SULFUR METABOLISM IN ESCHERICHIA COLI

I. Sulfate Metabolism of Normal and Mutant Cells

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Sulfur appears to be a universal constituent of living matter. Its metabolic importance and major modes of utilization by microorganisms have been established by direct chemical analyses of cellular protoplasm and associate culture media. A few of the detailed processes of its incorporation by living systems have recently become more clearly defined through the use of mutant molds and bacteria (Tatum, 1949). However, the mechanisms during initial stages of transformation in which inorganic sulfur is converted to organic sulfur compounds are not established, nor are the relationships between bound and unbound sulfur clear. Studies of potassium and phosphorus metabolism have shown that it is necessary to distinguish quantitatively between bound and unbound fractions of a nutritive element. This fundamental requirement for metabolic studies should be met by analytical methods applied to sulfur metabolism.

In view of the universal occurrence of sulfur in living systems and the need for knowledge concerning many facets of its metabolic role, further investigation is indicated. This paper reports results of studies of sulfate metabolism of *Escherichia coli* in which normal and mutant cells, radioactive tracer techniques, and competing biochemical reactions have been used.

MATERIALS AND METHODS

The culture methods and media used for growth of *E. coli*, strain B,[1] and the techniques of sampling and measurement of radioactivity have been previously reported (Cowie *et al.*, 1949). Alterations made in these methods when necessary to suit experimental requirements are described in appropriate sections below.

Mutant cells were obtained from M. J. Jones, American Cyanamid Company, Stamford, Connecticut. All amino acids used were DL-forms. S^{35}, half-life 87.1 days,[2] was used as the tracer isotope. Since this isotope has low beta energy, it was routinely necessary to correct radioactivity measurements for self-absorption.

Quantitative determinations of amounts of bacteria were conveniently made by employing a Beckman spectrophotometer operating at 650 mμ. The optical density so determined for culture samples was correlated with cell volume by calibration data.

[1] Obtained from the Department of Genetics, Carnegie Institution of Washington.

[2] Obtained from the Radioisotope Division, Atomic Energy Commission, at Oak Ridge.

EXPERIMENTAL RESULTS

Growth of E. coli in Synthetic Media

The sulfur requirements for growth of normal *E. coli* cells were first established for three inorganic compounds, Na_2SO_4, Na_2SO_3, and Na_2S. Aerobic and semiaerobic growth were determined as a function of sulfur concentration.

The M-9 medium[3] of Anderson (1946) was distributed to three series of eight culture tubes. Sulfur as Na_2SO_4, Na_2SO_3, or Na_2S was added to each tube to final concentrations varying from 0.0001 to 0.5 mg sulfur per ml medium. To the sulfite and sulfide series was added a culture tube containing Na_2SO_4 (0.01 mg sulfur per ml), which served as a reference standard for direct comparison with other tubes of the series.

Each tube of the array was seeded with *E. coli*, which had previously been grown in a medium low in glucose. The introduction of "low glucose grown" cells into an adequate nutrient medium ensured the initiation of logarithmic growth almost immediately. The tubes were placed in water baths at 37 C and aerated by means of bubbler tubes operating from the laboratory air supply. Another array of cultures was prepared, identical with that above except that aeration was withheld. Samples for optical density determinations were taken at 0, 1, 2, and 3 hours. Density measurements made at 19 hours showed little change from those at 3 hours.

The relationship among growth of cells, chemical form, and concentration of sulfur in the synthetic medium is shown in figure 1. These data reveal that growth is a maximum at 0.005 to 0.01 mg sulfur per ml of medium irrespective of the form of sulfur or degree of aeration. There appears to be little difference in the amount of growth supported by the various forms of sulfur for concentrations below 0.005 mg sulfur per ml, whereas, for concentrations above 0.01 mg sulfur per ml, sulfite and sulfide inhibited growth. Inhibition was more marked under semiaerobic conditions. The question whether inhibition of growth is the result of direct toxicity of sulfite and sulfide, an accumulation of toxic by-products, or an increased oxygen requirement in the presence of reduced forms of sulfur is under investigation.

Figure 1 demonstrates that in the case of Na_2SO_4 the yield of organisms from the synthetic medium employed is a function of sulfur concentration for low levels of sulfur, i.e., the organisms are limited in their capacity to develop new cells by the amount of sulfur present. For the higher concentrations of sulfur as sulfate, the yield of cells is independent of the amount of sulfur supplied. These observations confirm those generally found for metabolites supplied over wide concentration ranges.

Cell Permeability in Relation to Metabolism

The radioactive tracer technique allows further investigation of the sulfur needs of the cell. In particular, a distinction between metabolized and non-

[3] M-9 medium contains 0.1 g $MgSO_4$, 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 1 g NH_4Cl, 100 ml 4 per cent glucose, and 900 ml of water.

metabolized sulfur is made possible, allowing precise studies of the distribution of sulfur with reference to the cell membrane.

Immediate sulfate uptake. One to two ml of resting cells of *E. coli*, harvested after completion of growth in synthetic medium (Cowie *et al.*, 1949), were immersed in a mixture of 0.85 per cent NaCl and a known amount of $Na_2S^*O_4$[4] using S^{35} as the tracer element. The cells were immediately centrifuged at 20,000 g in an angle-head centrifuge. The supernatant solution was separated from the packed cells, and the centrifuge tubes were rinsed with 20 ml of NaCl solution. Aliquots of cells, supernatant, and rinse were taken for measurement of the radioactivity. The time elapsed between immersion of cells and collection of

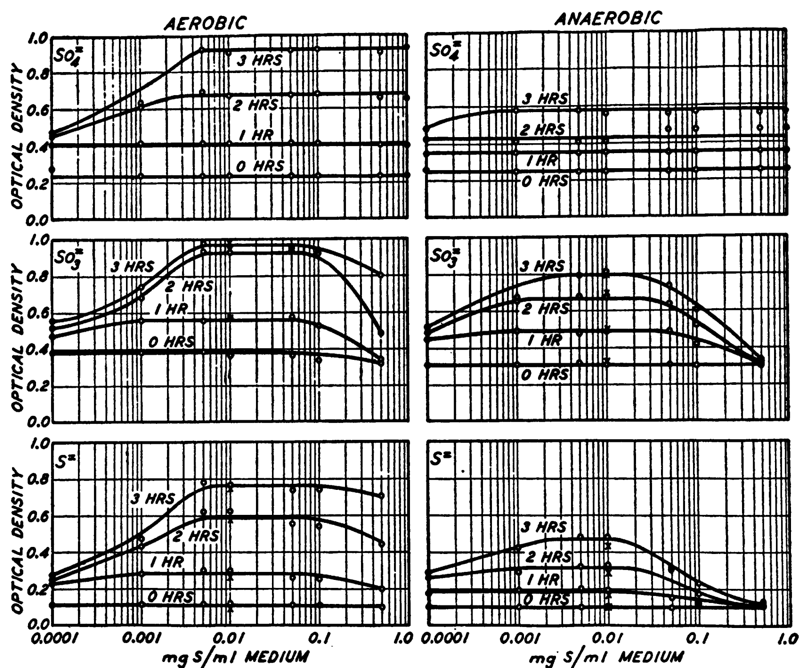

Figure 1. Growth of *E. coli* strain B in relation to sulfur concentration in synthetic media.

the supernatant was approximately 8 minutes. The measurements expressed as the ratio, $\frac{\text{radioactivity/ml cells}}{\text{radioactivity/ml medium}}$, recorded in table 1, indicate a fixed relationship between sulfate within the cells and sulfate in the medium. Immersion of these resting cells in the saline, radioactive sulfate solution, when continued for several hours, showed no additional uptake of sulfur.

The ratio given above is that part of a unit volume of cells which is in equilibrium with an equal volume of medium with respect to sulfur concentration. The term "water space" has been suggested to describe this quantity (Cowie *et al.*, 1949). It is evident that equilibrium of $S^*O_4^=$ is established across the cell

[4] S^* refers to mixtures of stable and radioactive isotopes of sulfur.

membrane within 8 minutes, indicating that the cell membrane of *E. coli* is highly permeable to sulfate ion.

Table 2 demonstrates that immediate sulfur uptake by resting cells depends solely upon the concentration of sulfur in the medium, a change in the latter being followed by an equal change in the water space sulfur content.

If the sulfur immediately taken into the cell is actually contained passively within a freely permeable cell membrane, then the radioactivity should be readily washed out by immersion of the resting cells in saline. Such is the case,

TABLE 1

Immediate $S^*O_4^=$ uptake by E. coli cells

EXPT. NO.	ACTIVITY/ML CELLS / ACTIVITY/ML MEDIUM	WATER SPACE
		per cent
VI	29.7 / 41.4	72
VIII	34.7 / 48.3	72
IX	27.5 / 36.4	76

TABLE 2

Immediate $S^*O_4^=$ uptake in resting E. coli cells

MgS*/ML MEDIUM[a]	MgS*/ML CELLS[a]	WATER SPACE
		per cent
0.0033	0.0024	72
0.0300	0.0180	60
0.0560	0.0340	61
0.1097	0.0780	71
0.2161	0.1280	59
0.4289	0.2750	64

[a] Computed from the specific activity of sulfur added.

for these cells immersed in a known volume of 0.85 per cent NaCl establish a new equilibrium with respect to sulfur concentration, the immersion fluid gaining in total radioactivity in proportion to the volume used, the cells correspondingly losing activity. Subsequent washes reduce the remaining radioactivity to low levels. Following each wash, however, there is established an equilibrium between the water space sulfur content and that of the surrounding medium.

It is concluded that the cell membrane of *E. coli* is highly permeable to $S^*O_4^=$ and that resting cells contain in their water space amounts of sulfate in equilibrium with the medium. This quantity of sulfate is passively held, i.e., is not chemically bound by protoplasm, as the ready removal by washing procedures

indicates. The large quantities of unbound sulfur held by these cells in a medium high in sulfur constitute a potential source of error in any studies concerned with measurements of sulfur content of *E. coli* or other microorganisms. Table 2 shows that no limit is approached in the incorporation of sulfur by resting *E. coli* cells over the range of concentrations of sulfur studied. This observation is in contrast to the results of experiments described below in which cells have been supplied an energy source.

Uptake of sulfate during growth. It has been demonstrated that resting cells take up sulfate sulfur only to the degree in which it is present in the medium surrounding the cells. During growth, however, there is a marked uptake of inorganic sulfate from the medium, probably by the creation of nondiffusible organic sulfur compounds in the cell. It is this sulfur fraction distinguished from the water space sulfur that is most directly concerned in these studies of sulfur metabolism in *E. coli.*

TABLE 3
Sulfur uptake during growth

EXPT. NO.	ML CELLS GROWN	MgS*/ML MEDIUM	MgS*/ML CELLS	S* ACCOUNTED FOR
				per cent
E	3.7	0.026	3.3	96
K	1.8	0.026	3.8	98
L	1.6	0.026	3.8	98
O	4.3	0.026	3.5	96
VIII	0.32	0.303	3.1	99
			Mean = 3.5	

Several ml of *E. coli* cells may be grown overnight in M-9 medium to which has been added a tracer quantity of carrier-free S^{35}. These cells contain, after three washes with 0.85 per cent NaCl, about 3.5 mg of sulfur per ml of cells. Table 3 presents the results of a series of experiments in which the total bound sulfur has been determined. It will be noted that if sufficient sulfate for maximal growth is present in the medium (>0.005 mg sulfur per ml medium) the sulfur uptake per ml cells is independent of the total volume of cells grown. It appears that for each unit of protoplasm grown there is incorporated a definite amount of sulfur.

A measure of the accuracy of each experiment is given in the last column in table 3 where the sum of the radioactivities of all of the wash solutions and washed cells is compared to the known total radioactivity in the original medium.

A quantitative examination of the extent to which the growth phase of the culture affects the direct uptake of sulfur from the medium is of interest. The determination of sulfate uptake during both the logarithmic and stationary growth phases in M-9 medium containing radioactive $SO_4^{=}$ shows that unless new protoplasm is produced little if any sulfate is taken up. Figure 2 demonstrates

that during logarithmic growth a direct correspondence between growth and sulfate uptake occurs, whereas in the stationary phase there is no additional uptake of the sulfate.

It is also of importance to examine the uptake of sulfur by growing *E. coli* cells in relation to the quantity of sulfur supplied. For this purpose the organisms were grown in M-9 medium to which various amounts of radioactive sulfate were added. Cells were harvested at 0, 2, 4, and 6 hours, and their sulfur content was

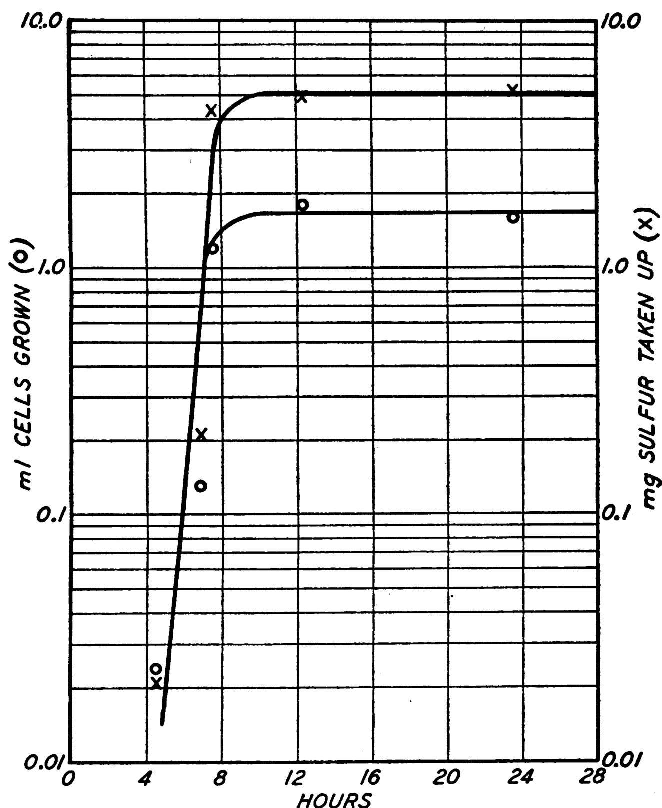

Figure 2. Growth and sulfate uptake by normal *E. coli* strain B. The upper curve represents mg of sulfur taken up (right-hand ordinate) by volume of cells grown (left-hand ordinate).

determined from radioactivity measurements. The data are presented in figure 3. It is noted that for low concentrations of sulfate in the medium, the amount of sulfur taken up per ml of cells (exclusive of water space sulfur) increases linearly with the amount of sulfate provided and, for high concentrations, is independent of the supply. Although the cells have grown continuously during the period of the experiment, the total cell volume does not influence the unit cell volume uptake of sulfur, for this uptake is independent of the time of sampling. These results confirm the fact pointed out above that the sulfur content of *E. coli* grown in an adequate sulfate medium is constant. In addition, they

show a relatively low uptake of sulfur for cells grown in sulfur-deficient media. The optical density measurements used to calculate cell volume are known only qualitatively in terms of cell mass and in terms of protein content. From the data of figure 3 we may not state with precision whether the bacteria grown in low sulfur contain sulfur-deficient protein or less protein per unit volume of cells, or whether there is a redistribution of sulfur among the variety of organic combinations possible. Nevertheless, figure 3 demonstrates clearly the variation in sulfur content that may be expected for *E. coli* grown in media differing in sulfur concentration.

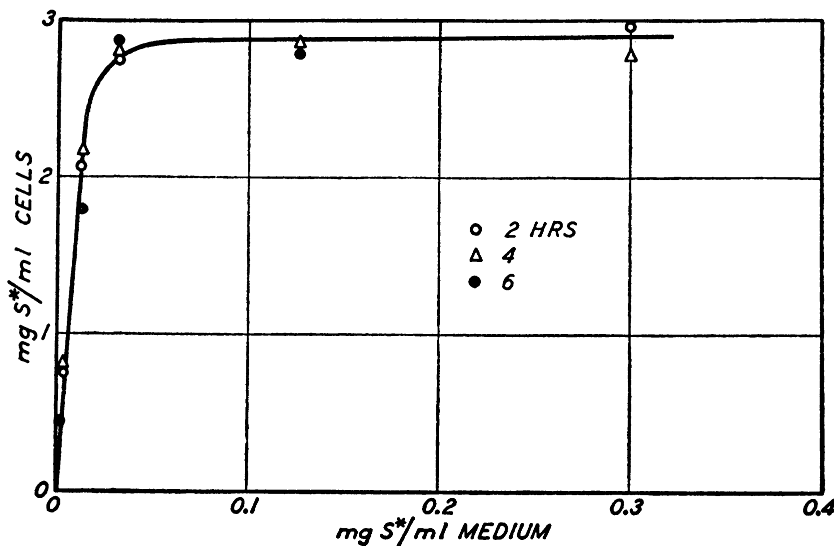

Figure 3. Sulfate uptake of *E. coli* strain B as a function of sulfate concentration in the medium.

The Effect of Glucose upon Sulfate Uptake

E. coli cells grown in a sulfur-deficient medium take up only a fraction of the sulfur utilized when grown in adequate sulfur media (figure 3). Examination of a cell-free low-sulfur culture medium after a 12-hour growth period reveals that there is nearly complete removal of the sulfur. Since the cellular uptake accounts for the removal of sulfur, and since growth is also less than for an adequate sulfur medium, it is apparent that low-sulfur-grown cells are sulfur-limited. These cells provide a means by which to study further sulfate uptake in adequate sulfur media in relation to energy sources available.

The results of some experiments on additional sulfate uptake by sulfur-deficient cells in the absence and presence of glucose are shown in figure 4. It is evident that these cells do not take up the sulfate provided in the medium until glucose is added. Upon the addition of glucose, growth proceeds, and there exists a direct correlation among growth, nitrogen content, and sulfate uptake. The nitrogen: sulfur ratio by weight for these cells is approximately 10.9, which

lies within the range of values for a wide variety of proteins reported by Block and Bolling (1945).

It is concluded that energy is required for sulfate to be utilized by *E. coli* and, to the extent that nitrogen uptake is a measure of protein synthesis, sulfate uptake measures the synthetic activity of these cells.

Exchange of Bound Sulfur

It has been shown that the sulfur composition of *E. coli* is essentially constant for cells grown in adequate sulfate media, that glucose is required for sulfate utilization, and that the nitrogen content of the cells is directly propor-

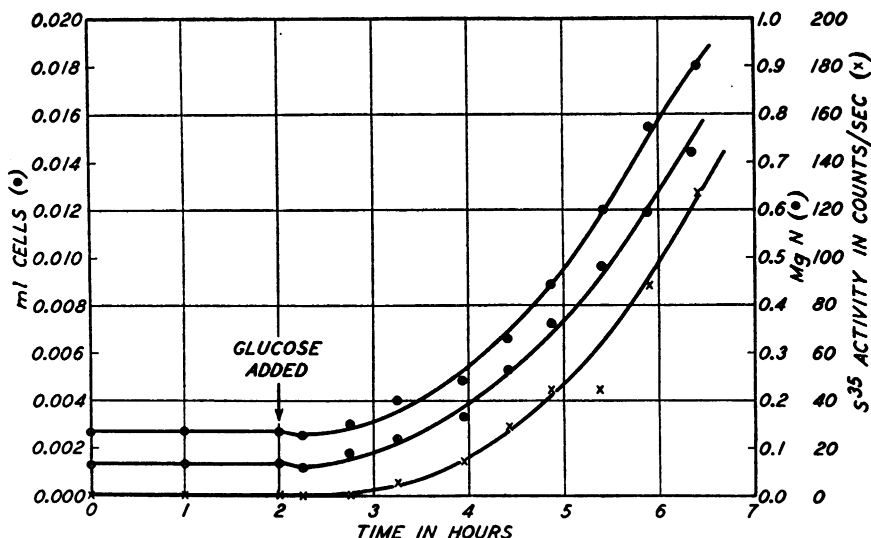

Figure 4. The effect of glucose upon sulfur uptake, growth, and nitrogen content of sulfur-starved *E. coli* strain B. The upper curve (left-hand ordinate) shows growth of cells before and after the addition of glucose, and the middle and lower curves (right-hand ordinates) show nitrogen content and sulfur uptake, respectively.

tional to the sulfur content. These facts suggest that sulfate is bound by incorporation in protein, presumably through synthesis of sulfur-containing amino acids. It is of interest and importance to ascertain the degree to which bound sulfur undergoes exchange with the free sulfur of culture media during the growth and reproduction of cells, for the degree of exchange is indicative of both synthetic activity and degradative processes.

Several experiments have been performed to measure exchange. (a) Cells grown overnight in M-9 medium (0.026 mg sulfur per ml) containing radioactive sulfate were harvested and washed three times with 0.85 per cent saline solution to eliminate all unbound sulfur. Wash losses in a typical experiment indicated that at the end of the first, second, and third washes there remained 4.06, 3.82, and 3.78 mg sulfur per ml of cells, respectively. After the final washing the cells were resuspended in 0.85 per cent NaCl, and equal samples of cells

were added to four Erlenmeyer flasks. Each flask contained 200 ml M-9 medium in which nonradioactive sulfur was added either as methionine, cystine, cysteine, or sulfate ion. The flasks were placed in a water bath at 37 C, cultures were aerated, and at 0, 1, 2, and 3 hours 6-ml samples were withdrawn, 5 ml for radioactivity measurements of the cells and 1 ml for turbidity measurements.

Table 4 demonstrates that little of the bound, labeled sulfur exchanged with the sulfur compounds in the medium despite continuing cellular growth. This method, as shown by the scatter of the data, is not reliable for the measurement of very low exchange processes. A more suitable method was provided through the measurement of the radioactivity lost to the medium as the cells

TABLE 4

*Sulfur exchange in growing E. coli cells**

TIME	CHEMICAL FORM OF NONRADIOACTIVE SULFUR IN MEDIUM	ML CELLS PER TUBE	Mg RADIOSULFUR IN CELLS
hours			
0	Na_2SO_4	0.284	1.17
1		0.441	1.06
2		0.753	1.14
3		0.880	1.14
0	Cystine	0.284	1.16
1		0.456	1.05
2		0.882	1.06
3		0.998	1.10
0	Cysteine	0.284	1.17
1		0.327	1.14
2		0.439	1.07
3		0.774	1.14
0	Methionine	0.284	1.19
1		0.447	1.13
2		0.860	1.11
3		0.946	1.19

* The sulfur concentration in all tubes was 0.01 mg sulfur per ml medium.

continued to grow. The latter method, however, can only give an upper limit of the amount of the exchanged radioactive sulfur of the cells since dead cell debris or the presence of small amounts of cells incompletely removed from the medium by centrifugation would appear to be exchanged sulfur.

(b) In part to remedy this difficulty the radioactive cells were added to a dialysis bag, which in turn was suspended in a nonradioactive medium. Samples of the dialyzate and of the contents of the dialysis bag were removed during the experiment to determine the radioactivity that had been lost from the metabolizing cells. In one such experiment, 1.34 ml of cells containing 4.53 mg of bound radioactive sulfur were resuspended in a dialysis bag containing 100 ml M-9 medium. The bag and its contents were placed in a large flask with 700 ml

M-9 medium and 200 ml 10 per cent glucose, the dialyzate being actively aerated throughout the experiment. The entire system was suspended in a water bath kept at 37 C. At 0.3, 1, 2, 4.3, and 7.2 hours 6-ml samples of the contents of the dialysis bag were withdrawn, 5 ml for radioactivity measurements of cell-free medium and 1 ml for turbidity measurements. Samples of the dialyzate were also removed for radioactivity determinations. The results showing the low exchange of the bound sulfur are given in table 5.

The supernatant of the culture in the dialysis bag showed an immediate gain in radiosulfur, 0.058 mg of the radiosulfur being found at the end of 15 minutes. This sulfur, however, must have been in nondialyzable or extremely slowly dialyzable forms because equilibrium with the dialyzate did not rapidly occur despite vigorous mixing. Therefore it is believed that cell fragments initially released when the cells were placed in the nutrient medium account for this loss of radiosulfur. As growth continued, the activity of the supernatants in the dialysis bag decreased, for slow diffusion of part of the sulfur through the cellophane bag to the dialyzate occurred. At the end of 7.2 hours 0.099 mg of radiosulfur of the 4.53 mg initially bound by the cells could be shown to have been lost or exchanged.

TABLE 5

Loss of bound radiosulfur from E. coli cells during growth

TIME	ML CELLS PER ML OF DIALYSIS BAG CONTENTS	RADIOSULFUR IN DIALYSIS BAG MEDIUM	RADIOSULFUR IN DIALYZATE
hours		*mg*	*mg*
0.3	0.63×10^{-2}	0.058	0.010
1.0	0.93×10^{-2}	0.058	0.023
2.0	0.99×10^{-2}	0.036	0.045
4.3	0.99×10^{-2}	0.026	0.048
7.2	1.14×10^{-2}	0.032	0.067

(c) We have observed that cells grown in radiosulfate media, after three washes with saline solution, do not continue to lose sulfur with additional washes. In the transfer, however, of these washed cells to fresh synthetic medium containing glucose, varying amounts of sulfur are invariably lost from the cells to the medium within the first few minutes after the inoculation. The quantity of sulfur lost seems to depend upon several factors: the growth phase of the cells when harvested and washed; the time between the final wash and the addition to the new culture medium; and the kind of wash solution used.

In order to minimize this loss the cells were harvested during the logarithmic growth phase, washed in nonradioactive synthetic medium containing glucose, and immediately added to the final medium in order to measure the exchange of the bound sulfur during cellular growth. Figure 5 shows that despite these precautions 0.077 mg of the total 2.5 mg of radiosulfur initially bound in the transferred cells appeared in the medium at the end of one-half hour. This quantity represented 3.1 per cent of the total bound sulfur of the original labeled

cells. After this initial loss, however, the metabolizing cells lost only an additional 1.0 per cent of the bound sulfur during the next 4.5 hours of growth. Growth had continued as shown in figure 5 to the early stationary phase. These findings confirm results shown in table 5, where the immediate loss of the bound sulfur from the radioactive cells exceeded the later losses observed as the cells continued to grow.

(d) In an experiment designed to allow maximal exchange of the bound sulfur of the cell with the sulfur of the medium, radioactive cells were harvested and washed in saline solution several hours after growth had ceased. The cells were then maintained at 3 C for 4 hours. At this time the cells were transferred

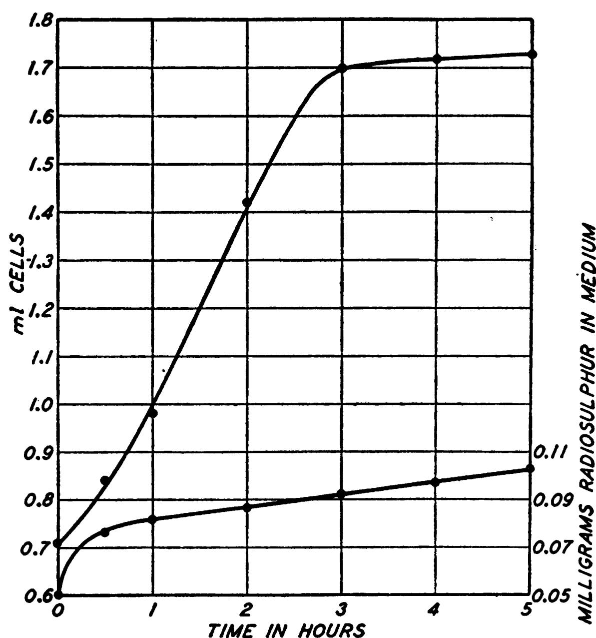

Figure 5. Exchange of bound radiosulfur of the cell with nonradiosulfur of the medium during growth. The upper curve (left-hand ordinate) shows growth, and lower curve (right-hand ordinate) represents loss of radiosulfur from the cells to the medium.

to a new culture medium, which contained three sulfur sources, cystine, methionine, and Na_2SO_4 at equal sulfur concentrations, the total concentration being 0.075 mg sulfur per ml medium.

The results of this experiment were qualitatively similar to those reported in section (c) above. However, a more pronounced lag in growth was observed. During this lag a greater initial loss of sulfur from the cells was measured, about 10 per cent being lost instead of the 3 per cent for the cells shown by figure 5. The rate of exchange or loss of sulfur from the cells during growth was not markedly different in the two cases. The low exchange of the sulfur of the cell with the sulfate or amino acid sulfur of the medium in these experiments supports the suggestion that for *E. coli* cells, and possibly other microorganisms, protein synthesis can be measured by sulfate uptake.

Sulfate Metabolism of Methionine Mutant Cells

The combined use of mutant *E. coli* cells and the radiosulfur tracer technique provides a new and valuable method for quantitative biochemical assay of sulfur metabolism. Lampen and his co-workers (1947) have produced a series of mutant strains of *E. coli* which require for growth the presence of preformed sulfur containing amino acids. The nutritional requirements of these cells differ from the normal strains in their inability to carry out certain definite synthetic reactions. Lampen *et al.* have observed that a methionine-requiring mutant will not grow in media containing sulfate as the sole sulfur supply. With methionine as the sulfur source, the mutant strain grows rapidly. By supplying radioactive Na_2SO_4 and nonradioactive methionine with the sulfur of each form in equal concentrations (approximately 0.025 mg of sulfur per ml of medium), growth readily occurs. The methionine mutant, *E. coli* culture no. 1-344, obtained from the American Cyanamid Company,[5] was used to investigate whether sulfate is in any way metabolized by the growing mutant cell.

Two tubes of 40 ml of M-9 medium containing sulfur as $Na_2S^*O_4$ at a concentration of 0.024 mg sulfur per ml were inoculated with the methionine-requiring mutant. One tube had in addition DL-methionine added at a concentration of 0.027 mg sulfur per ml medium, and both cultures were allowed to grow overnight, aerated in a water bath at 37 C. No cells grew in the methionineless medium, whereas 0.148 ml of cells were harvested from the methionine-sulfate medium. After three washes with 20 ml 0.85 per cent saline solution, in which the radioactivity of the cells after each wash was measured, it was found that at the end of the first, second, and third washes there were 1.63, 1.38, and 1.31 mg of sulfur per ml of cells, respectively. A repetition of this experiment yielded a value at the end of three washes of 1.55 mg of sulfur per ml of cells.

If the methionine mutant cells have a total sulfur requirement of 3.5 mg of sulfur per ml of cells as do the normal sulfate-grown cells, then 1.31 mg of sulfate-derived bound sulfur represents approximately 37 per cent of the total sulfur needs. Since both sulfur sources were present in the medium in about equal concentrations, sufficient to provide all sulfur needs for maximal growth, the remaining sulfur (63 per cent) must have been provided by the methionine. There can be no doubt that sulfate is utilized by these mutants whenever protein synthesis and subsequent cellular reproduction are guaranteed by the presence of methionine in the medium. It is evident that the use of radioactive tracers together with biochemical mutants provides a method for the simultaneous study of the metabolism of sulfur derived from different chemical sources. Thus the usefulness of the biochemical mutant is complemented and extended.

Sulfate Metabolism of Cystine Mutant Cells

The cystine mutant no. 508-462, another of Lampen's mutant strains, was used to study the utilization of sulfate ions in the presence of an equal quantity of sulfur as cystine.

[5] The mutant cultures were provided through the courtesy of M. J. Jones of the American Cyanamid Company, Stamford, Connecticut.

No growth was observed with only radioactive sulfate present in the medium. However, if an equal concentration (0.026 mg sulfur per ml) of sulfur as cystine is added, growth is observed. Little if any sulfate is taken up by the growing mutant cells. After three washes, 0.0068 of a mg of sulfate sulfur per ml of cells was retained. Only 0.019 per cent of the 3.5 mg of sulfur required per ml of normal sulfate-grown cells is incorporated from sulfate.

Competitive Utilization of Sulfur Compounds by Normal E. coli

Tables 6 and 7 summarize the results of a number of experiments in which growth and radiosulfate uptake have been investigated in relation to the form

TABLE 6

*Sulfur uptake in normal E. coli cells**

TUBE NO.	NONRADIOACTIVE SULFUR COMPOUNDS IN MEDIUM	CONCENTRATION OF NONRADIOACTIVE SULFUR COMPOUNDS IN MEDIUM (MG PER ML MEDIUM)	ML CELLS GROWN	Mg RADIOSULFUR PER ML OF CELLS	PER CENT OF TOTAL† SULFUR AS RADIO-SULFUR
1	Methionine	2.6	0.000	0.00	0
2		0.26	0.162	1.17	33
3		0.026	0.130	1.28	36
4		0.0026	0.128	2.62	73
5	Homocysteine	0.026	0.162	1.30	37
6		0.0026	0.126	2.54	74
7	Taurine	0.026	0.110	3.60	100
8	Cysteic acid	0.026	0.110	3.60	100
9	Na_2SO_3	2.6	0.000	0.00	0
10		0.26	0.080	0.00	0
11		0.026	0.096	0.09	0.25
12		0.0026	0.158	2.60	73
13	Cysteine	0.026	0.088	0.00	0
14		0.0026	0.110	2.60	73

* Sulfur (0.026 mg per ml medium) was present in each tube as $Na_2S^*O_4$.

† Control uptake ($Na_2S^*O_4$ the only sulfur source in the medium) was 3.6 mg of sulfur per ml of cells.

and amount of other sulfur compounds in M-9 medium. Table 6 shows that neither taurine nor cysteic acid suppresses sulfate uptake, 3.6 mg sulfate sulfur being found for each ml of cells grown. For the remaining sulfur compounds the amount of sulfate sulfur utilized depends upon the sulfur concentration ratio of the two forms supplied. Column 5 of table 6 shows the amount of sulfate sulfur actually taken up, and column 6 compares this uptake with that found for cells grown in media containing sulfate as the sole sulfur supply. The highest levels of methionine and Na_2SO_3 used completely inhibit growth and sulfate uptake. At the 0.026-mg level, where the nonsulfate sulfur is equal to the sulfate sulfur, Na_2SO_3 and cysteine nearly completely suppress sulfate uptake, whereas methionine and homocysteine allow about one-third of the sulfate uptake found for

sulfate-grown cells. For levels of nonsulfate sulfur one-tenth of the sulfate sulfur, radiosulfate uptake was more pronounced. It is evident that there is a preferential utilization of Na_2SO_3, cysteine, homocysteine, and methionine in the presence of sulfate.

Preferential utilization of some of the sulfur-containing amino acids is strikingly demonstrated by the data of table 7. Cells grown for 19 hours in M9 medium containing 0.0004 mg sulfur per ml as radiosulfate and varying amounts of nonradioactive sulfur amino acids were harvested and analyzed for radiosulfur. These determinations are shown in column 5 of table 7. It may be observed that the largest sulfate uptake occurred in the presence of methionine, and that sulfate uptake is greater for the lower methionine concentrations. Except for the lowest level of cystine used, there is practically no sulfate uptake in the presence of cystine or cysteine. For a 100-fold concentration change in the methionine level there is less than a 2-fold change in sulfate uptake, while for cystine a 100-fold concentration change causes a 5,000-fold change in sulfate uptake. Furthermore, 0.01 mg cystine per ml allows only one-seventh the sulfate uptake that occurs for 0.01 mg methionine per ml. It would appear, then, that with reference to sulfate uptake cystine and cysteine are preferred to homocysteine and methionine. Since all these amino acids and also Na_2SO_3 depress sulfate uptake to some degree, it is evident that competition among simultaneously administered sulfur substrates occurs. Thus, competition and preferential utilization of sulfur compounds, one of which is radioactive, provide a way for study of sulfate metabolism that is analogous to the use of biochemical mutants.

TABLE 7

*Trace sulfate uptake in normal E. coli cells**

TUBE NO.	AMINO ACID PRESENT IN MEDIUM	AMINO ACID SULFUR PER ML MEDIUM	ML CELLS GROWN	MICROGRAMS RADIOSULFUR PER ML CELLS
		mg		
1	Cystine†	1.00	0.141	0.01
2		0.10	0.105	0.01
3		0.01	0.072	46.1
4	Methionine	1.00	0.058	206
5		0.10	0.058	237
6		0.01	0.041	328
7	Cysteine	0.10	0.079	0.02
8	Homocysteine	0.10	0.100	119

* $Na_2S^*O_4$ present in each tube at a concentration of 0.0004 mg sulfur per ml medium.

† This quantity of cystine was added to the culture tube as a powder, since cystine is highly insoluble.

DISCUSSION AND CONCLUSIONS

The experiments reported in this paper have demonstrated that growth of *Escherichia coli* may be controlled by altering the quantities of inorganic sulfur present in the synthetic media employed. Growth per se, however, is no simple

guide to the mechanism by which sulfur exerts its control of growth and provides for the reproduction of cells. Consequently it has been necessary to study sulfur uptake in relation to growth and reproduction and to the presence of organic sulfur compounds in the media as well as to the amounts of sulfur present. For this purpose sulfur as Na_2SO_4 has been employed since almost invariably sulfate serves as a satisfactory sulfur source for microorganisms.

The results shown in figure 1 permit the choice of an adequate sulfate medium for these investigations. These studies show that in an adequate medium sulfate uptake is directly proportional to growth and nitrogen content (figures 2 and 4). It is necessary, however, as the results given in tables 1 and 2 show, to distinguish between the bound and unbound sulfur of the cell. With this in mind it is possible to estimate the total sulfur retained per ml of cells grown in an adequate sulfate medium, 3.5 mg of sulfur per ml of cells being bound when sulfate was the sole sulfur source in the medium.

For sulfur-deficient media (figures 1 and 3) reduced growth and reduced sulfur content per ml of cells are observed. Figure 4 demonstrates that sulfur-starved cells take up additional sulfur from an adequate sulfur medium only in the presence of an energy source such as glucose. The early incorporation of sulfur and nitrogen by sulfur-starved cells proceeds at a rate in excess of that found for cells grown entirely in adequate sulfate media. This is indicative of sulfur utilization through (a) the usual uptake by new cells immediately produced, and (b) the simultaneous fulfillment in the starved cells of the uncompleted requirements necessary for optimal growth.

Bound radiosulfur undergoes exchange to a very limited degree with the nonradioactive compounds in the media (figure 5; tables 4, 5, and 6). These exchange experiments suggest that as protoplasm is produced and new cells are grown, the dividing cells pass on intact many of the sulfur compounds originally synthesized by the parent cells. These findings support the hypothesis that sulfate utilization serves as a measure of protein synthesis in *E. coli* when grown in sulfate medium (Block and Stekol, 1950).

The use of mutant *E. coli* cells in the study of sulfate metabolism coupled with the radioactive tracer techniques described above demonstrates that the methionine- and homocysteine-requiring mutants utilize inorganic sulfate supplied to the medium, whereas cystine-requiring mutants do not. The results of these experiments, when compared with the sulfate uptake by the normal strain B of *E. coli* in the presence of sulfur amino acids in the medium, show that sulfate metabolism of mutant and normal cells is qualitatively similar. Thus, the use of a labeled metabolite in the presence of a nonlabeled metabolite during the course of metabolism by either mutant or normal *E. coli* yields additional information as to the biochemical significance of the compounds investigated. The studies of sulfur metabolism of microorganisms by Lampen *et al.* (1947), Hockenhull (1949), Horowitz (1947), and Simmonds (1948) have suggested possible synthetic reactions involving sulfur. The methods used in the present investigations have provided several new facts and may confirm certain results previously reported: in particular, (a) methionine- and homocysteine-requiring mutants utilize inorganic sulfate; (b) no sulfate-derived bound sulfur could be

detected in cystine-requiring mutants; and (c) for normal cells, the quantity of sulfate-derived sulfur when cells are grown in media containing equal amounts of sulfur as sulfate and methionine, homocysteine, or cystine is approximately the same as that found for mutants *requiring* the amino acid used; and (d) Na_2SO_3 and cysteine as well as cystine suppress almost entirely the utilization of sulfate sulfur. Preliminary paper chromatographic analyses of cell hydrolyzates, to be reported, reveal that normal cells grown on methionine and radiosulfate do not contain radiomethionine, whereas radiomethionine is found in cell hydrolyzates from *E. coli* grown on sulfate as the sole sulfur source.

From the facts presented it is concluded:

(1) The cell membrane of *E. coli* is highly permeable to sulfate ions.

(2) Sulfate uptake is directly proportional to growth.

(3) An energy source, such as glucose, is necessary for the binding of sulfate sulfur.

(4) In adequate sulfur medium the sulfur content per ml of *E. coli* cells, strain B, is approximately 3.5 mg and may be considerably less for cells grown in sulfur-deficient media.

(5) Exchange of bound sulfur with environmental sulfur during growth is very low, being of the order of 0.5 per cent per hour.

(6) Although these results may confirm some of the hypotheses regarding the biosynthesis of sulfur by microorganisms, it is emphasized that the total sulfur content of *E. coli* has been measured only for media in which sulfate provides the sole sulfur source. The total sulfur uptake and sulfur compounds formed may be quite different in media containing additional sulfur sources.

(7) The application of biochemical mutants, radioactivity, competitive utilization of metabolites, and chromatography in the present studies indicates the usefulness of a combination of methods for the study of sulfate metabolism.

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