Levels of coenzyme A – glutathione mixed disulfide in *Escherichia coli*[1]

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The pool of coenzyme A – glutathione mixed disulfide (CoASSG) rapidly increased 2.0 times in response to oxygen starvation and 1.5 times in response to glucose starvation but did not change following ammonia starvation. The increase in the CoASSG pool resulted from an increase in the CoASSG fraction of the CoA pool from 42 to 66–93%. Fluoride, cyanide, chloramphenicol, and rifampicin all caused similar increases. Aerobic growth on fermentable sugars resulted in CoASSG making up 40–55% of the CoA pool while growth on nonfermentable carbon sources or anaerobic fermentation resulted in CoASSG replacing acetyl CoA and free CoA to make up 85–95% of the CoA pool. The CoASSG:ATP ratio varied inversely with the growth rate in two groupings of carbon sources made up of either fermentable or nonfermentable molecules. Cultures grown aerobically on fermentable sugars exhibited a lower CoASSG:ATP ratio reflecting the lower proportion of CoASSG in the CoA pool.

Introduction

CoASSG (or DS I) and a modified CoA dimer (DS II) have recently been isolated from formic acid extracts of *Escherichia coli* and identified (1, 2). CoASSG has also been shown to be present in other bacteria (1), yeast (3), and liver (4, 5). The nucleotides were present throughout cell growth in *E. coli*, and upon shift to anaerobic growth or growth into stationary phase, the pool size of CoASSG increased.

The role of the disulfide forms of CoA remains obscure although it has been shown that they can inhibit *E. coli* RNA polymerase when they have been freshly isolated from cell cultures or when they are combined with iron (2, 6). A distinction between whether the increased pool of CoASSG was a signal affecting other aspects of metabolism or simply a response to the cessation of growth could not be made from these preliminary experiments. Since a number of other nucleotides including ppGpp have been implicated in the regulation of cellular metabolism (7–14), changes in the pool size of a CoA nucleotide in response to changes in metabolism were of obvious interest. In addition, the relationship of CoASSG to the remainder of the CoA pool needed clarification.

This work describes the rate and extent of change in the CoASSG pool and the fraction that it makes of the CoA pool (free CoA, acetyl CoA, and CoASSG) in response to metabolic changes brought on by metabolite depletion and by metabolic inhibitors. These experiments show that CoASSG levels increase only when carbon metabolism is disrupted in cultures growing aerobically on glucose or other fermentable sugars.

Materials and Methods

Formic Acid Extracts

Batch cultures of *E. coli* B at 37°C in baffled flasks were grown using Tris–salts minimal medium containing 0.1 *M* Tris–Cl (pH 7.6), 0.16 m*M* sodium sulphate, 1 m*M* magnesium sulphate, 10 m*M* sodium chloride, 0.2 m*M* calcium chloride, 0.001 m*M* ferric chloride and 0.5 m*M* potassium phosphate supplemented with 10 μCi (1 Ci = 37 GBq) ^{32}Pi per millilitre (New England Nuclear), 0.2% carbon source, and 7.5 m*M* ammonium sulphate unless otherwise stated. Oxygen depletion was achieved by stopping the vigorous swirling (1). Samples of 5 ml were removed at various times and pipetted directly into 0.5 ml of 1 *M* formic acid on ice. After 30 min, the cell debris was removed by centrifugation and the supernatant was neutralized with ammonium hydroxide.

CoASSG Assay

ATP, GTP, and CoASSG were separated by chromatography on 48-cm orange ribbon paper (Schleicher and Schuell) in a solvent containing ammonium sulphate – 0.1 *M* sodium phosphate (pH 7.0) – *n*-propanol (12 g : 10 ml : 0.2 ml) (1). The radioactivity was localized by autoradiography with X-ray film (Kodak) and then quantitated by cutting the chromatogram and counting the pieces in a liquid scintillation counter (Packard). The material in the formic acid extracts which migrated with an R_f value of 0.14 was composed of CoASSG, acetyl CoA, and free CoA. The 5.5-ml crude extracts were therefore charged on a 1 × 3 cm A-25 DEAE-Sephadex column equilibrated with water. The column was washed with 50 ml 0.2 *M* TEAB (pH 8.0), eluted with 10 ml of 1.5 *M* TEAB (pH 8.0), and the eluate was concentrated to dryness and redissolved in 1 ml of water. When aliquots of these A-25 Sephadex fractions were subjected to paper chromatography as described above, only CoASSG remained in the material migrating at R_f 0.14. Glutathione eluted from the column in the 0.2 *M* TEAB washing. The recycling assay for CoA was carried out as described by Allred and Guy (15). ppGpp was quantitated by paper chromatography (1) of the crude extracts as described above and by tlc on PEI-cellulose plates (Brinkmann) in 1.5 *M* potassium phosphate (pH 3.6) as previously described (7). The unit, 1 pmol/ml · Klett unit, used in this work corresponds to 140 pmol/(5 × 10^8) cells.

To show that CoASSG was not formed during the extraction and fractionation steps, the following control experiment was carried out. ^{32}P-Labelled CoA was isolated by DTT treatment of labelled DS I as described in Fig. 2 of Ref. 2 followed by desalting and removal of the excess DTT using G-10 Sephadex (also in Ref. 2). This ^{32}P-labelled CoA was added to the formic acid extract immediately after the addition of formic acid to unlabelled cells. Following A-25 Sephadex fractionation, either with or

ABBREVIATIONS: CoA, coenzyme A; CoASSG, coenzyme A – glutathione mixed disulfide; TEAB, triethylammonium bicarbonate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PEI, polyethyleneimine; tlc, thin-layer chromatography.

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without prior neutralization, and concentration, 97% of the ^{32}P label was found in oxidized CoA while less than 3% was found in CoASSG. In a supplementary experiment, incubation of CoA and GSH (0.4 m*M* and 10 m*M* respectively) at 0°C for 3 h in either 0.1 *M* formic acid or 0.1 *M* Tris–Cl (pH 7.6) or 0.2 *M* TEAB (pH 8.0) did not result in the formation of any CoASSG. Overnight incubation with oxygen gas bubbling through the mixture was necessary for significant CoASSG production.

Results

CoASSG Assay

During work on the characterization of DS I (CoASSG), it was noted that CoASSG and other CoA nucleotides chromatographed erratically on PEI-cellulose tlc plates developed with 1.5 *M* potassium phosphate (pH 3.6). For example, both CoASSG and CoA dimer were originally found to migrate slower than GTP (1) but were occassionally found to migrate much faster even when 0.25 *M* potassium phosphate (pH 3.6) was used for development and gave the appearance of having broken down. In attempting to circumvent this problem, it was found that development of the tlc plates with 1.2 *M* LiCl resulted in uniform and reproducible migration of the CoA nucleotides with the following R_f values: CoA dimer, 0.15; DS II, 0.17; authentic CoASSG, 0.63; DS I, 0.63; CoA, 0.47; AMP, 0.61; ADP, 0.39; and ATP, 0.16. When a comparison of the total charge is made between the CoA nucleotides and the other adenosine nucleotides, the CoA nucleotides migrate faster than expected. The CoA dimer contains six phosphate groups but migrates with ATP while CoASSG, containing three phosphate groups and one effective carboxylic acid group, migrates faster than AMP and CoA. This discrepancy and the erratic behaviour in phosphate buffer must be caused by the pantetheine chain and the glutathione tripeptide which in some way modify the chromatographic properties of the CoA nucleotides. It was therefore decided that PEI-cellulose was not a reliable system with which to assay changes in CoASSG pools. Instead, paper chromatography which clearly separated what will be referred to as the CoA pool, composed of free CoA, acetyl CoA, and CoASSG (R_f, 0.14; Ref. 1), from the other nucleotides was used for most of the work described in this paper. In order to remove free CoA and acetyl CoA from the CoASSG spot, the crude extracts were fractionated using A-25 DEAE-Sephadex at pH 8.0, a procedure which hydrolyzed the acetyl CoA and oxidized free CoA to CoA dimer (R_f, 0.02). Only the original CoASSG remained to chromatograph with a R_f value of 0.14 and could be quantitated independently.

The recycling assay of Allred and Guy (15) was employed as a supplementary means of quantitating the CoA fractions in the crude extracts. By assaying for CoA activity in the presence or absence of either DTT or NEM, it was possible to differentiate between acetyl CoA, free CoA, and CoASSG. The level of CoA dimer (DS II) was indistinguishable from CoASSG but paper chromatography showed that CoA dimer was usually present in amounts less than 10% of the CoASSG pool. The result obtained with the recycling assay agreed with the results from paper chromatography. The relative proportions of CoASSG, free CoA, and acetyl CoA in the crude extract of mid-log phase cells were 1.6:1.1:1.0 (0.32:0.23:0.20 pmol/ml · Klett unit).

Incorporation of ^{32}Pi into CoASSG

The incorporation of ^{32}Pi into the pools of ATP, GTP, ppGpp, and CoASSG (CoA) of exponentially growing *E. coli* is shown in Fig. 1. ATP which is required for the biosynthesis of CoA was labelled very quickly whereas label appeared in CoASSG and the CoA pool slowly after a lag of several minutes. Label appeared in ppGpp at a similar rate in agreement with an earlier report of the incorporation of [^{3}H]guanosine in ppGpp (16). Both CoASSG and ppGpp were fully labelled only after 0.9 generations whereas both ATP and GTP were fully labelled within 0.5 generations.

Induced Changes in the CoASSG pool

In order to study the response of CoASSG to various metabolic changes, the growth of batch cultures of *E. coli* was disrupted either by metabolite depletion or by the addition of metabolic inhibitors.

Metabolite Depletion

The variations in nucleotide pools resulting from oxygen, glucose, and ammonia starvations are shown in Fig.

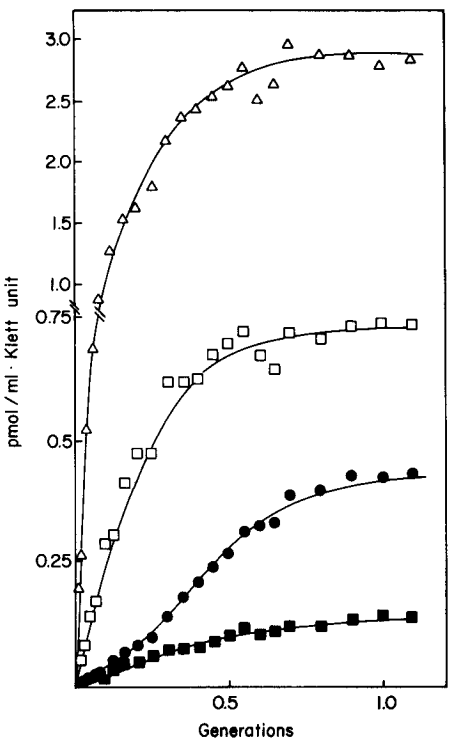

FIG. 1. Incorporation of ^{32}Pi into the pools of CoASSG, ATP, GTP, and ppGpp of mid-log phase cells growing at 37°C. The ^{32}Pi was added at time 0 and one generation was 55 min. The label appeared in the CoA pool at the same rate that it appeared in CoASSG. Symbols: ATP, △; GTP, □; ppGpp, ■; and CoASSG, ●.

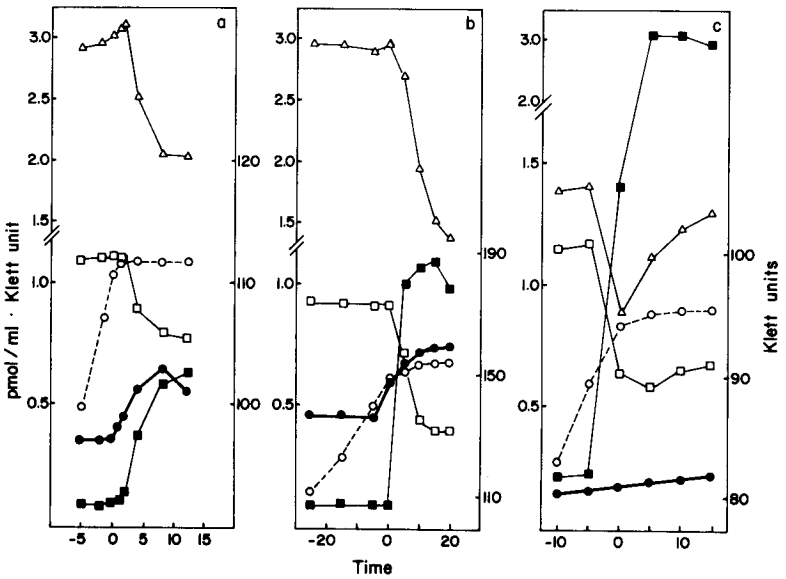

FIG. 2. Nucleotide levels before and after (*a*) oxygen depletion, (*b*) glucose depletion, and (*c*) ammonia depletion. The cultures contained (*a*) 0.2% glucose and 7.5 m*M* ammonium sulphate, (*b*) 0.09% glucose and 7.5 m*M* ammonium sulfate, and (*c*) 0.2% glucose and 0.5 m*M* ammonium sulfate in the Tris–salts medium. Aliquots were removed and assayed as described in Materials and Methods. Symbols: ATP, △; GTP, □; ppGpp, ■; CoASSG, ●; and Klett units, ○.

2. In agreement with previous reports (11, 17), the cessation of growth and RNA accumulation occurred simultaneously and the Klett colorimeter measure of cell density provided in all three cases is representative of both. The 30–50% drop in both the ATP and the GTP pools coupled with the 6- to 15-fold increase in the ppGpp pool is also consistent with previously reported data (11, 17) and the levels of these nucleotides are supplied here for reference. Depletion of either oxygen or glucose resulted in an increase in the CoASSG pool of 2.0 or 1.5 times respectively and in both cases the increase started prior to any change having occurred in the other nucleotide pools. The response of ATP, GTP, and ppGpp seemed to require a nearly complete cessation of growth whereas the CoASSG pool responded to the slow down in growth just prior to the complete cessation of growth brought on by either anaerobiosis or glucose starvation.

Perhaps more significant than the absolute increase in the CoASSG pool was the proportional increase of CoASSG in the CoA pool composed of acetyl CoA, free CoA, and CoASSG (Table 1). During anaerobiosis, the CoA pool increased by 25% and the CoASSG fraction of the CoA pool rose to 66% of the total from the original 42%. Anaerobiosis would result in a faster excretion of acetate via the phosphoroclastic system (18) which may account for the reduced levels of acetyl CoA. In addition, since less CoA would be required for slower anaerobic growth, the unused CoA may then be channelled into CoASSG possibly for storage.

After glucose starvation, the total CoA pool decreased by 28%, possibly the result of CoA being degraded as a substitute carbon source. Furthermore, the CoASSG component increased to 92% of the CoA pool, another indication that when the need for CoA in carbon metabolism decreases, much of the CoA is converted into CoASSG.

TABLE 1. Percentage changes in the CoA pool and the percentage fraction of CoASSG in the CoA pool following metabolic disruption

Type of disruption	Percentage change in CoA pool	CoASSG fraction as percentage of CoA pool
None	0	44
Glucose depletion	−28	92
Oxygen depletion	25	66
Ammonia depletion	0	42
KCN, 0.04 m*M*	0	46
KCN, 0.4 m*M*	−18	88
KCN, 4 m*M*	20	72
KF, 10 m*M*	30	93
Chloramphenicol	30	85
Rifampicin	31	67

As shown in Fig. 2, depletion of ammonium ion, the nitrogen souce, did not significantly affect either the proportion of CoASSG in the CoA pool or the level of the CoA pool. Variations in CoASSG and the CoA pool are therefore linked to carbon metabolism and not nitrogen metabolism in spite of the fact that cellular metabolism stops in response to both carbon and nitrogen starvation.

Metabolic Inhibitors

The inhibitors were chosen to selectively inhibit respiration (KCN and NaN_3), glycolysis (KF), transcription (rifampicin), and translation (chloramphenicol), although each of the inhibitors, when added to cell cultures, may affect several different processes directly and indirectly making a clear definition of their effects difficult.

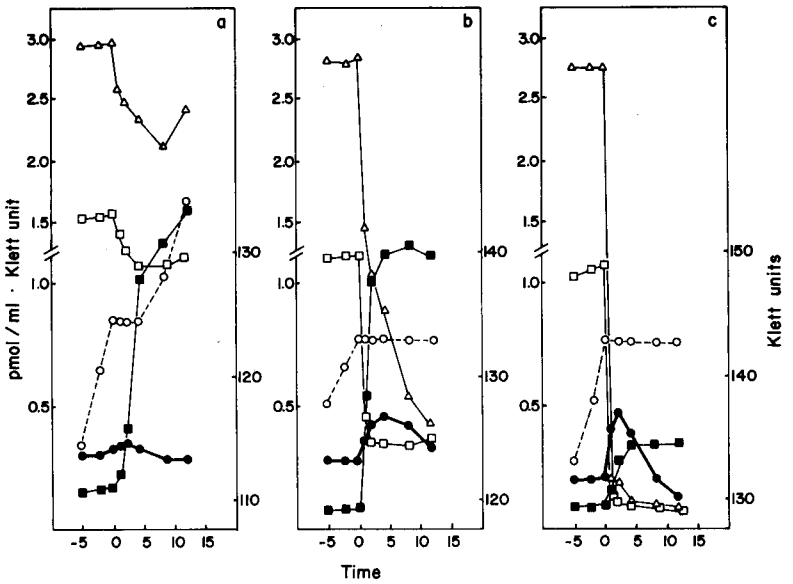

FIG. 3. Nucleotide levels before and after the addition of KCN at concentrations of (*a*) 0.04 m*M*, (*b*) 0.4 m*M*, and (*c*) 4 m*M* at time 0. Aliquots were removed and assayed as described in Materials and Methods. Symbols: ATP, △; GTP, □; ppGpp, ■; CoASSG, ●; and Klett units, ○.

Potassium cyanide, when added to cultures at 0.04, 0.4, and 4 m*M*, resulted in an immediate (within 1 min) cessation of growth and RNA accumulation (Fig. 3). ATP and GTP pools decreased very rapidly and recovered only in the case of 0.04 m*M* KCN when growth and RNA accumulation had restarted after a pause of 4 min. These observations are consistent with previous reports of the effect of cyanide on cultures of *E. coli* (19, 20). The pool of ppGpp increased 3- to 12-fold and did not fall even when growth had restarted in 0.04 m*M* KCN.

The pool of CoASSG increased at all three cyanide concentrations but the extent of the increase varied from 2.5 times at 4 m*M* to almost no increase at 0.04 m*M* KCN and the pool tended to fall after reaching a peak between 2 and 4 min after the addition of cyanide. Neither the CoA pool nor the increase in the CoASSG fraction of the CoA pool varied consistently with the KCN concentration (Table 1), indicating that the different KCN concentrations had different effects upon cellular metabolism ranging from complete inhibition of many different enzymes at the high concentration to a transient inhibition of electron transport at the low concentration (19–21). However, there is a general increase in CoASSG at the expense of the other forms of CoA which may reflect the decreased need for CoA as a result of a general metabolic slowdown. Sodium azide, when added at 4 m*M*, resulted in changes in the nucleotide pools identical to those exhibited after the addition of 0.04 m*M* KCN consistent with an earlier report (19).

Potassium fluoride at 10 m*M* resulted in a transient cessation of growth lasting 4-min with no change in either the ATP or the GTP pools (Fig. 4). The pool of ppGpp rose by 2.5 times within 1 min and then decreased gradually. The pool of CoASSG increased by two times over a period of 8 min while the CoA pool increased by 30% and was converted to 93% CoASSG. KF is known to selectively inhibit the glycolytic pathway which indirectly stops the conversion of pyruvate to acetyl CoA and allows subsequent glucose metabolism to occur using the hexose monophosphate shunt. As with glucose and oxygen starvation, the decreased use of CoA is reflected in the increase in CoASSG.

The antibiotics rifampicin and chloramphenicol caused respectively a 3.0-times and a 1.5-times increase in both the ATP and GTP pools as well as a decrease in the ppGpp pool to 50% of its original level (Fig. 4). The pool of CoASSG increased in both cases (2.5 times for rifampicin and 1.8 times for chloramphenicol), becoming the predominant fraction in the CoA pool which had increased by 30% in both cases. CoA is not directly involved in either RNA or protein biosynthesis and the effects of the antibiotics on the CoA pool may be indirect, resulting from the inhibition of glycolysis and the tricarboxylic acid cycle by the elevated levels of ATP.

Relationship of CoASSG and ATP

It has been shown that the adenylate energy charge, the anabolic reduction charge ($NADPH/(NADP^+ + NADPH)$), and the catabolic reduction charge ($NADH/(NAD^+ + NADH)$) are invariant for growth on both fermentable and nonfermentable carbon sources over a broad range of growth rates (22). In a similar survey of carbon sources, it was found that the CoASSG pool increased with generation time but in an irregular fashion. However, the CoASSG:ATP ratio was found to increase linearly with the generation time in two groupings of carbon sources (Fig. 5), suggesting that there is a close relationship between the CoA pool and ATP or the 'energy content' of the cell. The group with the lower ratio was composed entirely of fermentable sugars while

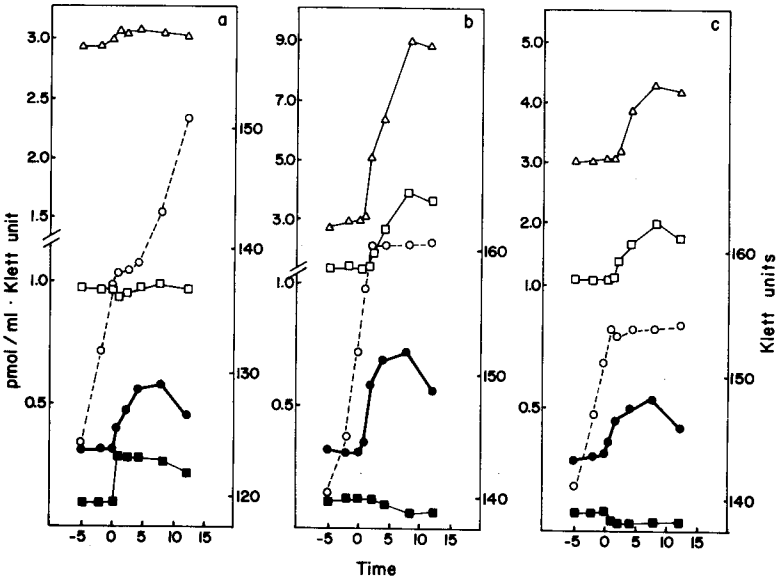

FIG. 4. Nucleotide levels before and after the addition of (*a*) 10 m*M* KF, (*b*) 12.5 μg rifampicin per millilitre, and (*c*) 125 μg chloramphenicol per millilitre at time 0. Aliquots were removed and assayed as described in Materials and Methods. Symbols: ATP, △; GTP, □; ppGpp, ■; CoASSG, ●, and Klett units, ○.

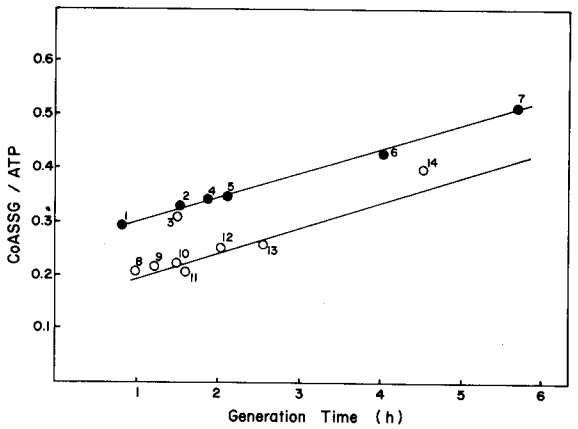

FIG. 5. CoASSG:ATP ratio in mid-log phase cultures grown on 0.2% of various carbon sources. Aliquots were removed at various times and assayed as described in Materials and Methods. The values represent an average of four determinations. The carbon sources as numbered in the figure were (1) casamino acids; (2) α-ketoglutarate; (3) sorbitol; (4) glutamate (37°C); (5) glutamate (34°C); (6) acetate; (7) pyruvate; (8) glucose (37°C); (9) galactose; (10) glucose (32°C); (11) glucose (30°C); (12) fructose; (13) mannose; and (14) glucose (anaerobic). Open circles (points 3, 8–14) represent fermentable sugars while closed circles (points 1, 2–7) represent nonfermentable carbon sources.

the group with the higher ratio was composed primarily of nonfermentable carbon sources.

It was also observed that the CoASSG fraction of the CoA pool was larger in cells grown on the nonfermentable carbon sources varying from 84 to 97% while fermentable sugars resulted in a lower proportion between 42 and 55% (Table 2). There were two exceptions to these generalizations. The glucose culture grown anaerobically had a large proportion of its CoA pool in the form of CoASSG, in agreement with the earlier observation that the CoASSG fraction of the CoA pool increased during anaerobiosis. The cultures grown on sorbitol were found in the grouping of nonfermentable carbon sources both on the basis of the CoASSG:ATP ratio and the large CoASSG fraction, perhaps reflecting the importance of the initial oxidation step in its metabolism.

Discussion

These experiments demonstrate that significant increases in the pool of CoASSG in cells grown aerobically on glucose result when the pyruvate to acetyl CoA conversion and subsequent excretion of acetate are affected. Fluoride is a specific inhibitor of glycolysis and causes an increase in CoASSG at the expense of free CoA and acetyl CoA. Anaerobiosis, glucose starvation, cyanide, rifampicin, and chloramphenicol, all of which can directly or indirectly affect carbon metabolism by glycolysis and the tricarboxylic acid cycle, cause similar increases in CoASSG. Such increases occur only in cells grown aerobically on glucose or other fermentable carbon sources since only under these conditions does CoASSG make up less than 55% of the CoA pool. Cells grown on glucose have been reported to have a very low level of oxidative phosphorylation and since most of the ATP must therefore come from fermentation and the phosphoroclastic system, the tricarboxylic acid cycle is active primarily for biosynthesis (23). Free CoA and acetyl CoA under these conditions may arise from the metabolism of pyruvate to acetyl CoA which is then hydrolyzed by the phosphotransacetylase – acetate kinase enzyme pair to be excreted as acetate (18). During

TABLE 2. CoASSG and CoA pool levels before and 8 min after oxygen depletion during growth on different carbon sources[a]

Carbon source	Before oxygen depletion			After oxygen depletion		
	CoA pool	CoASSG	CoASSG–CoA pool	CoA pool	CoASSG	CoASSG–CoA pool
Casamino acids	0.136	0.128	0.94	0.138	0.132	0.96
Pyruvate	0.630	0.594	0.94	0.530	0.528	0.99
Acetate	0.376	0.288	0.77	0.344	0.272	0.79
Glutamate	0.296	0.264	0.89	0.262	0.204	0.78
α-Ketoglutarate	0.376	0.310	0.82	0.384	0.310	0.81
Sorbitol	0.404	0.400	0.99	0.424	0.402	0.95
Glucose (anaerobic)	0.384	0.336	0.88	—	—	—
Glucose	0.624	0.304	0.47	0.780	0.504	0.64
Galactose	0.498	0.250	0.50	0.508	0.298	0.59
Fructose	0.564	0.310	0.54	0.630	0.380	0.60
Mannose	0.700	0.388	0.55	0.666	0.516	0.77

[a]Values expressed in picomoles per millilitre·Klett unit.

anerobic growth on glucose, acetate is excreted in greater amounts and the specific activity of acetate kinase and phosphotransacetylase is higher (18), resulting in lower levels of acetyl CoA. It is not clear why there is no free CoA under these conditions. Since excess acetate will not be produced during growth on nonfermentable carbon sources, the levels of acetyl CoA and free CoA are depressed.

How is CoASSG produced? In spite of the preponderance of CoASSG in both yeast and *E. coli* and its presence in liver, no explanation of how CoASSG arises has yet been proposed. Random oxidation of CoA with glutathione is one plausible route but some other mechanism is suggested by the fact that CoASSG increases after oxygen depletion and predominates in aerobically grown cells.

What role does CoASSG play in metabolism? Again no firm conclusions have been drawn although it has been reported to inhibit *E. coli* RNA polymerase, a property inconsistent with it being present at all times during cell growth. Since a majority of the small CoA pool is in the form of CoASSG, it may be a means of protecting the metabolically valuable CoA molecule from other random oxidations and also as a means of storing excess CoA. The enzyme CoAS–Sglutathione reductase (NADPH) (NADPH:CoAS–Sglutathione oxidoreductase; EC 1.6.4.6) identified in yeast and liver (24–26) and also shown to be present in *E. coli* (2) is probably responsible for creating a supply of free CoA. The specific activity of this reductase in crude extracts of *E. coli* was shown to increase both during anaerobiosis and during growth into stationary phase coinciding with the rise in the pool of CoASSG. The reductase uses NADPH as an electron source and it might be expected that changes in the NADPH pool would coincide with changes in the CoASSG pool. However, Andersen and von Meyenburg (22) have shown that the anabolic reduction charge ($NADPH/(NADP^+ + NADPH)$) is constant regardless of the carbon source and that there is very little change in the ratio during glucose and oxygen starvation. Rifampicin and chloramphenicol caused a slight increase in the anabolic reduction charge coincident with the increase in CoASSG. This is inconsistent with the concept that increased NADPH would allow faster reduction of CoASSG. Unfortunately, absolute values of the NADPH pool were not presented and there may be a better correlation between the actual levels of NADPH and CoASSG. The adenylate energy charge also showed very little response to rifampicin and chloramphenicol (22) but there was a significant change in the ATP and GTP pools when these antibiotics were added (Fig. 4).

Until very recently, there have been few reports of changes in the CoA pools resulting from nutrient changes and none of these discussed CoASSG. A detailed study of the yield of CoA produced by *Sarcina lutea* during growth on various carbon sources has been reported (27) and no consistent variation of CoA yield with fermentable or nonfermentable carbon sources was evident. Stadtman and Kornberg (3) found that the majority of CoA isolated from bakers yeast was in the form of CoASSG and recently it has been shown that there are significant changes in the form of CoA depending upon the stage of sporulation in *Bacillus megaterium* (28. 29). The levels of acetyl CoA and reduced and oxidized forms of CoA have been shown to vary between dormant and germinating spores and between growing and sporulating cells. In addition, CoA has been shown to be the predominant low molecular weight sulfhydryl compound in both spores and cells.

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