarnitines formed *in vivo*. The data in Table 12 show that, when assayed enzymatically and specifically for acetylcarnitine, essentially all of the esterified carnitine in extracts of *T. bovina* is acetylcarnitine.

Acetylcarnitine and the synthesis of N-acetylglutamate

Besides forming citrate in the TCA cycle, acetylCoA participates indirectly in the formation of arginine in yeast and fungi (163). Coincidently, arginine stimulates *T. bovina* growth (214). Therefore, the ability of acetylcarnitine to act as the acetyl-donor for N-acetylglutamate synthase was investigated. This enzyme was assayed by incubating cell-free extracts with acetylcarnitine and [U-^{14}C]glutamate (see ref. 215) but N-acetyl[^{14}C]glutamate was not formed even when acetylcarnitine was replaced by acetylCoA. Another possibility is that CAT functions in the cell to transfer the acetyl group from N-acetylornithine to glutamate but again, when N-acetylornithine was used in the assay for N-acetylglutamate synthesis, no N-acetyl[^{14}C]glutamate formed.

Utilization of [^{14}C]acetylcarnitine by growing yeast

Since acetylcarnitine was the only acylcarnitine detected in *T. bovina*, its metabolic fate was determined. [1-^{14}C]acetylcarnitine was synthesized and added to cultures of the yeast growing in the absence of carnitine. For comparative purposes, [1-^{14}C]acetate was added to identical cultures. It has already been shown that *T. bovina* does not synthesize significant quantities of carnitine and thus [^{14}C]acetate

Figure 16. Incorporation of [^{14}C]acetylcarnitine into growing yeast cells. Cells were grown in basal media lacking L-carnitine but containing in A, 500 nmol of 0.5 Ci/mol [^{14}C]acetylcarnitine or in B, 500 nmol of 1.46 Ci/mol [^{14}C]acetate. ^{14}C was measured in samples of the media and then each flask was inoculated with 40,000 cells. Cell density was monitored by turbidity measurements at 600 nm and the ^{14}C distribution determined at the indicated points. The culture ^{14}C was measured directly on a 1 ml sample of the culture. Another 1 ml sample of the culture was placed into a boiling water bath for 3 min, cooled, and centrifuged to remove debris and the ^{14}C in the supernatant fluid measured. Cell associated ^{14}C was measured after filtering a 2 ml sample of the culture through a Whatman GF/C glass fiber filter using a Millipore apparatus. The cells and filter were assayed together for ^{14}C. The filtrate ^{14}C was also measured and its value subtracted from the amount of ^{14}C released by boiling the culture to obtain a measure of the cell associated ^{14}C extracted by hot water. All values are expressed as a percentage of the ^{14}C present in the cultures at the time of inoculation. At least 24 h elapsed before the cultures reached 0.1 absorbance after which only 4 and 6 h of incubation time were required for the cultures to reach 0.5 absorbance in A and B respectively.

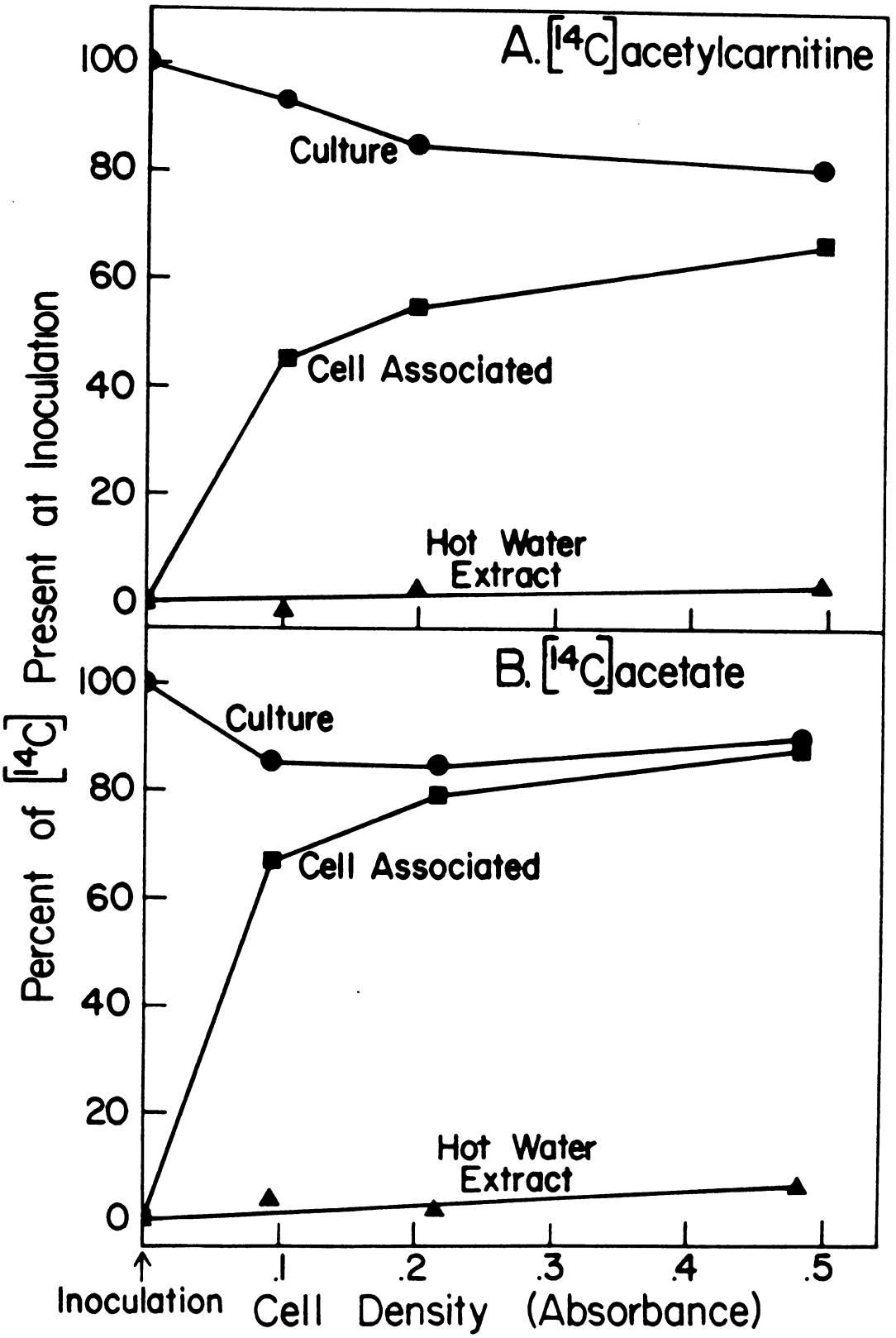

A. [14C]acetylcarnitine
Culture
Cell Associated
Hot Water Extract
B. [14C]acetate
Culture
Cell Associated
Hot Water Extract
Percent of [14C] Present at Inoculation
100
80
60
40
20
0
Inoculation
.1
.2
.3
.4
.5
Cell Density (Absorbance)

Figure 16

serves as a control. Figure 16A presents the data for the uptake of [^{14}C]acetylcarnitine as a function of cell density over the exponential phase of growth while Figure 16B presents the results obtained with [^{14}C]acetate. The figure indicates that a substantial portion of the ^{14}C from both acetylcarnitine and acetate were taken y the cells during the period of analysis. The percentage of cell associated ^{14}C in this figure was calculated relative to the amount of ^{14}C added to the culture. Due to the small decrease in total ^{14}C in the cultures during the course of the incubations, somewhat higher percentages for cell associated ^{14}C would be obtained if they were calculated relative to the ^{14}C in the culture at the time of measurement. At any given cell density, a slightly greater quantity of [^{14}C]acetate was taken up than was [^{14}C]acetylcarnitine. From other data it was calculated that acetylcarnitine stimulated the yeast growth rate to the same extent as carnitine. The cultures represented in Figures 16A and B took 24 and 28 h respectively to reach cell densities having an absorbance of 0.1; subsequent growth to 0.5 absorbance occurred within 4 to 6 h respectively. Thus very little ^{14}C was lost from the cultures during the period when most of the cell mass was produced and during which other studies showed the amount of glucose consumed increased from 6% to nearly 60%. These results indicate that a significant portion of the acetylcarnitine was not oxidized to gaseous CO_2 during the incubation period. Moreover, very little of the cell-associated ^{14}C was extracted by hot water indicating that [^{14}C]acetate and [^{14}C]acetylcarnitine were metabolized by the cell and that the ^{14}C was incorporated into cellular components.

Distribution of ^{14}C among cellular components

Homogenates prepared from the cultures shown in Figure 16 were separated into cell walls, lipids, and acid-insoluble protein plus nucleic acids. The term cell walls refers to all the cell debris sedimenting at 700 x g in 2 min. A large amount of ^{14}C derived from [^{14}C]acetate was recovered in this cell wall pellet, see Table 13, whereas this fraction was poorly labeled by [^{14}C]acetylcarnitine. Table 14 shows that a combination of 6 N KOH and 1% sodium dodecylsulfate are required to completely extract the ^{14}C in the cell wall pellet from cells labeled with [^{14}C]acetate. In other experiments, the proportion of ^{14}C in the cell wall fraction varied with the preparation, probably due to the heterogeneous nature of this fraction. As expected, very little ^{14}C was found in the acid soluble fraction of either sample after denaturing the homogenates with 6% $HClO_4$, see Table 13.

The lipids extracted from the acid insoluble pellets were labeled with ^{14}C especially from cells incubated with [^{14}C]acetylcarnitine, see Table 13. Samples of these lipid extracts were analyzed by TLC on silica gel and as shown in Figure 17, both [^{14}C]acetate and [^{14}]acetylcarnitine contributed ^{14}C to the 3 major classes of lipids, i.e., polar, non-polar, and acidic lipids. Since O-acetyl esters are hydrolyzed by 0.1 N KOH, the lipid-extracted protein was treated with this reagent to determine the extent to which cell protein was O-acetylated. Almost none of the ^{14}C remaining in the lipid-extracted protein pellet was solubilized by 0.1 N KOH in the culture labeled with [^{14}C]acetate while 25% of the label derived from [^{14}C]acetylcarnitine that was in this pellet was solubilized by 0.1 N

TABLE 13. DISTRIBUTION OF ^{14}C IN CELLS GROWN WITH [^{14}C]ACETYLCARNITINE

	% of Total	
	[^{14}C]acetate	[^{14}C]acetylcarnitine
Cell associated	98	83
700 x g cell wall pellet	42.7	2.6
Water soluble	2.5	4.7
Soluble in chloroform/methanol	15.8	34.4
Soluble in 0.1 N KOH	0.4	25.6
Insoluble in 0.1 N KOH	11.1	4.2

Cells were grown in 100 ml of media containing 5 μM [1-^{14}C]acetate or [1-^{14}C]acetyl-L-carnitine. Specific activities were 2 μCi/μmole for [1-^{14}C]acetate and 0.48 μCi/μmole for [1-^{14}C]acetyl-L-carnitine. Cells were fractionated and analyzed for ^{14}C as described in Methods.

TABLE 14. SOLUBILIZATION OF ^{14}C FROM [^{14}C]ACETATE-LABELED CELL WALLS

Solubilizing Agent	% Soluble
0.1 N KOH	24.4
6 N KOH	36.5
1% SDS[a]	39.1

The cell wall pellet from cells grown with [^{14}C]acetate (see Table 13) was treated in sequence with the solutions shown and the percentage of ^{14}C removed each time with the supernatant fluid after centrifugation of the sample reported. The pellet was incubated 30 min at 65°C with 0.1 N KOH and 20 h at 65°C with 6 N KOH.

[a]Sodium dodecylsulfate in 0.25 M phosphate buffer, pH 8.0.

Figure 17. Distribution of ^{14}C in cell lipids. Cell lipids were isolated as indicated in Table 13 and separated by TLC on silica gel developed with petroleum ether:ether:acetic acid, 70:30:1. Cholesterol and oleate were run simultaneously as shown. Condiolipin, which remained at the origin, and olive oil triglyceride, which migrated with R_f>0.8, were run separately (not shown). Lipids were located by exposing the plates to iodine after which segments of silica gel were removed to scintillation vials and analyzed for ^{14}C as shown above each chromatogram. The numbers above the segments represent the percentage of total ^{14}C recovered. A, cells grown with [^{14}C]acetylcarnitine; plate developed 30 min. B, cells grown with [^{14}C]acetate; plate developed 2.5 h.

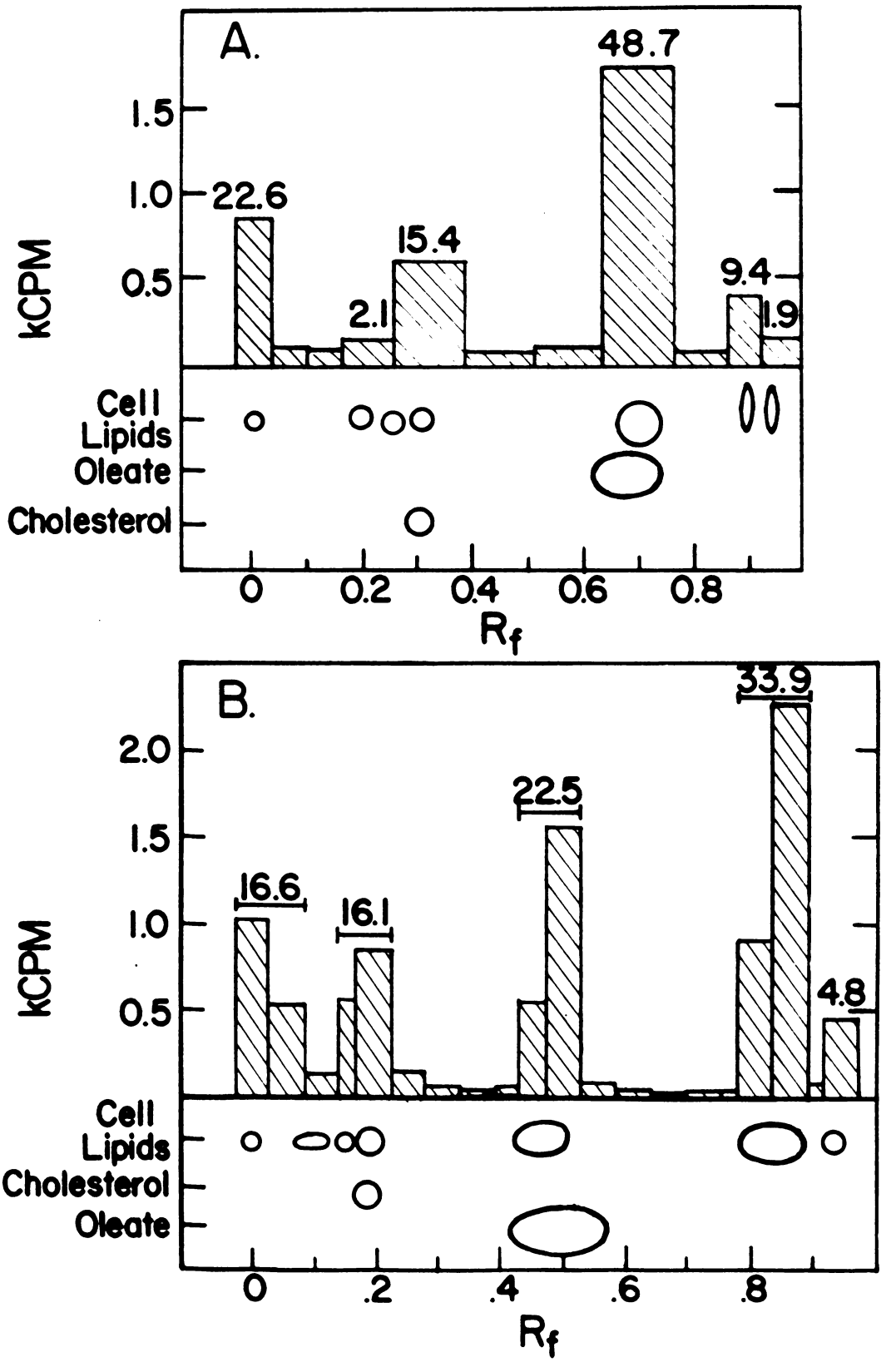

A.
48.7
22.6
15.4
9.4
2.1
1.9
1.5
1.0
0.5
kCPM
Cell
Lipids
Oleate
Cholesterol
0
0.2
0.4
0.6
0.8
Rf
B.
33.9
22.5
16.6
16.1
4.8
2.0
1.5
1.0
0.5
kCPM
Cell
Lipids
Cholesterol
Oleate
0
.2
.4
.6
.8
Rf

Figure 17

KOH. Although the ^{14}C solubilized by this treatment was presumed to be derived from O-acetylated cell components, it did not co-chromatograph on paper with authentic [^{14}C]acetate.

The distribution of [^{14}C]acetylcarnitine in this experiment was markedly different from that produced by [^{14}C]acetate. About 85% of the cell-associated [^{14}C]acetylcarnitine was acid precipitable with one-third of the label being found in the lipids fraction. Since lipids are easily extracted, the amount of acetylcarnitine incorporated into lipids was determined. A series of cell cultures were grown to an absorbance of 0.2, treated 2 h with [^{14}C]acetate, [^{14}C]acetate + carnitine, or [^{14}C]acetylcarnitine and the cells harvested. The cells were saponified in ethanolic KOH at 80°C until the ethanol evaporated. This basic solution was extracted with petroleum ether to remove sterols and then it was acidified to pH <2 and the saponifiable fatty acids extracted with petroleum ether. The material remaining after ether extraction was treated with warm water and insoluble debris was removed by centrifugation. The data in Table 15 present the results of two experiments that were averaged together. A negligible amount of ^{14}C remained insoluble after the ether and aqueous extractions. Most of the ^{14}C derived from acetylcarnitine appeared in the ether extracted aqueous fraction although a significant amount of ^{14}C was recovered in the saponifiable fatty acids fraction. In contrast, [^{14}C]acetate mostly labeled sterols, i.e., the non-saponifiable lipids extracted from basic solution. The saponifiable fatty acids fraction is not the predominantly labeled fraction although [^{14}C]acetylcarnitine contributes more acetyl units to the fatty acid pool than does acetate. The combination [^{14}C]acetate + carnitine

TABLE 15. DISTRIBUTION OF ^{14}C IN SAPONIFIED CELLS

Fraction	Percent of Total ^{14}C in Cells		
	Cells grown with [^{14}C]Acetate	Cells grown with [^{14}C]Acetate + carnitine	Cells grown with [^{14}C]Acetylcarnitine
Saponified Lipids extracted from basic solution	59.3	17.0	4.4
Saponified Lipids extracted from acid solution	10.0	28.6	19.0
Ether extracted, water soluble, saponified cell material	27.2	52.2	75.6

Cells were grown without carnitine to an absorbance of 0.2 and then incubated with either [^{14}C]acetate or [^{14}C]acetylcarnitine added to a concentration of 5.6 μM for 2 h after which the cells were saponified and analyzed for ^{14}C as described in Materials and Methods. Each value represents the average of two experiments. [^{14}C]Acetate treated cultures received 1.40 x 10^6 DPM in one experiment and 2.58 x 10^6 DPM in the second of which 57.6% and 43.8% respectively became cell associated. [^{14}C]Acetylcarnitine treated cultures received 0.886 x 10^6 DPM in the first experiment and 1.000 x 10^6 DPM in the second of which 57.9% and 55.6% respectively became cell associated.

produced intermediate values for the amount of ^{14}C incorporated into two of the fractions listed in Table 15 while significantly increasing the amount of [^{14}C]acetate incorporated into the saponifiable fatty acid fraction.

Incubation of cells with [^{14}C]acetylcarnitine might have affected the synthesis of different species of sterols but this could not be determined directly due to the low specific radioactivity of the saponified lipids fraction extracted from basic solution (see Table 15). There was enough ^{14}C in this sterol containing fraction when cells were incubated with [^{14}C]acetate + carnitine to analyze the component sterols by TLC and compare the labeling pattern with that produced by cells incubated with [^{14}C]acetate alone. The results are shown in Figure 18 where indeed, carnitine stimulated the synthesis of an unidentified lipid component with a migration coefficient (R_f) similar to a sterol ester although the sample was saponified.

Carnitine may have also affected the synthesis of different species of fatty acids but this was not investigated. Instead, the ether extracted aqueous fraction containing ^{14}C derived from [^{14}C]acetylcarnitine was characterized. The ^{14}C in this fraction was greater than 90% bound to a cation exchange resin at pH 2 but only 50% bound at pH 7. It was nearly 22% bound to an anion exchange resin at pH 7. The net charge distributions derived from these data suggested that ^{14}C was associated with cellular amino acids, although the nature of the association is ambiguous because the extent of hydrolysis was not known.

To determine the cellular origin of the ^{14}C in the ether extracted aqueous fraction, cells were again incubated with

Figure 18. Effect of carnitine on [^{14}C]acetate incorporation into neutral lipids. Neutral lipids were isolated as described in Table 15 and separated by TLC on silica gel using petroleum ether:ether:acetic acid, 70:30:1 for development. Run time 45 min with cholesterol and cholesterol oleate included as standards. ^{14}C was detected using a Berthold TLC scanner and then the lipids were visualized by exposing the plate to iodine. A, cells received 5 μM carnitine plus [^{14}C]acetate. B, cells received only [^{14}C]acetate.

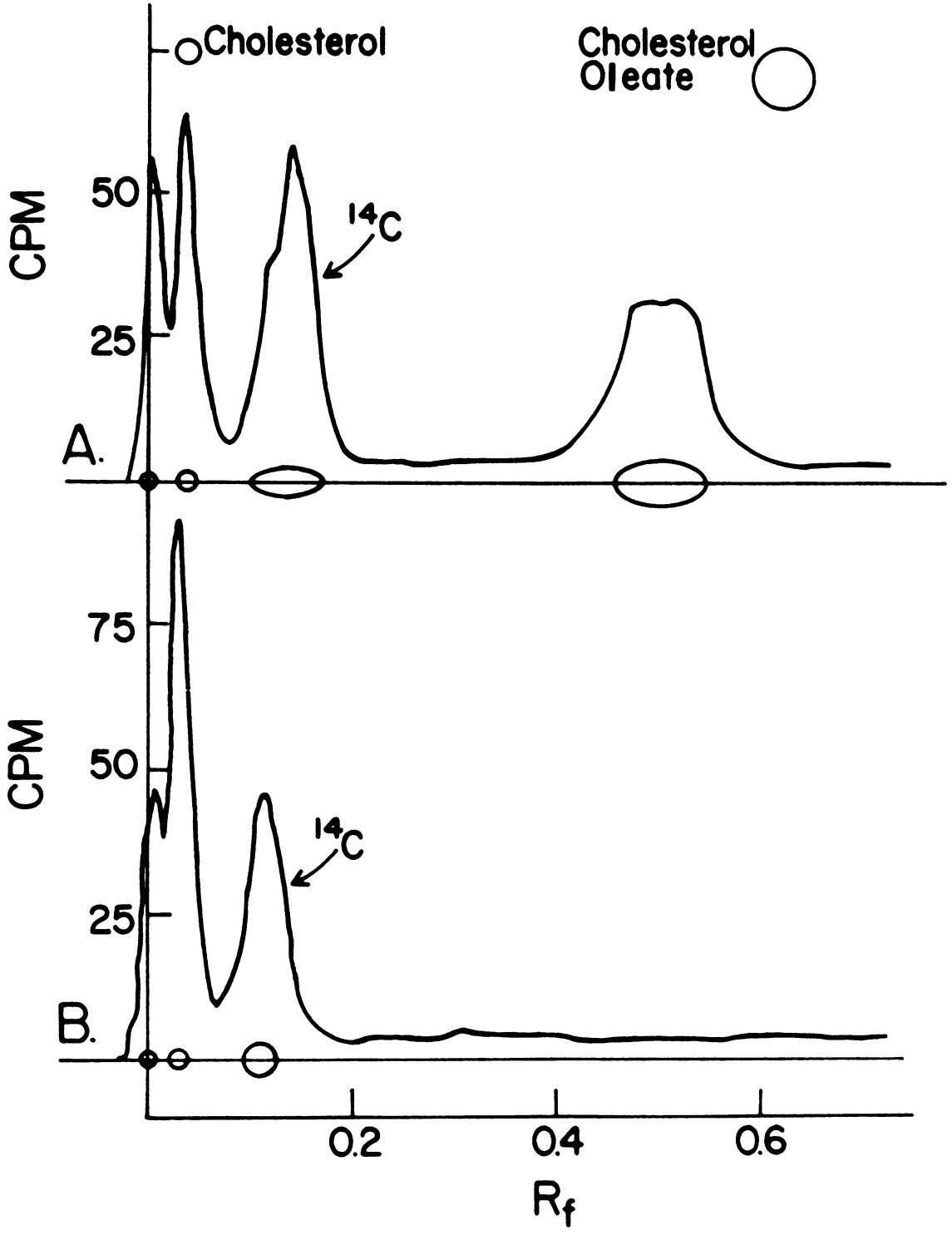

Cholesterol
Cholesterol Oleate
CPM
50
25
A.
^{14}C
75
50
25
B.
^{14}C
0.2
0.4
0.6
R_f

Figure 18

[^{14}C]acetate or [^{14}C]acetylcarnitine for 2 h after which the cells were collected and homogenized with the aid of glass beads in cold, distilled water. The homogenates were then fractionated into cell walls, lipids, ribonucleotides and proteins. The results of one experiment are summarized in Table 16. In general, the ^{14}C derived from [^{14}C]acetylcarnitine had a much different quantitative distribution among the various cell fractions than did the ^{14}C derived from [^{14}C]acetate. Again [^{14}C]acetate + carnitine appeared to produce intermediate results. In particular, these data illustrate three points, namely (1) the cell wall fraction from cells incubated with [^{14}C]acetylcarnitine typically contains low amounts of ^{14}C and thus formation of N-acetylglucoseamine does not appear to be a major function of acetylcarnitine, (2) the shortened incubation time (2 h) with [^{14}C]acetylcarnitine produces a relatively larger pool of acid soluble ^{14}C than was found in the hot water extracts or acid soluble fractions of cells grown long term with [^{14}C]acetylcarnitine (see Table 13), and finally (3) the major portion of [^{14}C]acetylcarnitine is incorporated into the protein fraction and the percentage incorporated is always about three times higher than the corresponding percentage in [^{14}C]acetate grown cells.

This last item is not strictly apparent from Table 16 because the 0.3 N KOH treatment used to hydrolyze RNA caused a 10.6% loss of ^{14}C from the protein pellet of cells incubated with [^{14}C]acetylcarnitine but, as shown in Figure 19, none of this solubilized ^{14}C was actually incorporated into any of the RNA nucleotides. Most of the ^{14}C eluted in the column wash and just prior to the elution of cytidylate during the chromatographic analysis of the RNA nucleotides, Figure 19. Thus no

TABLE 16. DISTRIBUTION OF ^{14}C IN FRACTIONS FROM HOMOGENIZED CELLS

Fraction	Percentage of Cell Associated ^{14}C[1]		
	[^{14}C]Acetate	[^{14}C]Acetate + Carnitine	[^{14}C]Acetylcarnitine
1. Cell Walls	24.9	15.7	8.2
2. Acid Soluble	3.6	2.1	16.5
3. Acid Insoluble			
3a. Lipids	26.6	23.1	13.4
3b. RNA Nucleotides	1.3	4.4	10.6
3c. Phenol Soluble Protein	13.7	30.9	29.2
3d. Phenol Insoluble Residue	14.1	16.3	14.8

Growing cells (100 ml cultures) were incubated 2 h with 5.3 µM [^{14}C]acetate (2.49 Ci/mol) with or without 5 µM carnitine present or, 5.3 µM [^{14}C]acetylcarnitine (1.35 Ci/mol). The cells from each culture were collected, resuspended in cold water and samples of the cell suspensions were taken for which 100% of cell associated [^{14}C]acetate was 1.933×10^6 DPM, and 100% of cell associated [^{14}C]acetylcarnitine was 0.79×10^6 DPM. Cell fractions were prepared and analyzed as described in the Materials and Methods.

Figure 19. Chromatography of RNA nucleotides. The RNA nucleotides obtained previously (see Table 16) from cells incubated with [^{14}C]acetylcarnitine were separated as described in the Methods by anion exchange chromatography. The nucleotides were identified by their absorbance at 260 nm (top panel) and ^{14}C analyzed as shown in the bottom panel. The numbers atop the peak fractions represent the 280 to 260 absorbance ratio of these fractions. The elution schedule is indicated by the arrows. The volume per fraction was 3 ml.

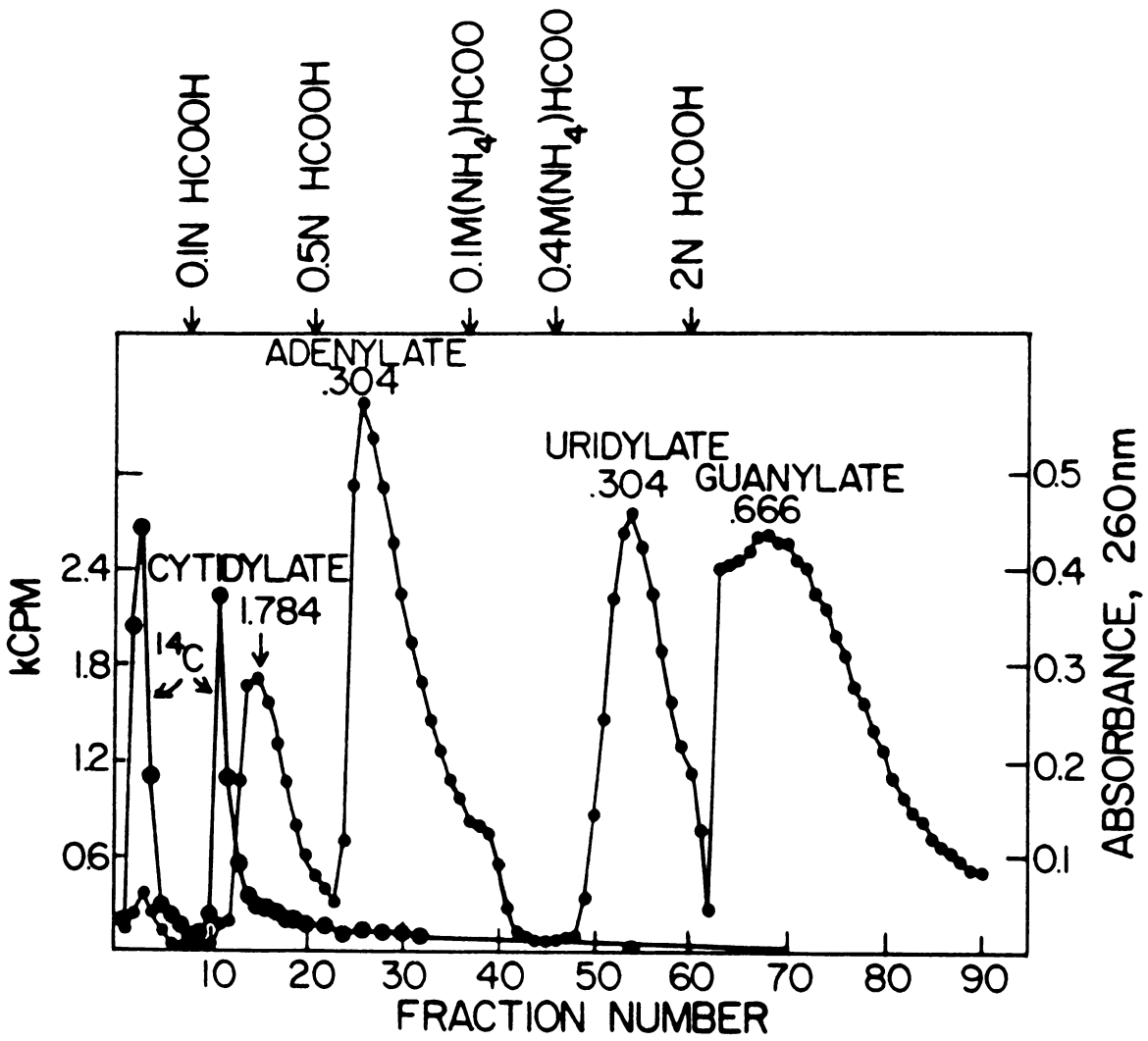

0.1N HCOOH
0.5N HCOOH
0.1M(NH4)HCOO
0.4M(NH4)HCOO
2N HCOOH
ADENYLATE
.304
URIDYLATE
.304
GUANYLATE
.666
CYTIDYLATE
1.784
14C
kCPM
2.4
1.8
1.2
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE, 260nm
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 19

evidence was found for the presence of ^{14}C in cellular RNA and the ^{14}C from [^{14}C]acetylcarnitine that contaminated the RNA fraction was probably derived from protein in which case 39.8% of the [^{14}C]acetylcarnitine was incorporated into protein. Furthermore, DNA, polysaccharides and glycoproteins are insoluble in phenol-DNA is probably not labeled because RNA nucleotides are not labeled while cell polysaccharides are removed with the cell walls in the first step of the fractionation procedure. Thus the ^{14}C in the phenol-insoluble fraction may also represent [^{14}C]acetylcarnitine incorporated into glycoproteins. For these reasons, it appeared that the acetyl group of [^{14}C]acetylcarnitine was being incorporated into cellular protein. This incorporation may be via O-acetyl ester or N-acetyl amide linkages, or integral wherein [^{14}C]acetylcarnitine provides carbons for the synthesis of amino acids.

Acetylcarnitine and protein acetylation

Two experiments were designed to test whether or not cell protein is directly or indirectly labeled by [^{14}C]acetylcarnitine. In the first, cell homogenates were incubated with [^{14}C]acetylcarnitine and the amount of ^{14}C incorporated into acid-insoluble material determined. When crude yeast extracts depleted of CoASH were incubated with [^{14}C]acetylcarnitine, ^{14}C was not incorporated into acid-insoluble material, Table 17, nor was ^{14}C incorporation into this precipitate stimulated by supplementing the crude extracts with CoASH and/or an energy source. The ^{14}C deficits in samples 2-6 compared to the boiled control sample indicated in Table 17 were too small to be significant. In addition, similar recoveries of soluble

TABLE 17. LACK OF PROTEIN ACETYLATION IN CRUDE CELL EXTRACTS INCUBATED WITH [^{14}C]ACETYLCARNITINE

Expt	CoASH	ATP, Mg^{++}	% ^{14}C Acid Soluble
1	-	-	100
2	-	-	97.7
3	+	-	98.0
4	-	+	94.6
5	+	+	97.7
6	-	-	99.2

Crude extracts were prepared from cells grown in basal media as described in the Methods. For experiments 1-5 the extract was treated for 2 min with 1 mM N-ethylmaleimide followed by 4.9 mM reduced glutathione. Experiment 6 was not so treated. Samples of the extracts (1.0 ml) were then incubated 60 min at 30°C with [^{14}C]acetylcarnitine (0.45 mM, 1.5 x 10^6 DPM) with other additions as shown (final concentrations; CoASH, 1.8 µM; glucose, 0.09 mM; ATP, 0.018 mM; $MgCl_2$, 0.0036 mM). The crude extract in experiment 1 was boiled 3 min prior to the incubation with [^{14}C]acetylcarnitine. The reactions were stopped with 6% perchloric acid and the denatured protein collected by centrifugation. The pellets were washed once with 2 ml of 0.5 M phosphate buffer, pH 6.5. The two supernatant solutions were combined and analyzed for ^{14}C. The acid soluble ^{14}C in experiment 1 was set equal to 100%. Not shown are an identical set of experiments in which [^{14}C]acetate replaced [^{14}C]acetylcarnitine.

^{14}C were obtained in an identical set of control incubations utilizing [^{14}C]acetate instead of [^{14}C]acetylcarnitine (not shown). The acid soluble material from experiment 4, see Table 17, was analyzed by Dowex chromatography and TLC and ^{14}C was only detected in [^{14}C]acetylcarnitine with a trace of ^{14}C in free acetate.

Identification of radioactive labeled amino acids in protein hydrolysates

In the second experiment, cells were again grown with [^{14}C]acetylcarnitine and the cell protein isolated, hydrolyzed, and the amino acids analyzed by HPLC. Table 18 summarizes the data for the protein isolation procedure and indicates that the phenol extraction method successfully recovered 56% of the acetylcarnitine-derived ^{14}C in a single fraction, i.e., the phenol soluble, acetone insoluble protein pellet. Three times as much radioactivity derived from [^{14}C]acetylcarnitine was recovered in this fraction as was recovered in the same fraction labeled with [^{14}C]acetate. The other fractions were not analyzed and the radioactivity unaccounted for in Table 18 was presumably in the phenol-insoluble cell debris. Assay of the amino nitrogen in the hydrolyzed protein samples yielded values of 8.7 and 9.4 μeq amino nitrogen/mg protein in the [^{14}C]acetylcarnitine and [^{14}C]acetate treated cultures respectively. The similarity of these two values suggested that the isolated protein fractions were not radically different from each other in terms of the percentage of lipid or glycosyl residue content.

The incorporation of radioactivity into cellular protein by N-acetylation was negligible. A weighed sample of radiolabeled protein

TABLE 18. ISOLATION OF ^{14}C LABELED PROTEIN

Fraction	[^{14}C]Acetate	[^{14}C]Acetylcarnitine
	Percentage of Cell Associated ^{14}C	
Cell Walls	5.6	5.1
Acid Soluble	1.7	8.9
Phenol, Acetone Soluble	54.0	25.3
Phenol, Acetone Pellet	18.1	55.9
mg Protein	166	243
Protein Specific Activity DPM/mg dry protein	49800	53100

Two identical 1L cultures having cell densities of 0.1 absorbance were supplemented with 7.4 µmol [^{14}C]acetate (21 µCi) or [^{14}C]acetylcarnitine (24 µCi) and incubated 5.5 h until the cell density had attained an absorbance of 0.4. Cells were collected by centrifugation, homogenized and fractionated as described in the Materials and Methods section. The acetone insoluble protein pellet was washed with methanol and dryed under a stream of N_2. Weighed samples were dissolved 24 h in "Protosol" (New England Nuclear), counted for ^{14}C and the specific activity calculated.

was hydrolyzed 72 h in constant boiling HCl, conditions adequate to hydrolyze N-acetyl bonds. The hydrolysate was evaporated under reduced pressure at 60°C and the vapors trapped in ice cold 1 N KOH solution placed into the condensing flask. Any free [^{14}C]acetic acid would have been removed from the hydrolysate during evaporation but no ^{14}C was detected in the condensing flask solution. In addition, all of the ^{14}C was recovered in the hydrolysate residue after evaporation.

Hydrolysates of the protein described in Table 18 were analyzed for the ^{14}C associated with specific amino acids by HPLC, see Figure 20. The top panels in Figures 20A and B record the fluorescence intensity (arbitrary units) of the phthaldialdehyde derivatized amino acids separated by reverse phase HPLC. The bottom panels show the quantity of ^{14}C present in the column eluates. Most of the peaks were identified by comparison of their retention times with those of standard amino acids. Tryptophan eluted at 25-26 min and probably appears as the shoulder on the left side of the leucine/isoleucine peak. Threonine eluted with glycine, and valine and methionine eluted together at about 23 min. The peaks at 16.8 and 43 min were not identified. The cells incubated with [^{14}C]acetylcarnitine contained radioactive glutamate, arginine, leucine, and lysine as shown in the bottom panel of Figure 20A, while leucine and lysine were primarily labeled by [^{14}C]acetate as indicated in the bottom of Figure 20B. The radioactive peaks at 2 min eluted in the void volume and represent underivatized amino acids. Proline does not react and histidine reacts poorly with phthaldialdehyde. Most of the radioactivity at 2 min was probably due to the amino acids being present in great excess during the derivatization reaction to favor

Figure 20. Identification of ^{14}C labeled amino acids by HPLC. Samples of the protein hydrolysates obtained previously (see Table 18) were derivatized with phthaldialdehyde, separated by HPLC, detected by fluorescence, collected in 0.5 min fractions in scintillation fluid and measured for ^{14}C (see Materials and Methods). In A, the protein hydrolysate was obtained from cells grown with [^{14}C]acetylcarnitine and in B, from cells grown with [^{14}C]acetate. In both A and B, the ^{14}C in each fraction is plotted just below the tracing of the fluorescence detected amino acid derivatives. Amino acid assignments were made by comparison of retention times with those of known standard amino acids.

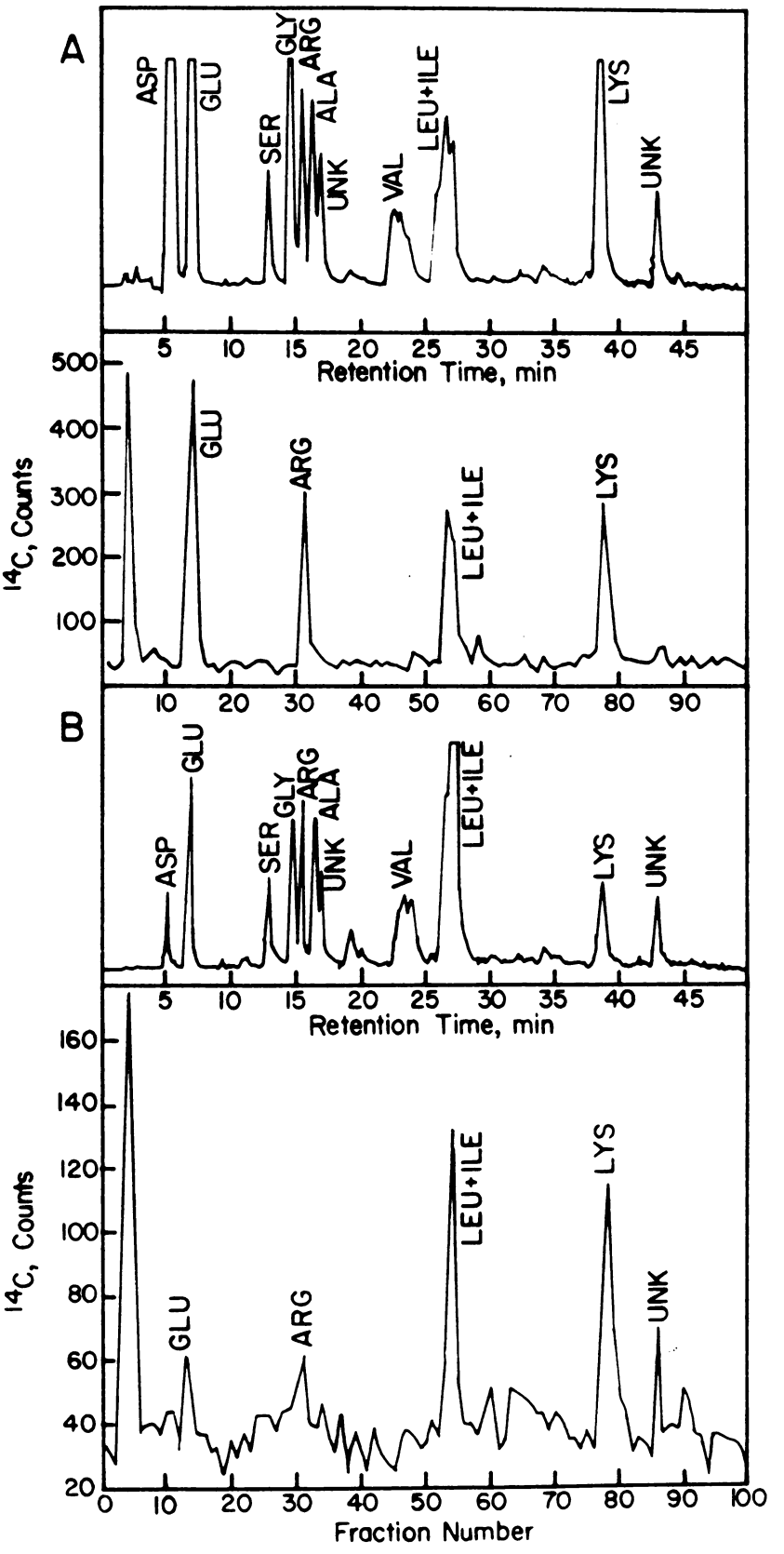

A
ASP
GLU
SER
GLY
ARG
ALA
UNK
VAL
LEU+ILE
LYS
UNK
5 10 15 20 25 30 35 40 45
Retention Time, min
^{14}C, Counts
500
400
300
200
100
GLU
ARG
LEU+ILE
LYS
10 20 30 40 50 60 70 80 90
B
ASP
GLU
SER
GLY
ARG
ALA
UNK
VAL
LEU+ILE
LYS
UNK
5 10 15 20 25 30 35 40 45
Retention Time, min
^{14}C, Counts
160
140
120
100
80
60
40
20
GLU
ARG
LEU+ILE
LYS
UNK
0 10 20 30 40 50 60 70 80 90 100
Fraction Number

Figure 20

detection of ^{14}C. For example, the area under the aspartic peak in Figure 20B was 1.5×10^7 units while the same peak still had an area of 1.3×10^7 units when only one-fifth as much sample was derivatized.

Although the chromatographic procedure used in Figure 20 effectively separated glutamate and arginine, leucine was not resolved from isoleucine or tryptophan, histidine was poorly derivatized and therefore inadequately resolved, and proline is inert to phthaldialdehyde derivatization. Therefore, a TLC system was used to specifically separate these amino acids. The protein hydrolysate from the [^{14}C]acetylcarnitine treated cells was passed through a column of Dowex 1-x8 (acetate) resin to remove glutamate. The eluate was evaporated under reduced pressure, dissolved in a small volume of water and chromatographed as indicated in Figure 21. Proline was identified by the characteristic yellow color it produces with ninhydrin. Leucine was resolved from both isoleucine and tryptophan, and lysine, histidine and proline were each individually isolated. The ninhydrin reactive spots were numbered as shown and used as a template for an identical sample chromatographed similarly. The numbered spots were scraped from the second plate, placed into scintillation vials and counted for ^{14}C. The radioactive amino acids were detected as shown in Table 19. Proline contained ^{14}C as expected if it was derived from glutamate. Histidine was not labeled. Separation of isoleucine from leucine demonstrated that only leucine was radiolabeled. Lysine also contained ^{14}C. The smear of radioactivity in spots 4-6 is attributed to the imperfect separation of arginine in this region of the plate by the TLC solvent systems selected. TLC analysis of the [^{14}C]acetate grown

igure 21. TLC analysis of radioactive labeled amino acids derived from
. bovina cell proteins. A sample (2 µl) of the [^{14}C]acetylcarnitine
abeled protein hydrolysate (described in the legend to Figure 20A) freed
f glutamate by treatment with Dowex 1-X8 (acetate) resin was applied to
he lower right corner of an AVICEL coated TLC plate. It was developed
n the first direction 3 h with isopropanol, formic acid (88%), water
160:9:39) (IPF). It was developed twice in the second direction for 3 h
sing pyridine, amylalcohol, water (35:35:30) (PA). Guide strips with
tandard amino acids were run as indicated. The amino acids were
isualized using ninhydrin spray reagent. The following spot numbers
orrespond to: 1, histidine; 2, lysine; 9, proline; 14, isoleucine; 15,
eucine.

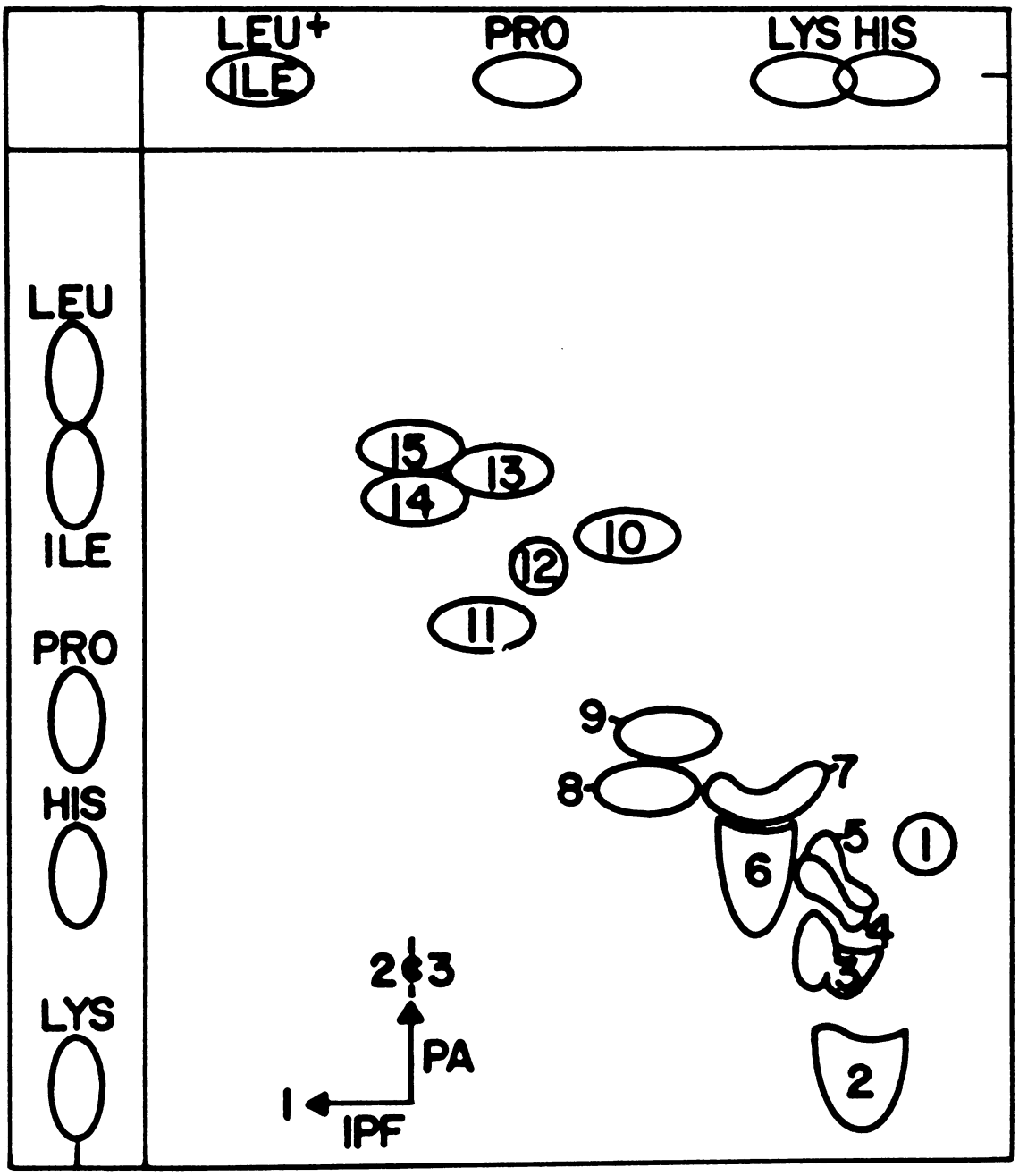

LEU+
ILE
PRO
LYS HIS
LEU
ILE
PRO
HIS
LYS
15
13
14
10
12
11
9
8
7
6
5
1
4
3
2
2&3
PA
1
IPF

Figure 21

TABLE 19. DISTRIBUTION OF [^{14}C]ACETYLCARNITINE DERIVED RADIOACTIVITY IN AMINO ACID FRACTIONS SEPARATED BY TLC

TLC Fraction	DPM	TLC Fraction	DPM
1 (HIS)	0	9 (PRO)	264
2 (LYS)	481	16	0
3	217	11	0
4	184	12	0
5	651	13	8
6	104	14 (ILE)	42
7	16	15 (LEU)	438
8	25		

A sample (2 µl, 5240 DPM) of the glutamate free protein hydrolysate derived from [^{14}C]acetyl-carnitine treated cells was analyzed by TLC as described in the legend to Figure 21. Some of the amino acids were visualized by spraying lightly with ninhydrin but all 15 numbered spots were removed from the plate using Figure 21 as a template and transferred to scintillation vials for detection of ^{14}C as shown. The numbered TLC fractions exactly correspond to those shown in Figure 21 with the amino acids of interest identified in parentheses.

cells' protein hydrolysate confirmed that leucine and lysine were the only amino acids containing measurable levels of radioactivity.

Glutamate is derived from the TCA cycle via citrate and 2-oxoglutarate. If the TCA cycle is fully operational in this yeast, then the α-carbon of glutamate should be labeled. Glutamate was isolated by anion exchange chromatography from the hydrolyzed protein sample labeled with [^{14}C]acetylcarnitine and the glutamate decarboxylated with glutamate decarboxylase. The liberated CO_2 was collected in 1 M hyamine which when analyzed did not contain detectable levels of $^{14}CO_2$.

These results demonstrate that acetylcarnitine contributes carbons for the synthesis of amino acids. Glutamate, proline, and arginine form a family of amino acids in E. coli labeled by radioactive acetate (222) because of their synthesis from a common precursor, 2-oxoglutarate derived from the TCA cycle and thus, ultimately from acetylCoA and oxaloacetate. The TCA cycle reactions are confined in the matrix of mitochondria in eucaryotes and so in T. bovina acetylcarnitine must enter the mitochondrial pool of acetylCoA whereas acetate does not. Certainly no evidence has been obtained with T. bovina to discourage the view that acetylcarnitine facilitates acetyl group transfer across acylCoA impermeable membranes and it is proposed here that acetylcarnitine shuttles acetyl groups into the mitochondria. If this is true, then CAT must be associated with mitochondria.

INTRACELLULAR LOCALIZATION OF CAT

Evidence for membrane bound (particulate) CAT

It had been observed earlier that during gel permeation chromatography of crude extracts (Sephadex G150), more than 50% of the CAT activity eluted in the void volume. In addition, the duration of cell homogenization did not affect the distribution of CAT during differential centrifugation. Subsequent experiments demonstrated that the sedimentation behavior of CAT depends on the phosphate concentration in the homgenization buffer, see Figure 22. The proportion of CAT in a 27,000 x g pellet fraction was about 50% when the phosphate concentration in the homogenization buffer was less than 50 mM, but less than 5% at 200 mM phosphate.

Evidence for compartmentalization of CAT is presented in Table 20. Cells were homogenized for 5 sec in a low phosphate concentration sorbitol buffer isotonic for yeast mitochondria which were collected by centrifugation at 20,000 x g for 10 min and assayed for CAT. Greater than 50% of the extracted CAT was particulate even though most of the mitochondrial matrix enzyme, citrate synthase, remained soluble. Virtually none of the cytosolic enzyme glucose-6-phosphate dehydrogenase was associated with the 20,000 x g pellets. Traces of malate dehydrogenase were found in the particulate fraction but 98% of the activity was soluble. Catalase, fumarase, NADH dehydrogenase, α-ketoglutarate dehydrogenase and isocitrate dehydrogenase (NAD^+-linked) were not detected.

Figure 22. Effect of phosphate concentration on sedimentation behavior of CAT. Crude extracts of T. bovina were prepared by the usual procedure (see Methods) in phosphate buffers at the indicated phosphate concentrations. CAT was assayed in the crude extract and then the extract was centrifuged 10 min at 27,000 x g and the supernatant fluid removed. The pellet was resuspended in buffer and assayed for CAT. CAT was assayed in Buffer II (see Methods).

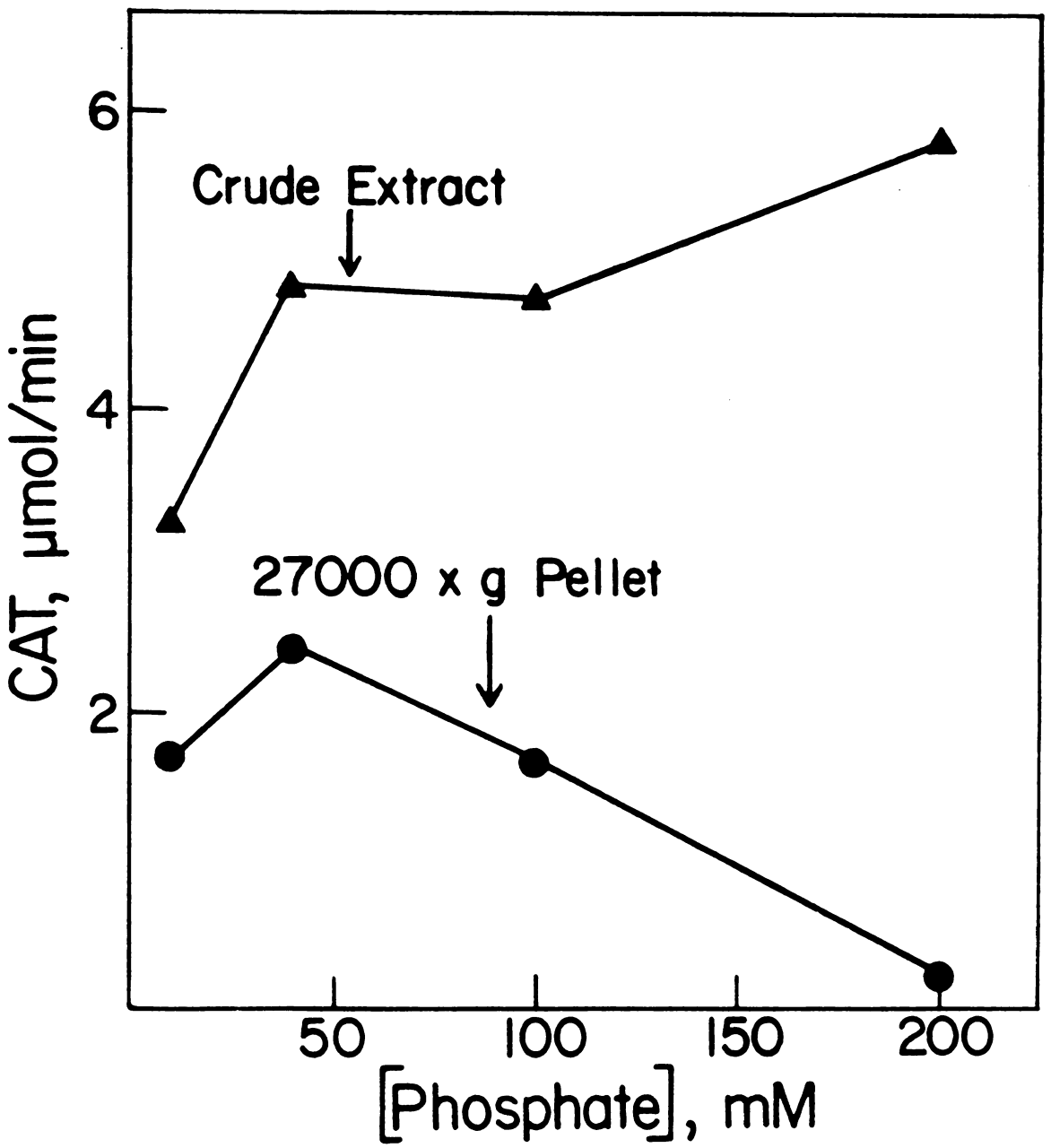

6
4
2
CAT, µmol/min
Crude Extract
27000 x g Pellet
50
100
150
200
[Phosphate], mM

Figure 22

TABLE 20. CAT DISTRIBUTION IN CRUDE EXTRACTS

	CAT	Citrate Synthase	Glucose-6-Phosphate Dehydrogenase
		µmol/min (%)	
500 x g supernate	0.96 (100)	.100 (100)	.350 (100)
20000 x g supernate	0.44 (46)	.079 (79)	.320 (91)
20000 x g pellet	0.53 (55)	.012 (12)	.004 (1)

Cells were homogenized for 5 sec with glass beads as described in the Materials and Methods. CAT was assayed in Buffer II supplemented with 0.1% (v/v) Triton X-100. Subcellular fractions were isolated by differential centrifugation. All centrifugations were performed for 10 min in a Beckman J2-21 centrifuge with the pellets being resuspended in the homogenization buffer. The values in parentheses represent the percent of the enzyme activity in the fraction.

Isopycnic density gradient analysis of CAT

When a cell homogenate (>95% of cells broken) prepared in 50 mM phosphate buffer was subjected to isopycnic centrifugation on a linear, 10-40% sorbitol gradient, more than 87% of the CAT activity in the gradient was particulate, see Figure 23. This figure shows that CAT has a bimodal distribution with peak activities at equilibrium densities of 1.13 and 1.09 g/cm^3 on either side of the ribosomal protein peaks. AcetylCoA hydrolase and glucose-6-phosphate dehydrogenase are located exclusively in the soluble portion of the gradient. A small but consistently measurable fraction of the total CAT activity overlapped into the soluble region. Specific activities of 4.4 and 9.8 μmol/min/mg protein for the lower and higher density CAT peaks respectively were obtained. These values represent, respectively, a 14-fold and a 31-fold increase in the enzyme activity compared to that in the crude extract.

The distribution of some other enzymes in cell-free homogenates are also shown in Figure 23. AcetylCoA synthetase migrates very nearly like a soluble enzyme as did NADPH-cytochrome c reductase although NADPH cytochrome c reductase was also present in low but measurable quantities near the bottom of the gradient. The higher density NADPH-cytochrome c reductase activity was not detected without adding sodium azide to the assay. The yeast mitochondrial membrane marker enzyme, NADH dehydrogenase, and the plasma membrane marker enzyme, α-glucosidase (maltase), were not detected. $NADP^+$-linked isocitrate dehydrogenase was detected and it remained in the soluble region. A portion of the citrate synthase also remained in the soluble region of the gradient indicating that mechanical disruption broke inner mitochondrial membranes. However, there is a second peak of citrate synthase, note

Figure 23. Isopycnic sorbitol density gradient analysis of CAT in extracts from mechanically disrupted T. bovina cells. Cell homogenization, gradient purification and enzymatic analysis were performed as described in the Materials and Methods section. The absorbance ratio is the ratio of the 260 and 280 nm absorbances. G6PDH, glucose-6-phosphate dehydrogenase. All enzyme activities are expressed in μmol/min.

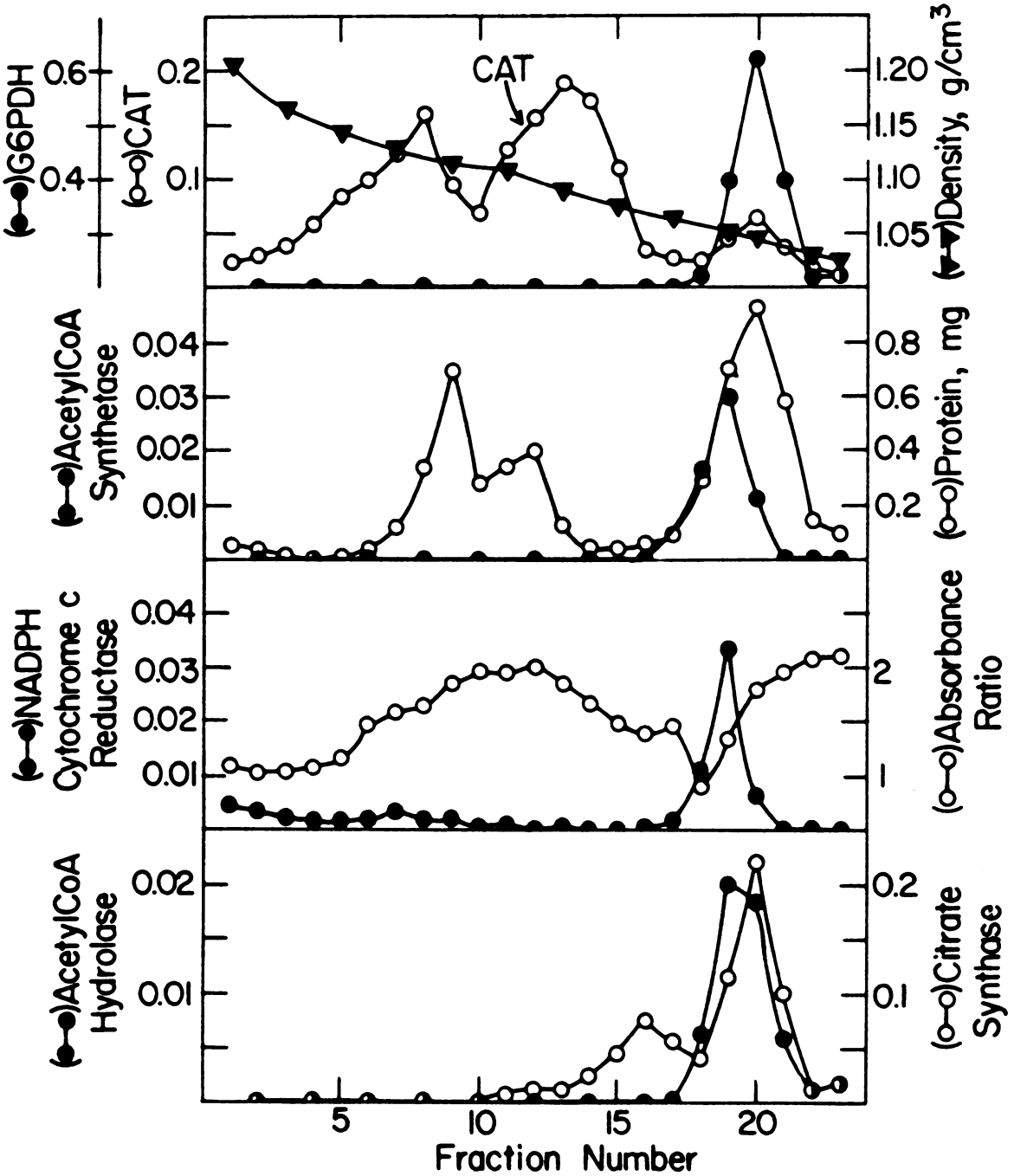

(●—●)G6PDH
(o—o)CAT
CAT
(▼—▼)Density, g/cm³
(●—●)Acetyl CoA Synthetase
(o—o)Protein, mg
(●—●)NADPH Cytochrome c Reductase
(o—o)Absorbance Ratio
(●—●)Acetyl CoA Hydrolase
(o—o)Citrate Synthase
Fraction Number

Figure 23

fraction 16, reminiscent of a novel citrate synthase particle reported previously (see ref. 217). It was found by gradient analysis of other cell homogenates that media carnitine did not alter the CAT distribution seen in control cells.

High concentrations of phosphate solubilize both the microsomal acetylCoA synthetase in S. cerevisiae (218) and CAT in mammals (46,80,117). When T. bovina was homogenized in 0.2 M phosphate buffer and then analyzed as described above by isopycnic density gradient centrifugation, see Figure 24, CAT remained mostly particulate. A typical amount of CAT activity was extracted from the cells but most of the activity migrated into the gradient as a peak at 1.07 g/cm^3 well separated from the soluble enzyme markers.

In another gradient (not shown) the higher density peak fractions of protein, CAT and NADPH-cytochrome c reductase were negatively stained and examined by electron microscopy. The NADPH cytochrome c reductase fraction (D_4^{20}=1.163) contained a large number of small vesicles with diameters less than 0.2 μm. The CAT fraction (D_4^{20}=1.138) contained both small and large vesicles with diameters approaching 1 μm, see Figure 25. The protein peak (D_4^{20}=1.120) contained only large, 1 μm diameter vesicles.

Association of CAT with mitochondria

Although these results indicated CAT was primarily a particulate enzyme in T. bovina, the cellular origin of this material was not identified with any of the marker enzymes assayed. The fairly large vesicles observed with the electron microscope suggested the particulate material was membrane ghosts of mitochondria. The homogenization

Figure 24. Effect of high phosphate concentration on the isopycnic density of CAT. Crude extracts were prepared in 0.2 M phosphate buffer and centrifuged through a sorbitol gradient. CAT was assayed in Buffer II and the other analyses were performed as described in the Methods.

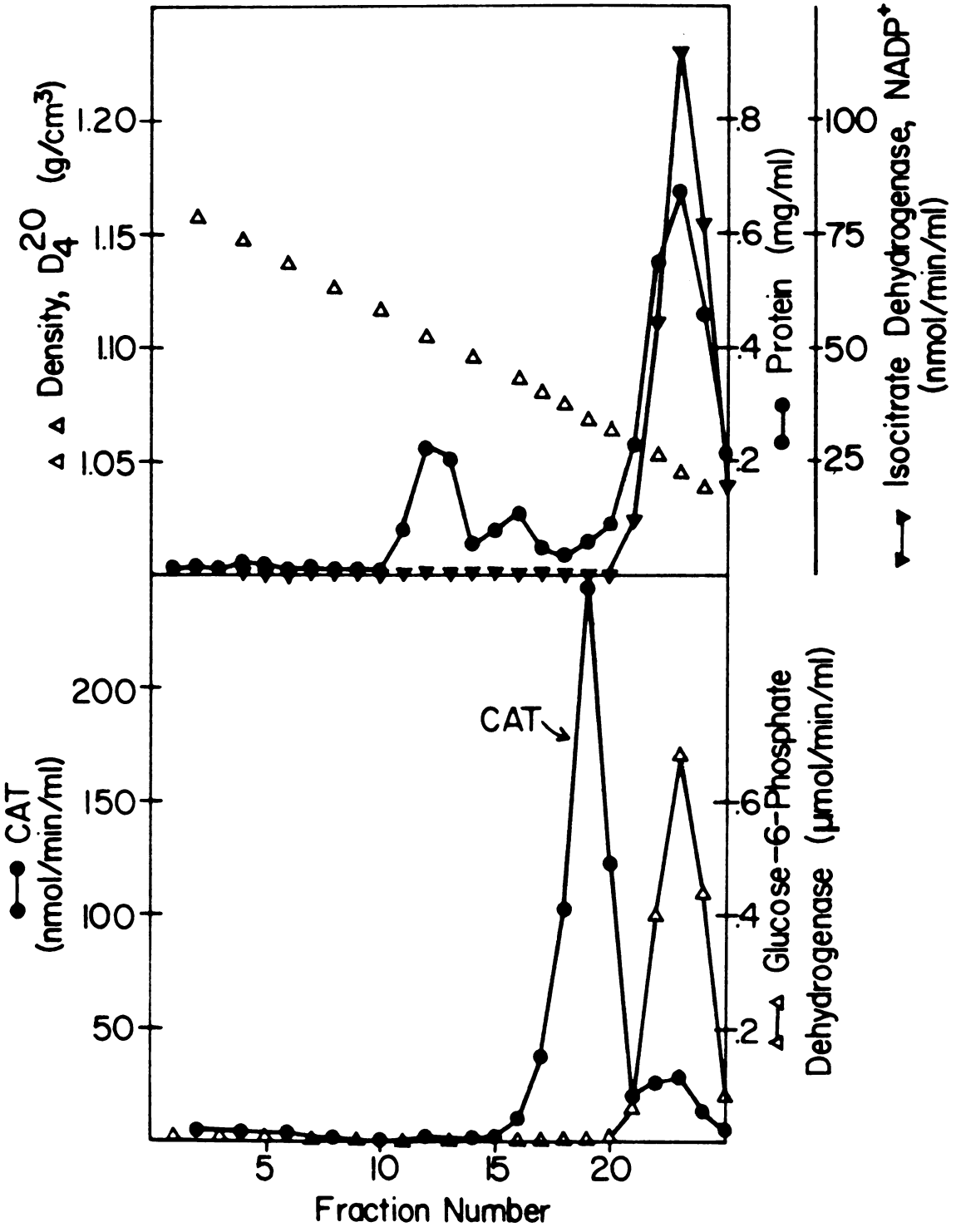

Density, D_4^{20} (g/cm³)
1.20
1.15
1.10
1.05
.8
.6
.4
.2
Protein (mg/ml)
100
75
50
25
Isocitrate Dehydrogenase, NADP⁺ (nmol/min/ml)
CAT
CAT (nmol/min/ml)
200
150
100
50
.6
.4
.2
Glucose-6-Phosphate Dehydrogenase (µmol/min/ml)
5
10
15
20
Fraction Number

Figure 24

Figure 25. Electron micrographs of peak CAT fraction (D_4^{20} = 1.138 g/cm^3). The particulate CAT fraction having an isopycnic density of 1.138 g/cm^3 (see Figure 23) in a sorbitol gradient was dialyzed against 50 mM phosphate buffer, pH 6.5, negatively stained with uranylacetate and examined with a Philips Model 201 electron microscope.

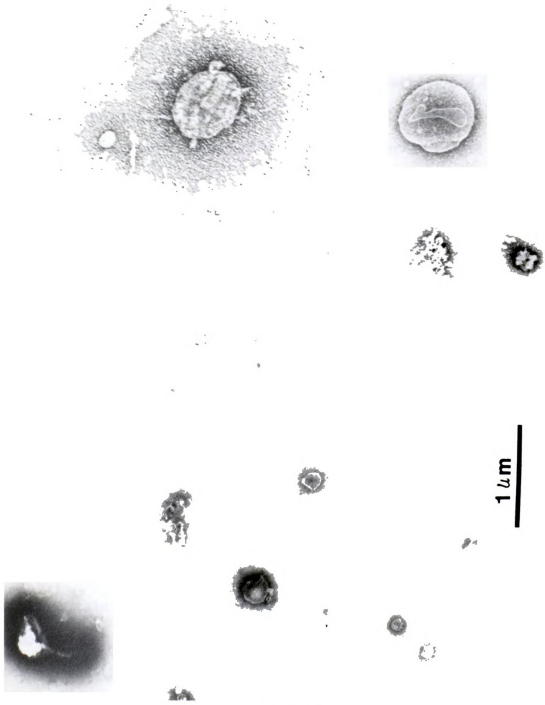


Figure 25

procedure using glass beads is described as a method for preparing mitochondria from microorganisms but it obviously did not work for I. bovina. Thus another method was selected to prepare intact mitochondria from these yeast, i.e., osmotic lysis of spheroplasts. A summary of the sedimentation behavior of CAT from spheroplast lysates is given in Table 21. In experiment 1, a significant portion of the citrate synthase activity and CAT was pelleted by a relatively low force of 8000 x g. In experiment 2, about 20% of the initial citrate synthase and CAT activities were not pelleted by centrifugation at 31,000 x g. The pellets contained only traces of the cytosolic marker enzyme in both experiments.

Since a large percentage of the mitochondria were left intact after osmotic lysis of the spheroplasts, they were purified by isopycnic density gradient analysis as shown in Figure 26. CAT has an identical distribution profile to citrate synthase throughout the gradient with the major peaks for both enzymes located at a density of 1.21 g/cm^3, a density typical of yeast mitochondria. Few mitochondria were broken in this preparation as indicated by the extremely low amount of citrate synthase located at the top of the gradient with the soluble glucose-6-phosphate dehydrogenase. The recovery of citrate synthase was 82% and the recovery of CAT was 93%. The lack of glucose-6-phosphate dehydrogenase activity in fraction 2 but the presence of CAT, citrate synthase and NADPH-cytochrome c reductase in this fraction suggests that it may contain spheroplasts that have lost their soluble, cytoplasmic components but not their intracellular organelles. The enzyme activities located near the top of the gradient are probably due to a heterogenous population of membrane particles. AcetylCoA synthetase is shown to be a

TABLE 21. SUBCELLULAR DISTRIBUTION OF CAT IN *T. bovina*

	Protein	CAT	Citrate Synthase	Glucose-6-Phosphate Dehydrogenase
	mg (%)		μmol/min (%)	
Expt. 1				
Spheroplast lysate	29.67 (100)	25.6 (100)	8.84 (100)	6.02 (100)
8000 x g supernate	25.30 (85)	15.3 (60)	6.65 (75)	6.27 (104)
8000 x g pellet	3.19 (11)	8.8 (34)	2.10 (24)	0.26 (4)
Expt. 2				
Spheroplast lysate	61.61 (100)	36.3 (100)	11.8 (100)	26.1 (100)
31000 x g supernate	54.67 (89)	7.36 (20)	3.03 (26)	25.6 (98)
31000 x g pellet	8.76 (14)	14.8 (41)	4.72 (40)	1.34 (5)

Spheroplast lysates were obtained as described in the Materials and Methods section. CAT was assayed in Buffer II supplemented with 0.1% (v/v) Triton X-100. Protein was assayed on samples treated with 1% (w/v) sodium dodecyl sulfate according to the method of Lowry *et al.* (205). Subcellular fractions were isolated by differential centrifugation. All centrifugations were performed for 10 min in a Beckman J2-21 centrifuge with the pellets being resuspended in the lytic buffer.

Figure 26. Subcellular localization of T. bovina CAT in mitochondria isolated by isopycnic sorbitol gradient centrifugation of a spheroplast lysate. Spheroplast lysate preparation, gradient centrifugation and enzymic analysis were performed as described in the Materials and Methods section. G6PDH, glucose-6-phosphate dehydrogenase; CAT, carnitine acetyltransferase. Enzyme activities are in μmol/min.

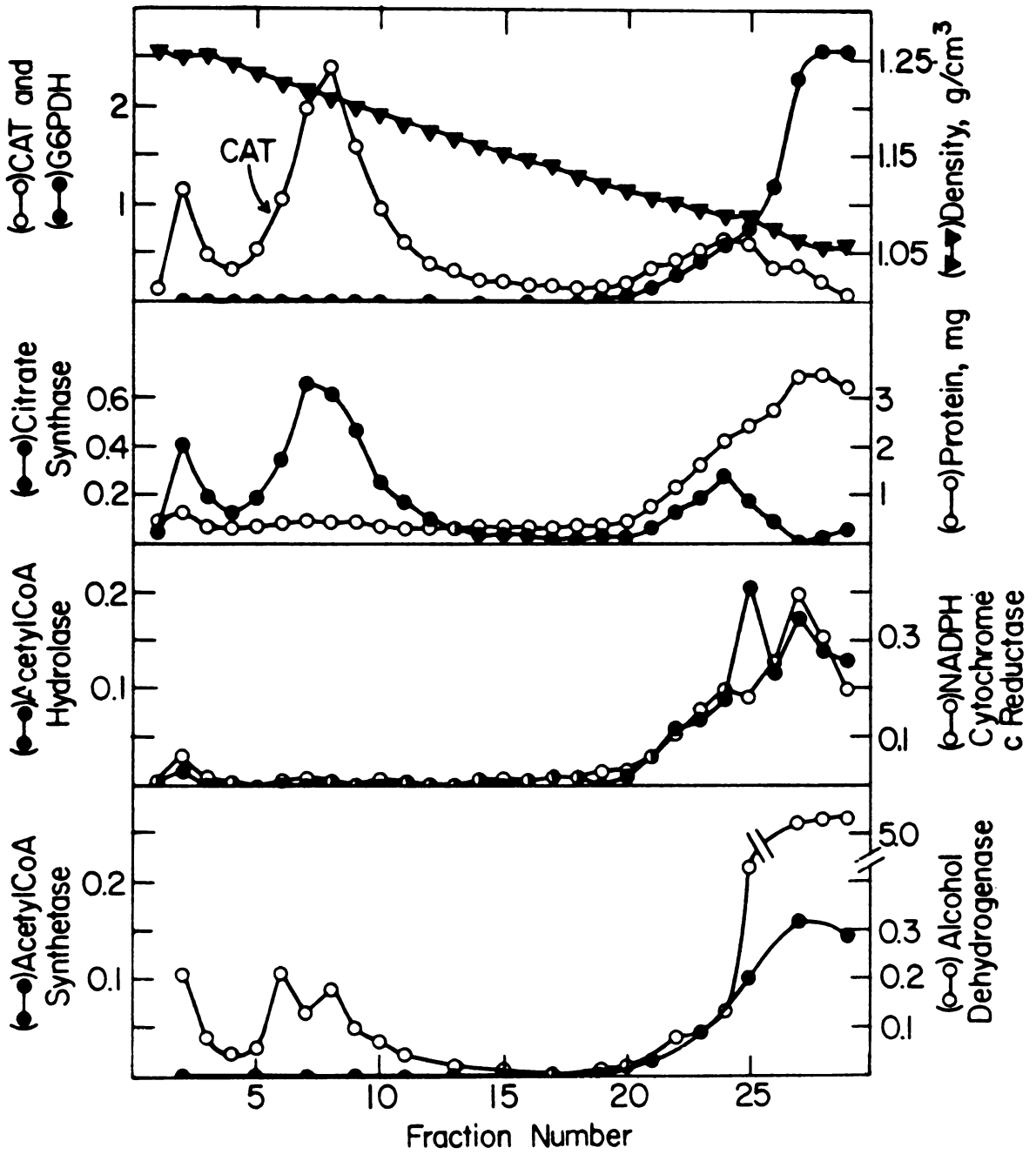

(o—o)CAT and
(●—●)G6PDH
CAT
2
1
1.25
1.15
1.05
(▼—▼)Density, g/cm³
(●—●)Citrate Synthase
0.6
0.4
0.2
3
2
1
(o—o)Protein, mg
(●—●)Acetyl CoA Hydrolase
0.2
0.1
0.3
0.1
(o—o)NADPH Cytochrome c Reductase
(●—●)Acetyl CoA Synthetase
0.2
0.1
5.0
0.3
0.2
0.1
(o—o) Alcohol Dehydrogenase
5
10
15
20
25
Fraction Number

Figure 26

cytosolic enzyme as is the majority of alcohol dehydrogenase although the 3% of alcohol dehydrogenase in the mitochondrial fractions represents a substantial amount of activity. No NAD-linked glutamate dehydrogenase activity was detected in the peak mitochondrial fractions although the enzyme appeared to be present in the spheroplast lysate. The specific activity of CAT in the peak fraction (fraction 8) is 5000 nmol/min/mg protein and must reflect an important function for CAT in the metabolism of T. bovina.

Utilization of Acetylcarnitine by Isolated Mitochondria

To determine if isolated mitochondria use acetylcarnitine for biosyntheses, intact mitochondria were prepared from spheroplast lysates by differential centrifugation and incubated with [^{14}C]acetylcarnitine or [^{14}C]acetate in isotonic buffer containing malate and NAD^+. In a typical preparation, the 15,000 x g mitochondrial pellet contained 44% and 42% respectively, of the citrate synthase and the CAT activity originally present in the lysate solution with no acetylCoA hydrolase activity detected in the pellet. In addition, the three cytochrome peaks detected in the difference spectrum shown in Figure 5 demonstrate that these preparations contained mitochondria.

The radioactive products formed during the incubation of the mitochondria with radioactive acetate or acetylcarnitine were isolated by anion exchange chromatography. Figures 27A and 27B show that the radioactivity derived from acetylcarnitine was incorporated into at least four separate compounds whereas acetate remained essentially unchanged. The elution profile of acetate and glutamate was established

Figure 27. Chromatography of mitochondrial preparations incubated with radioactive acetate or acetylcarnitine. Mitochondria were isolated as described in the Materials and Methods. The incubation solutions contained in 1.0 ml: 3 mg mitochondrial protein, 1 mM NAD^+, 7.5 mM malate, 50 mM Tris pH 7.4, 0.5 mM EDTA, 0.6 M sorbitol, and either 1.0 mM [1-^{14}C]acetate (2.356 x 10^6 DPM) or 1.0 mM [1-^{14}C]acetylcarnitine (3.033 x 10^6 DPM). After 30 min incubation at 30°C, the reactions were stopped by boiling the solutions 2 min. The samples were chromatographed on 1 x 7 cm columns of Dowex 1-x8 (Cl^-) eluted with a 240 ml linear gradient of 0.01 N HCl (conductivity 0.38 mS) to 0.075 N HCl (conductivity 50 mS). Fraction volumes were 2.85 ml; fractions 45 through 84 contained negligible radioactivity and are not shown in the figure. In C, the material in peak IV was converted to glutamate using glutamate dehydrogenase (231) and re-chromatographed on a similar column but eluted with a 0.01 to 0.0375 N HCl gradient. A, incubation with [^{14}C]acetate. B, incubation with [^{14}C]acetylcarnitine. C, peak IV after reaction with glutamate dehydrogenase.

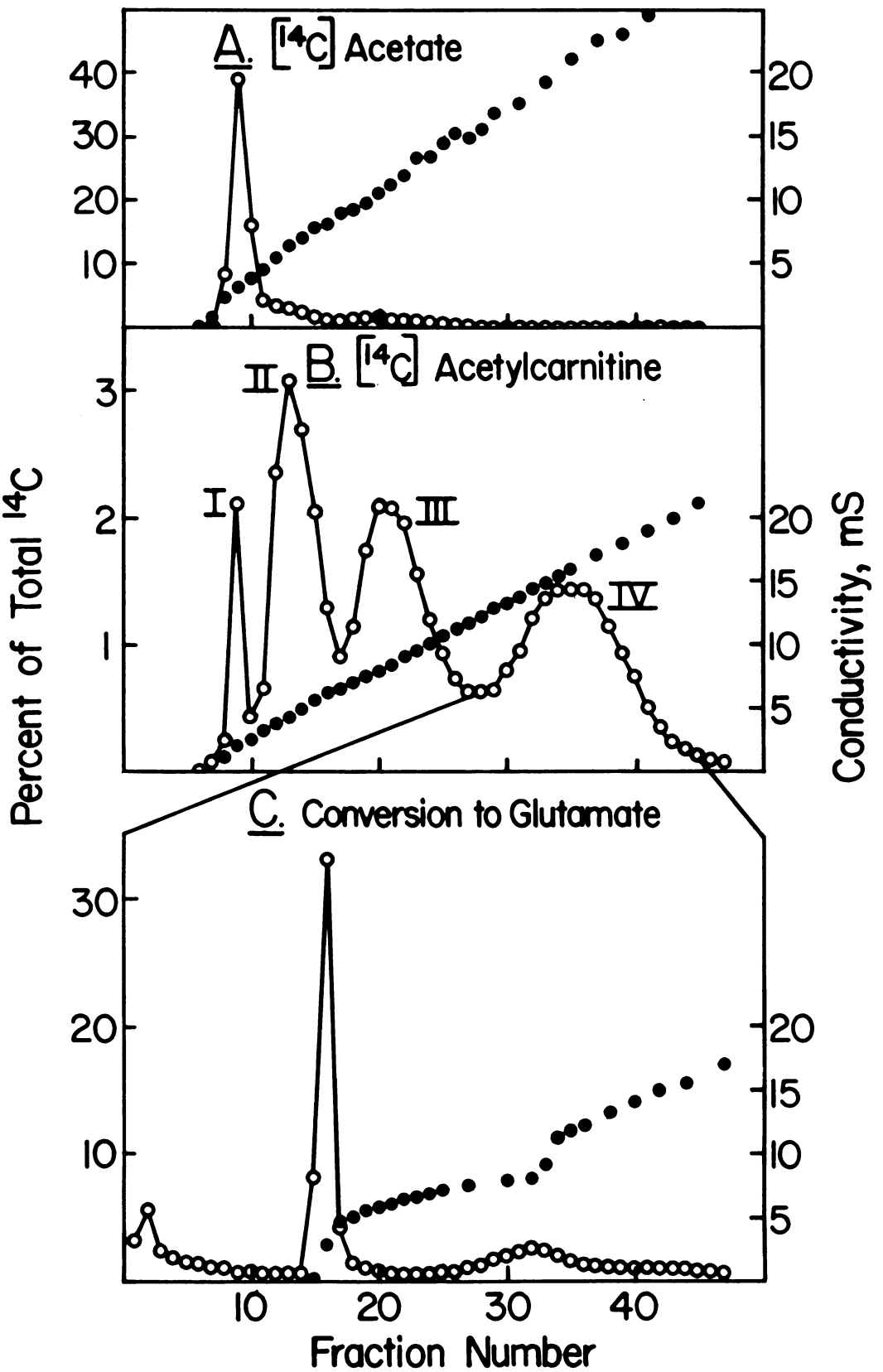

A. [14C] Acetate
B. [14C] Acetylcarnitine
C. Conversion to Glutamate
I
II
III
IV
Percent of Total 14C
Conductivity, mS
Fraction Number

Figure 27

independently using pure radioactive samples of each (Appendix I and data not shown) and both compounds appeared at the front of the HCl gradient used for elution. The conductivity at which peak III in Figure 11B eluted was typical of citrate (See Appendix I). Furthermore, peak III co-eluted with added citrate measured chemically. Based on its order of elution, peak IV appeared to be α-ketoglutarate (232) and, when assayed for this compound, peak IV (fractions 28-45 combined) contained 200 nmol of α-ketoglutarate. When the α-ketoglutarate in peak IV was converted to glutamate using glutamate dehydrogenase (231) and then re-chromatographed, the radioactivity in peak IV shifted its elution profile to the front of the HCl gradient where glutamate elutes.

Peaks I and II were not identified but acetate and glutamate co-elute with peak I. For both the [^{14}C]acetylcarnitine and [^{14}C]acetate incubations, 97% of the ^{14}C was recovered. The balance of the radioactivity not accounted for in Figure 27 was recovered in the washings obtained during application of the samples to the columns.

DISCUSSION

These studies demonstrate that carnitine nearly doubles the growth rate of glucose grown T. bovina under all the culture conditions tested, and that this growth rate is at least equal to the fastest doubling times recorded in the literature for Saccharomyces species. At 37°C the growth rate results in a generation time of less than an hour, an extremely fast generation time for any yeast. The mutant phenotype responds to carnitine similarly at 37°C and 30°C. However, the growth rate was strongly temperature dependent and deviations from the mean growth rates measured at 37°C and 30°C (see Table 3) could easily be due to 1 or 2 degrees variation in the temperature during incubation. The mutant phenotype is also stable over at least 30 generation times as deduced from data obtained for the experiment shown in Figure 6. This agrees with the similar finding by Miranda et al. (2) that the phenotype persisted over 4 passages through media lacking carnitine. Furthermore, the very low carnitine content of T. bovina (see Table 7) indicates that carnitine synthesis is not stimulated after growing cells without added carnitine.

This study clearly demonstrates that carnitine stimulates the growth rate of T. bovina equally aerobically or anaerobically. In addition, growing cells under a gas mixture of 5% CO_2 in nitrogen significantly reduced the lag time of batch cultures compared to air or nitrogen.

These findings suggest that trapping of CO_2 produced by yeast fermentation could account for the more rapid appearance of growth of T. bovina in the anaerobic portions of the agar plugs used in the early experiments on anaerobiosis. Similar reasoning, i.e., a lack of gaseous CO_2, might explain the long incubation time required for cultures grown in an atmosphere of pure nitrogen (see Figure 4).

Because the carnitine effect is measured as a stimulation of growth rate, it is difficult to ascertain just how quickly the cells respond to media carnitine. Several experiments indicated that carnitine stimulated growth within 3 hours of its addition. And, in one experiment (see Figure 6), the data indicated the cells responded to carnitine within 18 minutes of its addition. Assuming only L-carnitine is taken up by the cells, the data in Figure 9 show that nearly 40% or 0.2 μmol/g dry weight of L-carnitine was cell associated after 30 minutes of incubation, a 100-fold increase over the endogenous carnitine level. Thus it seems the response to carnitine depends primarily on how fast it is transported into the cell and does not appear to require a period of enzyme induction.

Early in the investigation, it was thought that because CAT is primarily localized in mitochondria in mammalian species it might also be associated with the mitochondria in T. bovina (which this study proves is so) and that the mitochondrial genome might direct the synthesis of this enzyme in T. bovina. If so, then chloramphenicol could be used to control the level of CAT in the yeast. However, actual experiments showed that mitochondrial peptide synthesis is not required for carnitine to stimulate growth and, because CAT is still very active in

chloramphenicol treated yeast, the mitochondrial genome is evidently not involved.

T. bovina mitochondria are not normal because the yeast is unable to grow on non-fermentable substrates, the classic description of a "petite" mutation. However, T. bovina contains cytochromes aa_3 and b and the "petite" characteristics of this yeast must be due to factors other than an inability to synthesize these respiratory chain components. Changes in mitochondrial DNA can alter the expression of nuclear genes such as those involved in the uptake and use of sugars (219).

The origin of T. bovina ATCC 26014 was described earlier and in experiments not shown, it was discovered that the wild-type T. bovina ATCC 22987 did not contain CAT. Thus the mutation that resulted in the isolation of T. bovina ATCC 26014 also caused CAT to be expressed and explains how the mutant (T. bovina ATCC 26014) became responsive to carnitine. The extremely high levels of CAT in T. bovina must compensate for the barely detectable level of carnitine in this yeast. The cells respond to 5 μM carnitine in the media by selectively lowering CAT levels, the specific activity of this enzyme falling 2- to 3-fold in crude extracts. This response is in direct contrast to the parellel increase in carnitine and carnitine acyltransferase activities that occur in livers of clofibrate treated or diabetic rats (43,73,220) and in contrast to the direct correlation of CAT with carnitine levels in mammalian tissues.

T. bovina cells contain remarkably high levels of CAT even under anaerobic culture conditions. Since carnitine also stimulates cell growth under anaerobic conditions, CAT does not appear to be necessary to aerobic metabolism although it probably functions under aerobic

conditions like it does under anaerobic conditions. CAT levels in *T. bovina* are high compared to mammalian tissues (11-13) but similar to the amount of CAT found in Baker's yeast (90) and *Candida tropicalis* (91). Furthermore, the kinetic properties of *T. bovina* CAT closely resemble those reported for the enzyme from pig heart (35), pigeon breast muscle (48), and Baker's yeast (90). Media carnitine did not affect the apparent K_m for acetylCoA of *T. bovina* CAT although it may have slightly lowered the K_m for carnitine.

CAT is the only carnitine acyltransferase activity present in crude extracts of *T. bovina*. As shown in Table 11, *T. bovina* CAT is specific for very short-chain acylCoA's whereas the commercial enzyme from pigeon breast muscle is considerably active with butyrylCoA and valerylCoA (67). In addition, yeast CAT is not solubilized by 50 mM or higher concentrations of phosphate as is mammalian CAT. The kinetic measurements and substrate specificity determinations of *T. bovina* CAT were made using the crude, particulate enzyme. Even without solubilization, crude *T. bovina* CAT was partially purified to 20,000 nmol/min/mg protein (20-fold purification) in one chromatographic step suggesting that pure yeast CAT might have a higher specific V_{max} than the mammalian enzyme if both enzymes are about the same molecular weight.

T. bovina CAT is stimulated by increasing ionic strength as is dog heart carnitine palmityltransferase I (212) although similar behavior has not been reported for mammalian CAT.

The carnitine content of the yeast grown in the absence of carnitine is much less than the carnitine content of mammalian tissues, even those in which it is least present (206). The intracellular carnitine

concentration seems too low to be active physiologically but the data in Table 7 showing 26% esterified carnitine suggest that this may not be so. It may ultimately provide the basis to explain how the yeast grows in the absence of carnitine. This study does show that traces of carnitine contaminating the synthetic medium might account for the carnitine content of this yeast emphasizing the exceptional capacity of this yeast to concentrate carnitine from dilute solutions. For example, intracellular concentrations of 0.1 to 2 μmol L-carnitine/g wet weight of cells are attained by cells suspended in a solution containing 5 μM carnitine (see Figure 8).

The percentage esterified carnitine in cells grown without carnitine (26%) is surprisingly close to the 34% esterified carnitine (Table 12) in cells grown with carnitine. Although CAT is able to form a variety of short-chain acylcarnitines *in vitro*, acetylcarnitine is the only acylcarnitine formed *in vivo*. Acetylcarnitine is also taken up by the cells, stimulating growth to the same extent as free carnitine. With these findings, this study came to focus on the effect of carnitine on acetyl group metabolism in this yeast. In no instance has the complete fate of the acetyl group of acetylcarnitine been followed directly in either *in vitro* or *in vivo* incubations. Thus the experimental approach described in this report, i.e., incubating cells with acetylcarnitine radiolabeled in the acetyl group, is a significant departure from the approaches taken by earlier investigations studying the metabolic role of acetylcarnitine.

This study shows that when cells are incubated with [^{14}C]acetylcarnitine nearly all of the cellular radioactivity is recovered in the lipids and protein of the yeast. This is the first

unequivocal demonstration that the acetyl group of acetylcarnitine is available for biosyntheses, contributing to the carbon skeleton of cell components such as amino acids and lipids. This is its function in this yeast and it may also be part of its function in other organisms but experiments have not been designed to show this. It is especially noteworthy that acetylcarnitine contributes to the synthesis of amino acids, a function totally unimagined prior to this investigation.

In yeast, Kohlhaw and Tan-Wilson (90) assume that ethanol produced by fermentation of glucose is oxidized to acetate and activated to acetylCoA thereby satisfying the cells' needs for "activated acetate". However, this scheme requires acetylCoA synthetase to be in every compartment of the cell in which acetylCoA is needed whereas, the conversion of acetylCoA to acetylcarnitine in this compartment would make it available to any other compartment in the cell. This might be especially important in fatty acid synthesis because most yeast including T. bovina (data not shown) lack ATP:citrate lyase and citrate lyase. In fact, this study shows that acetylcarnitine contributes more carbon to the synthesis of fatty acids than does acetate alone. On the other hand, a much lower percentage of acetylcarnitine entered sterols. The decreased amount of [^{14}C]acetylcarnitine in the sterols indicates that the acetylCoA pool from which they are synthesized is relatively heavily diluted by endogenous sources of acetate. Ergosterol, the major sterol in yeast (221), comprises about 0.1% of the dry weight while fatty acids comprise 1% or more of the dry weight. One would have expected, therefore, the fatty acids fraction to contain 10 times more radioactivity derived from [^{14}C]acetate than the sterol fraction although just the opposite was found. Thus free acetate appears

to be preferentially assimilated into sterols. This preference can be altered by carnitine. When carnitine is added to cells along with [^{14}C]acetate, the amount of ^{14}C incorporated into sterols decreases 3-fold while the ^{14}C in fatty acids increases 3-fold. Presumably this change helps increase the cells' growth rate. Isopycnic density gradient analysis showed that acetylCoA synthetase is only present in th cytosol of this yeast and the above results demonstrate that carnitine dramatically changes the relative proportion of free acetate activated by the synthetase that is incorporated into fatty acids and sterols even within this supposedly single compartment.

Most of the acetyl group of acetylcarnitine ended up in the cell protein in the amino acids glutamate, proline, arginine, leucine, and lysine. In contrast, [^{14}C]acetate only labeled lysine and leucine even though the HPLC chromatograms showed that glutamate and arginine had been synthesized. Thus carnitine promoted the entry of the acetyl group of acetylcarnitine into an acetylCoA pool inaccessible to [^{14}C]acetate. [^{14}C]Acetate enters this pool if carnitine is added simultaneously during the incubation period.

Glutamate, proline, and arginine form a family of amino acids in *E. coli* labeled by radioactive acetate (222) because of their synthesis from a common precursor, 2-oxoglutarate, derived from the TCA cycle and thus, ultimately from acetylCoA and oxaloacetic acid. The TCA cycle reactions are confined in the matrix of mitochondria in eucaryotes and thus acetylcarnitine enters the mitochondrial pool of acetylCoA whereas acetate does not. Based on this result, the scheme in Figure 28 was developed. Since cell membranes are permeable to acetic acid, the lack of acetylCoA synthetase in mitochondria explains the inability of acetate

Figure 28. Schematic representation of the metabolic role of carnitine in T. bovina. Cn, carnitine; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; CoASH, reduced Coenzyme A; AcCn, acetylcarnitine; AcCoA, acetylCoenzyme A; PEP, phosphoenolpyruvate; PYR, pyruvate; CAT, carnitine acetyltransferase; α-KGA, α-ketoglutarate; ATP, adenosine triphosphate; OAA, oxaloacetic acid; E.R., endoplasmic reticulum; CYTO, cytoplasm; MITO, mitochondria; amino acids are standard abbreviations.

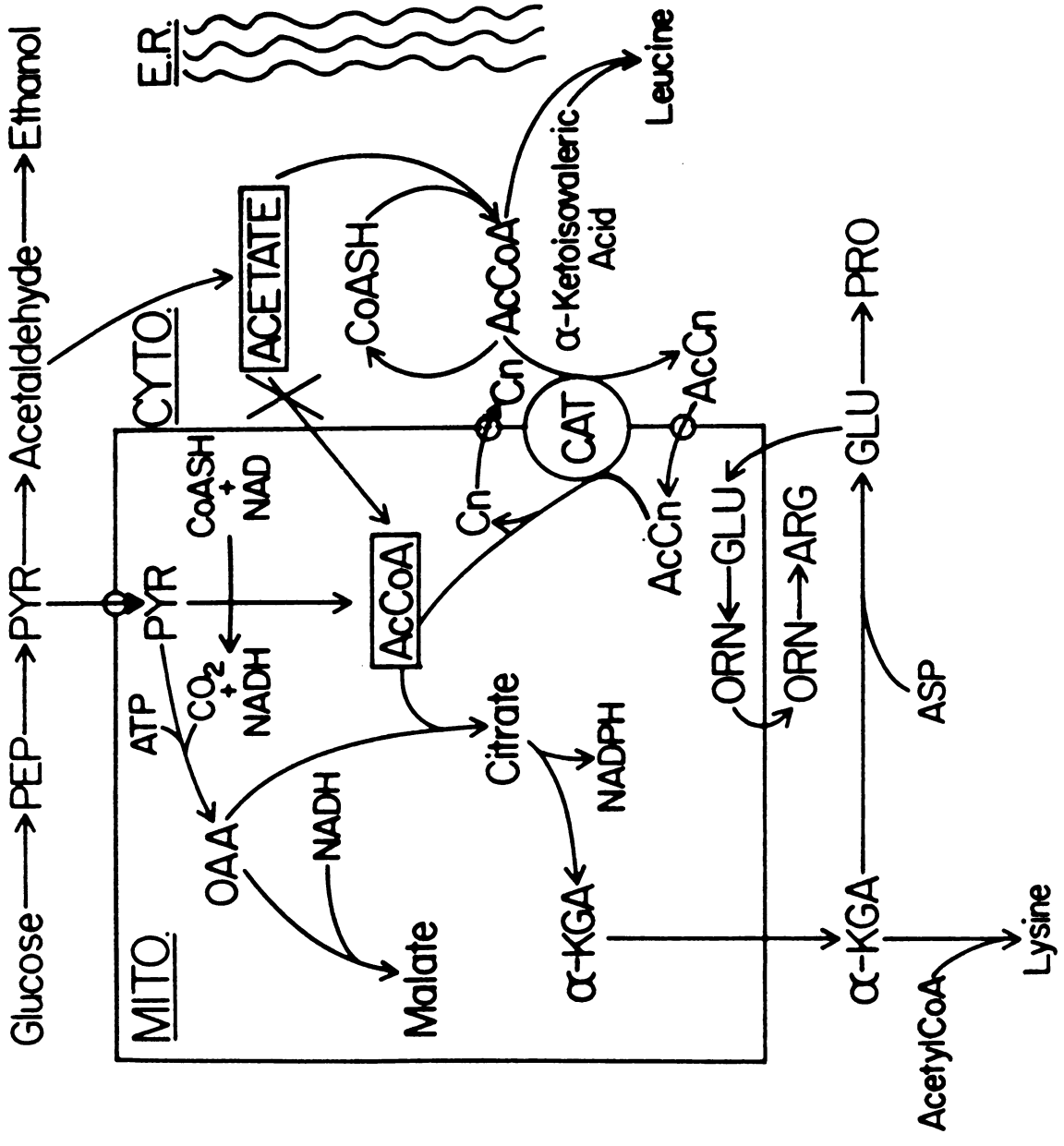

Glucose
PEP
PYR
Acetaldehyde
Ethanol
E.R.
CYTO.
ACETATE
CoASH
AcCoA
α-Ketoisovaleric Acid
Leucine
Cn
CAT
AcCn
CoASH
NAD
PYR
AcCoA
Cn
AcCn
ORN
GLU
GLU
PRO
ORN
ARG
ATP
CO_2
NADH
Citrate
NADPH
ASP
MITO.
OAA
NADH
Malate
α-KGA
α-KGA
Lysine
AcetylCoA

Figure 28

to enter the mitochondrial pool of acetylCoA. Acetate is activated to acetylCoA in the cytosol and transported into the mitochondrial matrix via acetylcarnitine. The acetyl group is then condensed with oxalacetate to form citrate. This citrate is converted to α-ketoglutarate as demonstrated in the studies with isolated mitochondria (see Figure 27). The α-ketoglutarate then passes out of the mitochondrion and is converted to glutamate. Asparagine was used as the nitrogen source in this study and glutamate oxaloacetate transaminase is a cytosolic enzyme in S. cerevisae (226). Glutamate dehydrogenase was not detected in mitochondrial preparations of T. bovina. Fumarase and α-ketoglutarate dehydrogenase were absent from T. bovina. The conversion of glutamate to arginine and proline accounts for the ^{14}C found in these three amino acids. Carnitine must accelerate the rate of biosynthesis of glutamate, arginine, and proline which explains why glutamate and arginine stimulate the growth rate of T. bovina without increasing the carnitine content of the yeast.

Pyruvate dehydrogenase complex was recently purified from top-fermenting Baker's yeast (227). This enzyme is probably present in T. bovina because yeast grown without carnitine form glutamate and arginine. The pyruvate dehydrogenase complex in yeast is not regulated by phosphorylation-dephosphorylation. The yeast complex is inhibited by NADH. The absence of NADH dehydrogenase in T. bovina probably causes NADH to inhibit pyruvate dehydrogenase and thus restrict the rate of acetylCoA production from pyruvate. This rate limiting reaction is circumvented by shuttling acetylcarnitine into the mitochondrion.

Oxaloacetate is presumably generated in the mitochondria from pyruvate by the action of pyruvate carboxylase. This enzyme is present

in yeast (228) and is included in Figure 28. The reaction catalyzed by malate dehydrogenase was detected in T. bovina. This reaction is probably driven to form malate and thus lower the NADH level in cells lacking NADH dehydrogenase.

In Figure 28, cytosolic acetylCoA is shown being incorporated into lysine and leucine. The pathway for leucine and valine biosynthesis is shown in Figure 29. Although both compounds have α-ketoisovaleric acid as an intermediate only leucine was labeled in this study. Thus ^{14}C must be incorporated into leucine during the condensation of acetylCoA with α-ketoisovaleric acid catalyzed by α-isopropylmalate synthase (step 5 in Figure 29). Since leucine is labeled by [^{14}C]acetate, α-isopropylmalate synthase has access to the cytosolic pool of acetylCoA. This enzyme is reported to be in the mitochondria of Baker's yeast (229) but in T. bovina it must be outside the inner membrane. The first intermediate in the pathway of lysine biosynthesis is homocitrate formed in an analogous manner to leucine by the condensation of acetylCoA with α-ketoglutarate catalyzed by homocitrate synthase. This enzyme is localized in mitochondria in Baker's yeast but since [^{14}C]acetate labels lysine, this condensation reaction must occur outside the mitochondrial inner membrane in T. bovina. Consequently, it appears that the acetyl group of acetylcarnitine is incorporated into leucine and lysine outside the mitochondrial matrix and into glutamate, proline, and arginine inside the mitochondrial matrix.

The proposition that acetylcarnitine shuttles "activated acetate" into or out of the mitochondrial matrix requires that CAT be present on both the cytosolic and matrix sides of the inner membrane of mitochondria. At least 80% of T. bovina CAT is associated with mitochondria and,

Figure 29. Pathway of biosynthesis of leucine and valine. "Active Acetaldehyde" refers to the 2-carbon derivative of thiamine pyrophosphate believed to be a reactant in the biosynthesis of the acetohydroxy acids (230).

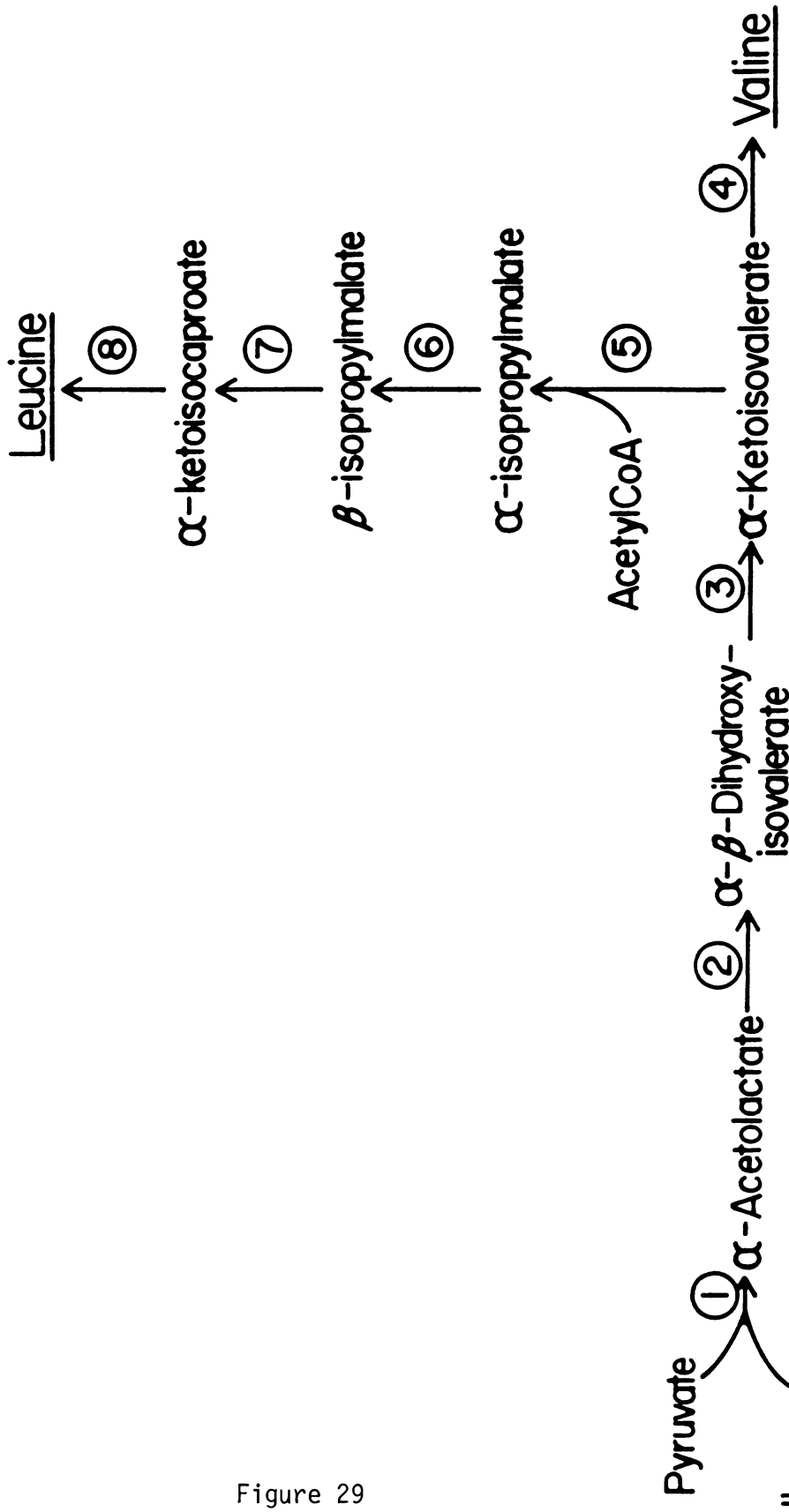

Pyruvate
"Active Acetaldehyde"
① α-Acetolactate
② α-β-Dihydroxy-isovalerate
③ α-Ketoisovalerate
④ Valine
AcetylCoA
⑤ α-isopropylmalate
⑥ β-isopropylmalate
⑦ α-ketoisocaproate
⑧ Leucine

Figure 29

except for a small percentage in the cytosol, CAT has the same density distribution in a sorbitol gradient as citrate synthase.

The data do not localize CAT to the inner and/or outer faces of the mitochondrial inner membrane. Instead, T. bovina CAT remains tightly bound to the mitochondrial membrane with less than 5% soluble activity in crude extracts. Mitochondrial outer membranes have an isopycnic density of 1.09 g/cm^3 while ruptured mitochondria band at a density of 1.14 g/cm^3 (223). Therefore, it seems that the two particulate CAT peaks in mechanically disrupted cells (see Figure 24) are derived from CAT bound to outer mitochondrial membranes at 1.09 g/cm^3 and CAT bound to the membranes of ruptured mitochondria in the 1.13 g/cm^3 region. In Figure 28, CAT is shown as an integral membrane protein with separate transporter proteins for carnitine and acetylcarnitine although a single translocase could perform both functions. These details require further experimentation.

The discovery that carnitine is involved in the synthesis of amino acids is both new and novel and was never imagined prior to its discovery. Carnitine also affects the proportion of exogenous acetate that becomes incorporated into sterols or fatty acids. Carnitine stimulates the synthesis of glutamate and arginine rather than vice versa, thus explaining how glutamate and arginine stimulate growth without increasing the carnitine content of the yeast. It seems likely that T. bovina is unable to synthesize carnitine but the yeast compensates by efficiently extracting carnitine from its environment. The yeast's carnitine transport system in conjunction with its exceptionally high levels of CAT help assure its survival.

T. bovina demonstrates that CAT and acetylcarnitine are not exclusively involved in fatty acid catabolism. In addition, the yeast system is now defined enough that its manipulation may provide new insight into how carnitine affects metabolism especially the compartmentation of acetyl group metabolism. The yeast might also provide a good model for carnitine transport studies and for determining the genetic factors governing the expression of CAT.

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LIST OF REFERENCES

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APPENDIX I

ANALYTICAL BIOCHEMISTRY 119, 261-265 (1982)

Preparation of Radioactive Acetyl-L-Carnitine by an Enzymatic Exchange Reaction

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A rapid method for the preparation of [1-^{14}C]acetyl-L-carnitine is described. The method involves exchange of [1-^{14}C]acetic acid into a pool of unlabeled acetyl-L-carnitine using the enzymes acetyl-CoA synthetase and carnitine acetyltransferase. After isotopic equilibrium is attained, radioactive acetylcarnitine is separated from the other reaction components by chromatography on Dowex 1 (Cl^-) anion exchange resin. One of the procedures used to verify the product [1-^{14}C]acetyl-L-carnitine can be used to synthesize (3*S*)-[5-^{14}C]citric acid.

Acetylcarnitine is found in many mammalian tissues (1), but its metabolic fate has not been clearly elucidated. Isolated mitochondria can oxidize acetylcarnitine (2). It can be formed outside the mitochondrion by peroxisomal beta-oxidation (3) or in the matrix of mitochondria via pyruvate oxidation (2,4), β-oxidation or oxidation of some amino acids. Acetylcarnitine can be exported from mitochondria and possibly used in the synthesis of fatty acids or biological acetylations (5). Carnitine acetyltransferase activity is associated with mitochondria, peroxisomes, and microsomes (6–8). Determining the fate of radioactive acetylcarnitine in tissues and cell preparations should help determine its function(s). However, a source of radioactive acetylcarnitine is required for such studies.

Previous methods for the preparation of labeled and unlabeled acetylcarnitine relied upon their net synthesis from carnitine and an activated acetyl group. The product obtained by enzymatic methods (9) is contaminated with free carnitine unless the reaction is forced to completion with a large excess of reactants. This also is expensive when radioisotopes are used. The same technique is needed to ensure high yields of acetylcarnitine synthesized by chemical procedures (10,11) but even then the chemical method is difficult to use when only a few milligrams of products of high specific radioactivity are desired. These problems are avoided by an alternative approach to the synthesis of radiaoctive acetylcarnitine that is analogous to the method of enzymatic synthesis of [γ-^{32}P]ATP from $^{32}P_i$ and ATP via glyceraldehyde-3-phosphate dehydrogenase and 1,3-diphosphoglycerate kinase (12). In this approach, radiolabeled acetate is exchanged into the unlabeled acetyl group of acetylcarnitine by coupling two enzymatic reactions as indicated below (* denotes a radioactive residue).

$$*\text{acetate} + \text{CoASH} + \text{ATP} \leftrightarrow *\text{acetyl-CoA} + \text{AMP} + PP_i \quad [1]$$

$$\text{acetyl-L-carnitine} + *\text{acetyl-CoA} \leftrightarrow *\text{acetyl-L-carnitine} + \text{acetyl-CoA} \quad [2]$$

Both reactions have equilibrium constants near one. At isotopic equilibrium, nearly all of the inexpensive radioactive isotope, acetate, exchanges into the product, acetyl-L-carnitine, when a large pool of acetyl-L-carnitine is used with a small pool of high specific activity radioactive acetate. In addition, contamination of acetylcarnitine with free

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carnitine is kept to a minimum by starting with analytically pure acetyl-L-carnitine as a reactant and using catalytic amounts of CoASH to effect the exchange reaction. A method is described herein using [1-^{14}C]acetate, although [^{3}H]acetate, [2-^{14}C] acetate, or [1,2-^{14}C]acetate would serve equally well.

MATERIALS AND METHODS

Materials. Sodium [1-^{14}C]acetate (58.3 Ci/mol) was obtained from New England Nuclear Corporation (Boston, Mass). CoASH was obtained from PL Biochemicals (Milwaukee, Wis.). The following chemicals and enzymes were obtained from Sigma Chemical Company (St. Louis, Mo.): glutathione (reduced), NAD^+, ATP, Trizma–HCl, L-malate, citrate synthase (85 U/mg), malic dehydrogenase (7150 U/mg), and carnitine acetyltransferase (85 U/mg). Acetyl-L-carnitine hydrochloride was kindly provided by Otsuka Pharmaceutical Company, (Naruto, Tokushima, Japan) and was judged to be >98% pure by assay of both free and total carnitine content. Acetyl-CoA synthetase was purchased from Boehringer-Mannheim (West Germany, 0.95 U/mg). The Dowex resins were obtained from Bio-Rad Laboratories (Richmond, Calif.). Other reagents were from Mallinckrodt (St. Louis, Mo.).

Preparation of [1-^{14}C]acetyl-L-carnitine. The final reaction volume of 1.0 ml contained: acetyl-CoA synthetase, 1 mg; sodium phosphate buffer, pH 7.6, 150 μmol; disodium ATP, 17.5 μmol; reduced glutathione, 2 μmol; CoASH, free acid, 0.3 μmol; $MgCl_2$, 6.6 μmol; acetyl-L-carnitine, 3.8–42 μmol (added from a neutralized solution); [^{14}C]-sodium acetate, 0.34–0.84 μmol. The exchange reaction was initiated by adding about 2.5 units of carnitine acetyltransferase and the reaction incubated 25 h at room temperature (22°C). The reaction was terminated by placing the reaction mixture into a boiling water bath for 3 min. Denatured protein was removed by centrifugation. [^{14}C]Acetyl-L- carnitine was purified by passing the solution through a 1 × 4-cm column of Dowex 1-X8 (Cl^- form, 100–200 mesh) resin, washing the column with 5 ml water, and collecting the product [^{14}C]-acetylcarnitine in the eluate. This solution was used to demonstrate that [^{14}C]acetate had exchanged into acetylcarnitine as described below. This solution can be stored frozen (−80°C) after adjusting the pH to 6.

The progress of the reaction was followed by removing 2-μl samples of the reaction mixture and applying them to 5 × 25-mm columns of the Dowex. The columns were washed twice with 0.5 ml of water, the total eluate collected in scintillation vials, and the radioactivity counted.

Verification that [^{14}C]acetate is in acetylcarnitine. Cation-exchange column chromatography was performed as described (10). A 0.2-ml sample of the [^{14}C]acetylcarnitine solution (2.886×10^6 dpm) was diluted to 2 ml with absolute ethanol, acidified with concentrated HCl to 0.05 N HCl, and applied to a 5 × 25-mm column of Dowex 50W-X8 (H^+ form, 100–200 mesh) resin. The sample was washed onto the column with 2 ml of 0.01 N HCl in 20% ethanol and eluted with 8 ml of 1 N NH_4OH in 20% ethanol. The eluate was collected in 1 ml fractions in scintillation vials, combined with scintillation fluid, and counted.

The radiochemical purity of the [^{14}C]-acetylcarnitine was checked by thin-layer chromatography on silica gel G plates (solvent: $CHCl_3$, CH_3OH, 17% NH_3; 2:2:1) developed to 16 or 17 cm. Radioactivity on the plate was detected with a Berthold thin-layer chromatography scanner.

Further verification that the [^{14}C]acetate had exchanged into the acetyl residue of acetylcarnitine was obtained using an enzyme-coupled spectrophotometric acetylcarnitine assay (13). The formation of citrate from acetylcarnitine in this assay is coupled to the reduction of NAD^+, the amount of NADH produced being proportional to the

amount of acetylcarnitine. To show that the ^{14}C was actually associated with acetylcarnitine, citrate was isolated and its ^{14}C-content measured. The assay tube contained 0.5 ml of 200 mM TRIS-HCl, pH 7.8, 60 mM L-malate, 4 mM Na_2EDTA; :0.2 ml of 2.5 mM NAD^+; 0.2 ml of 1.5 mM CoASH in a 10 mM solution of reduced glutathione; 1.0 ml water; 10 μl citrate synthase; 5 μl carnitine acetyltransferase; 1 μl malate dehydrogenase; and 75 μl of the [^{14}C]acetylcarnitine solution (1.0637×10^6 dpm). A control tube contained 0.125 μmol [^{14}C]-acetate in 25 μl. The reactions were terminated by placing the tubes in a boiling water bath for 5 min, after which they were cooled and centrifuged to remove denatured protein. The deproteinized solutions were combined with 1 ml of 8 mM citrate, pH 6.9, and a sample from each reaction mixture was chromatographed on a 1 × 5-cm column of Dowex 1-X8 (Cl^-). Acetate and citrate were retained by the resin and were eluted with an 80 ml, 0.01–0.075 N HCl linear gradient. Citrate was detected with acetic anhydride and pyridine (14) and the ^{14}C quantitated by scintillation counting of a small sample of each 1.9-ml fraction collected.

Radioactivity was measured in 10 ml of a Triton X-100 based cocktail (15) and the dpm calculated by the channels ratio method of correction for quenching.

RESULTS AND DISCUSSION

A time-course study for the exchange of [^{14}C]acetate into acetylcarnitine showed that within 25 h greater than 99% of the [^{14}C]acetate exchanged into the large pool of acetyl-L-carnitine (Table 1). In the experiment shown, the [^{14}C]acetylcarnitine had a specific activity of 0.476 mCi/mmol. Both the yield and specific activity of [^{14}C]acetylcarnitine depend on the relative amounts of [^{14}C]acetate and acetylcarnitine in the reaction mixture. In other experiments, [^{14}C]acetylcarnitine has been prepared with a specific activity as high as 6.24 mCi/mmol.

TABLE 1

EXCHANGE OF [1-^{14}C]ACETATE INTO ACETYL-L-CARNITINE

Incubation time (h)	[1-^{14}C]Acetyl-carnitine formed (μCi)	Percent exchange
1.5	6.2	31
3.5	10.8	55
25	20.3	102

Note. The reaction contained 343 nmol [1-^{14}C]acetate (58.3 μCi/μmol) and 42 μmol acetyl-L-carnitine. Samples were taken at the times indicated and assayed for [^{14}]acetylcarnitine as described under Material and Methods.

In the radiochemical assay for carnitine (16), acetylcarnitine is separated from acetyl-CoA by passing the reaction mixture over Dowex 1 anion exchange resin. Acetyl-CoA is retained by the resin and acetylcarnitine passes through. Phosphate was chosen to buffer the [^{14}C]acetylcarnitine synthetic reaction mixture and glutathione was used because Dowex 1 binds both these compounds and also binds the ATP, AMP, PP_i, and CoASH in the reaction mixture. This allows the [^{14}C]acetylcarnitine to be separated by passing the deproteinized reaction solution through a column of Dowex 1 (Cl^-). Controls have shown that Dowex 1 (Cl^-) retained greater than 99.8% of the ^{14}C in a solution of 0.0343 μmol [^{14}C]acetate in 0.2 ml water when the column was washed with either 1 ml of water or 1 ml of 0.1 M phosphate buffer, pH 8.0. Greater than 99.5% of the ^{14}C was also retained by the resin when the reaction mixture lacking carnitine acetyltransferase was applied to and washed through the column. Thus the amount of ^{14}C in the Dowex 1 eluate represents the amount of [^{14}C]acetylcarnitine formed. The [^{14}C]acetylcarnitine prepared as described above contains NaCl and $MgCl_2$, which are not removed by the resin. [^{14}C]Acetylcarnitine could be separated from these salts by passing the solution through a column of Dowex 50 (NH_4^+) prior to column

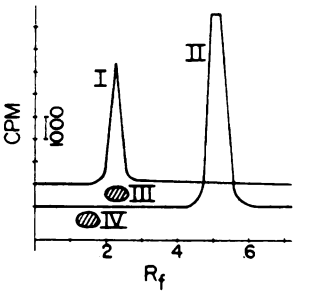

FIG. 1. Thin-layer chromatography of [^{14}C]acetylcarnitine prepared by the exchange reaction described under Materials and Methods. A 2-μl aliquot of each of the following solutions were applied to a silica gel G plate: I, [^{14}C]acetylcarnitine, 6.5 μCi/ml; II, [^{14}C]acetate, 10 μCi/ml; III, acetyl-L-carnitine, 0.39 mmol/ml; and IV, D,L-carnitine-HCl, 1 mmol/ml. The plate was developed to 16 cm in $CHCl_3$, CH_3OH, 17% NH_3; 2:2:1, dried in air, and exposed to I_2 to visualize III and IV. The ^{14}C-labeled compounds (I and II) were measured with a thin-layer chromatography scanner.

chromatography on Dowex 1 (HCO_3^-) and evaporating the eluate containing acetyl-L-carnitine under reduced pressure at 50°C to eliminate $(NH_4)HCO_3$. This procedure avoids extremes of pH that might otherwise hydrolyze the ester linkage of acetylcarnitine.

At pH values less than 4, acetylcarnitine has a net positive charge causing it to bind to a Dowex 50 cation-exchange resin. When a sample of the [^{14}C]acetylcarnitine solution was acidified, 98% of the ^{14}C bound to Dowex 50 and subsequently eluted with ammonium hydroxide (data not shown). This behavior is typical of short-chain acylcarnitines (10) and demonstrates that [^{14}C]acetate had exchanged into the acetyl residue of acetylcarnitine. Thin-layer chromatography of a sample of the [^{14}C]-acetylcarnitine solution showed that the ^{14}C comigrated with acetylcarnitine as a single peak well separated from reactant [^{14}C]-acetate (Fig. 1).

The amount of acetylcarnitine in the [^{14}C]acetylcarnitine solution was also measured by an enzyme-coupled assay that forms citrate from acetylcarnitine (13). One ^{14}C-labeled compound was formed from [^{14}C]acetylcarnitine in this assay which eluted with carrier citrate (Fig. 2B). [^{14}C]Acetate was unchanged in the control assay and its elution pattern was different from citrate (Fig. 2A). The trail of ^{14}C in Fig. 2B after the apparently complete elution of citrate was due to the high specific activity of citrate in this sample. The data in Fig. 2A indicate that the trail of citrate could be chemically detected when more carrier citrate is applied to the column. In the experiment, the amount of citrate produced was 0.364 μmol (determined by column chromatograph); the amount of acetylcarnitine was 0.366 μmol (measured by the amount of NAD^+ produced in the assay). Both these independently measured values agree to within experimental error with the calculated amount of 0.361 μmol of [^{14}C]acetylcarnitine present in the assay. These results also demonstrate that the assay could be used to prepare (3*S*)-[5-^{14}C]citrate in nearly quantitative yield from [^{14}C]acetylcarnitine.

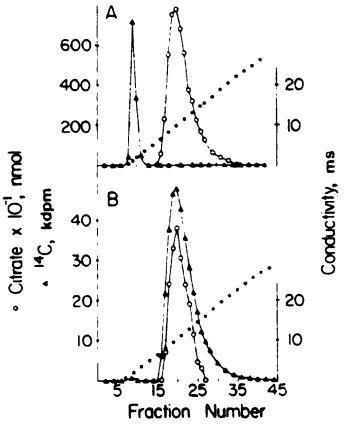

FIG. 2. Dowex 1 elution profile of [^{14}C]citrate formed from [^{14}C]acetylcarnitine. [^{14}C]Acetylcarnitine was converted to [^{14}C]citrate as described under Materials and Methods. Samples of the boiled reaction mixtures were applied to Dowex 1 (Cl^-) columns and washed onto the column with 20 ml water (not shown). No more than 0.5% of the ^{14}C-label appeared in the wash. The samples were eluted with a linear HCl gradient. Sixty-drop (1.9 ml) fractions were collected and conductivity was measured on a type CDM3 radiometer, Copenhagen instrument. (A) 2-ml sample of [^{14}C]acetate control reaction mixture; (B) 1-ml sample (354,556 dpm) of the [^{14}C]acetylcarnitine assay solution. ●, conductivity; ▲, ^{14}C-label; ○, citrate.

The procedure for the preparation of radioactive acetylcarnitine is rapid, quantitative, and simple. The data show that [^{14}C]acetate is converted to [^{14}C]-acetylcarnitine and that the [^{14}C]acetylcarnitine can be purified to greater than 98% radiochemical purity in a single step. Contamination of the radiolabeled acetylcarnitine by free carnitine was minimized by using a small, catalytic amount of CoASH in the reaction mixture.

Another advantage of this enzymatic procedure is that if D,L-acetylcarnitine is substituted for the pure L-isomer, only the biologically active L-isomer will be labeled. This is desirable because D,L-acetylcarnitine is cheap and readily available and it has been our experience that at equimolar concentrations the D-isomer does not effectively compete with the L-isomer.

The method could be adapted as a general method for the synthesis of short-chain acyl-L-carnitines since other aliphatic short-chain radioactive acyl groups could be substituted for acetate by using the appropriate thiokinase and acylcarnitine derivative. Pigeon breast carnitine acetyltransferase is active with acyl residues up to 6 carbons in length (17).

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