SULFUR ISOTOPE FRACTIONATION AND KINETIC STUDIES OF SULFITE REDUCTION IN GROWING CELLS OF *SALMONELLA HEIDELBERG*

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ABSTRACT A pulsed feeding technique was used during studies of sulfite reduction by *Salmonella heidelberg* in order to realize large percentages of $SO_3^=$ conversion while simultaneously maintaining a reasonably stable cell population. As a consequence, much data for conventional kinetic and sulfur isotope fractionation computations were obtained in any one experiment. Under the conditions of supplying 150 μg glucose per ml of medium every 6 hr, anaerobiosis, and varying the $SO_3^=$ concentration, the following observations were made: 1. Below 0.01% w/v Na_2SO_3, the reduction strictly followed first order kinetics with respect to $SO_3^=$ concentration. At higher concentrations, the rate of $SO_3^=$ reduction fell below that predicted by first order kinetics suggesting that a saturation effect was occurring. 2. At lower concentrations, the ratio of the isotopic rate constants k_1/k_2 was 1.02 whereas at higher $SO_3^=$ levels, k_1/k_2 values of 1.04 were found. These latter effects are much higher than those obtained in the equivalent chemical reduction. On the basis of these observations, a model is considered which features two isotopically dependent steps and an intermediate reservoir which forms at higher $SO_3^=$ concentrations. Results of an experiment under aerobic conditions and an experiment wherein the reduction rate was thermally altered, are also presented.

INTRODUCTION

Krouse et al. (1) recently demonstrated that several species of *Salmonella* are capable of significantly altering the $^{34}S/^{32}S$ abundance ratio during $SO_3^=$ reduction to H_2S. It was further noted that the attending iostope effects were much larger than those associated with the inorganic chemical reduction of $SO_3^=$, (4).

In these preliminary studies, the media components were not replenished throughout the experiments. Subsequent attempts to derive more exact information from this procedure were not successful because of the following factors:

1. The cell population did not stabilize during any one run.

2. The H_2S production per unit cell varied over a large range in any one experiment.

3. The ratio of the isotopic rate constants, k_1/k_2, was not constant throughout any individual reduction.

Some of the fluctuations were possibly due to the different carbon sources present in the medium (Trypticase Soy Broth, Baltimore Biological Laboratories, Ltd., Baltimore, Md.). In addition, there appeared to be a superimposed toxicity effect of the $SO_3^=$.

Therefore, the procedure was altered so as to introduce a single energy source (glucose) into the medium at regular time intervals during the reduction. This pulse feeding resulted in marked stabilization of the cell population, H_2S production rate, and the accompanying isotope effects. Furthermore, large percentages of conversion of $SO_3^=$ to H_2S were easily realized. As a result, more quantitative relationships between the kinetics and the isotope fractionation were obtainable.

Theory

Bigeleisen (2) has developed from statistical mechanics and Eyring's reaction rate theory (3) a theoretical expression for the ratio of isotopic rate constants in chemical conversions. In our case, this ratio is designated k_1/k_2 where

$$^{32}SO_3^= \xrightarrow{k_1} H_2{}^{32}S$$

$$^{34}SO_3^= \xrightarrow{k_2} H_2{}^{34}S.$$

A simple argument suggests that $k_1/k_2 > 1$. The lighter isotopic species have higher vibrational frequencies than the heavier ^{34}S species. As a result, the more energetic ^{32}S bonds tend to rupture more readily in chemical reactions.

Quantitative agreement between the kinetic isotopic effects predicted by Bigeleisen's expression and those measured experimentally is hampered by the lack of information concerning the "activated complex" of Eyring's theory. Nevertheless, comparisons of theoretical and experimental isotope fractionations have elucidated many chemical mechanisms.

Harrison and Thode (4) found k_1/k_2 to be 1.022 at room temperature in the case of inorganic chemical reduction. The experimental temperature variation of this ratio was negligible up to 100°C. This kinetic isotope effect was identified with the initial S-O bond rupture during the reduction. This interpretation is further verified by the fact that k_1/k_2 for both $SO_4^=$ and $SO_3^=$ reduction are essentially the same.

If only a small percentage conversion (<5%) is carried out, k_1/k_2 can be approximated by:

$$\frac{k_1}{k_2} \approx \frac{(^{32}S/^{34}S)\ \text{Product}\ H_2S}{(^{32}S/^{34}S)\ \text{Initial}\ SO_3}.$$

For larger percentage conversions, the unreacted $SO_3^=$ becomes increasingly enriched in ^{34}S owing to preferential loss of ^{32}S during reduction.

In the case of first order kinetics, an expression for calculating k_1/k_2 for any percentage reaction can be derived as follows: Let A and X represent the $SO_3^=$ and H_2S concentrations respectively, at any time. Then the two competing isotopic reactions may be written:

$$^{32}A + \_\_\_\_ \xrightarrow{k_1} {}^{32}X + \_\_\_\_$$

$$^{34}A + \_\_\_\_ \xrightarrow{k_2} {}^{34}X + \_\_\_\_.$$

For a reaction, which is first order with respect to $SO_3^=$ concentration:

$$\frac{d^{32}X}{dt} = k_1[(^{32}A)_0 - {}^{32}X][\text{——}]$$

$$\frac{d^{34}X}{dt} = k_2[(^{34}A)_0 - {}^{34}X][\text{——}]$$

where $(^{32}A)_0$ and $(^{34}A)_0$ represent the concentration of $^{32}SO_3^=$ and $^{34}SO_3^=$ respectively at zero time. $^{32}X = {}^{34}X = 0$ at time $t = 0$. Therefore integration and division yields:

$$\frac{k_1}{k_2} = \frac{\ln\left[\dfrac{(^{32}A)_0}{(^{32}A)_0 - {}^{32}X}\right]}{\ln\left[\dfrac{(^{34}A)_0}{(^{34}A)_0 - {}^{34}X}\right]}.$$

If the fraction of molecules which have reacted is designated as

$$f = \frac{^{34}X + {}^{34}X}{(^{32}A)_0 + (^{34}A)_0}$$

and the ratio $r = \dfrac{^{34}X/{}^{32}X}{(^{34}A)_0/(^{32}A)_0}$,

then

$$\frac{k_1}{k_2} = \frac{\ln\left[1 - f \cdot \dfrac{1 + (^{34}A/{}^{32}A)_0}{1 + (^{34}X/{}^{32}X)}\right]}{\ln\left[1 - rf \cdot \dfrac{1 + (^{34}A/{}^{32}A)_0}{1 + (^{34}X/{}^{32}X)}\right]}.$$

Since natural ^{34}S is approximately 4% abundant compared to an abundance of ^{32}S of 95%, the approximation $\dfrac{1 + (^{34}A/{}^{32}A)_0}{1 + (^{34}X/{}^{32}X)} \approx 1$

introduces little error so that

$$\frac{k_1}{k_2} \approx \frac{\ln (1 - f)}{\ln (1 - rf)} \tag{1}$$

The value of "f" is obtained by measuring the amount of S in the product at any time and dividing by the initial S concentration. "r" is the $^{34}S/^{32}S$ composition of the product at any time divided by the $^{34}S/^{32}S$ ratio for the initial $SO_3^=$. This ratio is determined mass spectrometrically. Whereas the accuracy in determining absolute $^{34}S/^{32}S$ ratios is about 1%, two specimens can be compared isotopically with much better precision. Therefore r can be measured with a standard deviation of ($\pm$) 0.2% or better. This represents the smallest source of error in the experiment.

Equation 1 is only valid for first order kinetics. Since f and r are ratios, k_1/k_2 is not

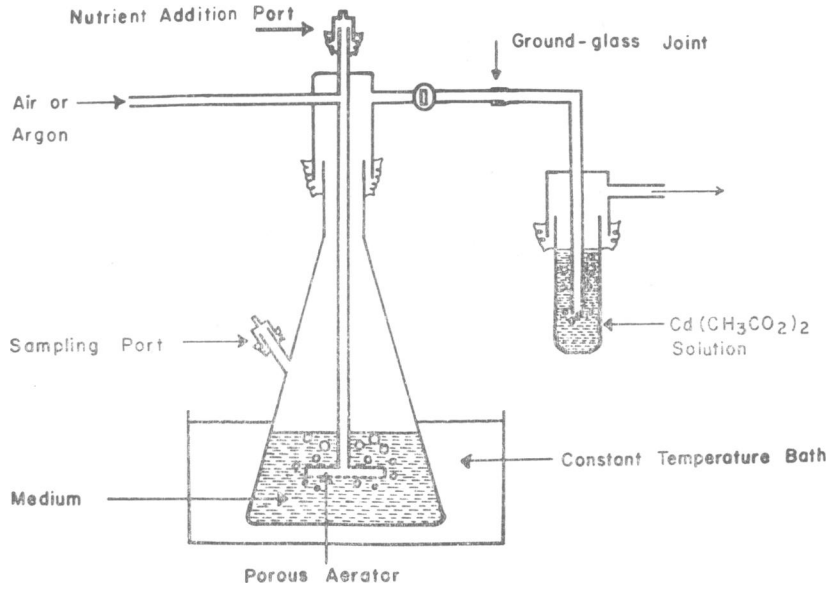

FIGURE 1 Sulfite reduction vessel.

dependent upon the reactant concentration. This is a characteristic of reactions which exhibit first order kinetics with respect to the reactant containing the isotopes of interest. Thus, it is seen that measurements of k_1/k_2 can complement more conventional studies in determining reaction order. This has been demonstrated in this study.

The standard method of expressing relative $^{34}S/^{32}S$ abundances is in terms of a δ_{34} scale defined as follows:

$$\delta_{34} = \left[\frac{(^{34}S/^{32}S) \text{ sample}}{(^{34}S/^{32}S) \text{ standard}} - 1 \right] \times 1000 \tag{2}$$

In our studies, the standard was the initial $SO_3^=$ while the samples comprised H_2S product fractions and unreacted $SO_3^=$ reservoir at selected reaction times.

EXPERIMENTAL

The reaction vessel, a modification of that described previously (1) to facilitate nutrient addition, without loss of evolved gas, is shown in Fig. 1. Sterile nutrient solution was added via the extension tube and the sweeping gas (argon or air) pushed the nutrient solution through the aerator for immediate dispersal in the medium.

Sterile Na_2SO_3 solution was added aseptically to 1 liter of autoclaved Trypticase Soy Broth (Baltimore Biological Laboratories, Ltd.) to give final concentrations ranging from 0.0025 to 0.1% w/v Na_2SO_3. The media were inoculated with 1 ml of an 18 hr culture of *Salmonella heidelberg* grown in Trypticase Soy broth and incubated at 37°C.

Sterile glucose was added every 6 hr to yield a final concentration of 150 μg/ml (8.32×10^{-4} M).

Viable cell population was measured by doing plate counts of aliquots of the culture sampled at the designated times.

The product H_2S was swept continuously from the reaction vessel with either argon (anaerobic conditions) or air (aerobic conditions) which had been passed through a bacteriological filter. Blank determinations showed that the sulfide contamination from all sources (flushing gases and medium) would not exceed 1% of the total sulfide collected at the lowest concentration studied. The trapping of the H_2S and mass spectrometric procedures were described previously (1). In any one run, several H_2S product fractions were collected over chosen time intervals for kinetic studies and isotopic analyses. Data for conventional kinetic calculations were obtained by weighing Ag_2S quantitatively prepared from the H_2S fractions (1).

RESULTS AND DISCUSSION

Growth Determinations

A typical curve of viable cells vs. time under the conditions of pulse feeding glucose is shown in Fig. 2. The cell population, for example, peaks at about 3.2×10^3 cells/ml and drops rapidly to a relatively stable level of 1.75×10^9 cells per ml (5.5% of peak level) under anaerobic conditions. In previous experiments, where glucose was not added, the population steadily decreased with time after the initial peaking. Therefore, the glucose was utilized by the organism with the result that a reasonably stabilized population existed for the major part of any one reduction. The population peaking has two possible explanations.

1. A primary energy source was utilized and depleted in the Trypticase Soy Broth. Obviously, the ability of the organism to utilize alternate energy sources in the medium, as well as depletion of other essential metabolites with time, introduce immeasurable variations under these conditions (6).

2. The rapid decline of the population to the plateau may have been contributed to by a toxic effect of the high initial $SO_3^=$ concentration. Kaplan (5) has stated that metabolic intermediates, thus preventing their further metabolism.

In reality, the population has a "saw-tooth" dependence with time as a consequence of the pulse feeding. The positive results, obtained under these crude conditions for stabilizing cells, support the validity of refinement to obtain a fully stabilized culture more rigidly controlled under chemostat conditions. This is currently being attempted.

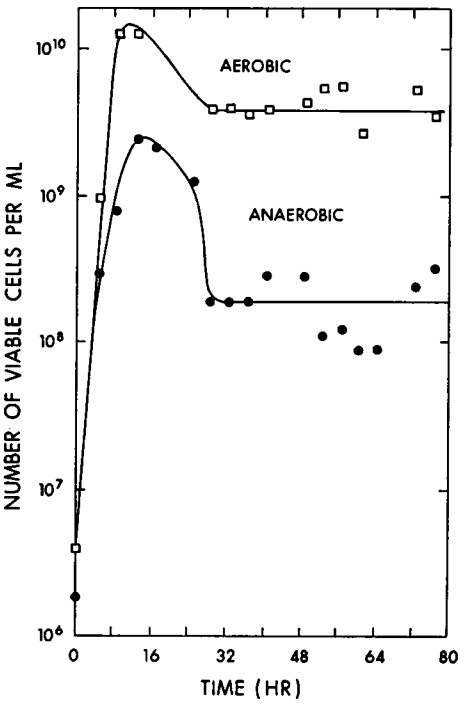

FIGURE 2 Growth of *S. heidelberg* under aerobic and anaerobic conditions. Growth conditions: Trypticase Soy Broth + 0.1% w/v Na_2SO_3 + 150 μg glucose per ml of medium every 6 hr. Incubation temperature 37°C.

Kinetics and Isotope Effects during Anaerobic Reduction

In Table I, the reaction kinetics and attending isotope effects are tabulated for some representative experiments in the concentration range 0.005 to 0.1 % w/v Na_2SO_3. In all cases, glucose was added every 6 hr. Therefore, it is emphasized that all observations and interpretations below apply to conditions wherein the energy source is periodically replenished and should not be generalized to results obtained from batch cultures where the energy source is being continuously depleted.

For $SO_3^=$ concentrations less than 0.01 %, the systems strictly obeyed first order kinetics with respect to $SO_3^=$ concentration. This is shown in Fig. 3 where the plot of $\ln \frac{[SO_3^=]_0}{[SO_3^=]_t}$ vs. time yields straight lines of the same slope for the three lowest concentrations. Therefore, in this region, the kinetics obey the rate law $R = k[SO_3^=]$, where R is the rate and k (the slope of the plot in Fig. 3) is the rate constant and has the value 0.05 hr^{-1}.

The behavior at concentrations above 0.01 % w/v Na_2SO_3 is shown in Fig. 4. It is remarkable that the $\ln \frac{[SO_3^=]_0}{[SO_3^=]_t}$ vs. time plots yield straight lines at all concentrations. Although this is consistent with first order kinetics, k steadily decreases with increasing concentration. Therefore the total system does not obey the fundamental requirement for first order kinetics that $R = k[SO_3^=]$ where k is constant. In fact as

shown in Fig. 5, the fractional change in R as a function of the fractional change in concentration, is comparatively small at higher concentrations.

The results suggest that the rate is determined by $SO_3^=$ concentration below a

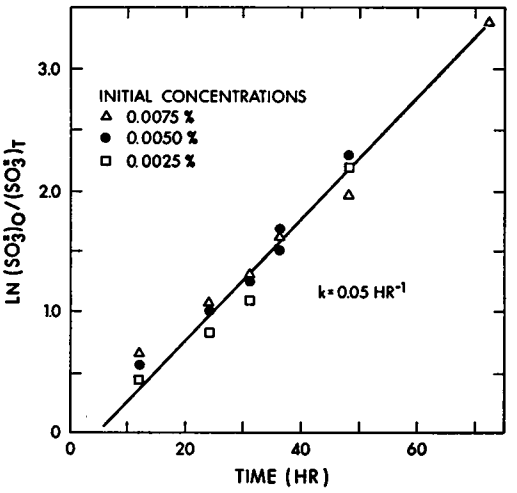

FIGURE 3 Plot of ln $[SO_3^=]_0/[SO_3^=]_t$ vs time for an initial concentration of $Na_2SO_3 \leq 0.0075\%$ w/v. Growth conditions: anaerobic, as described in Fig. 2.

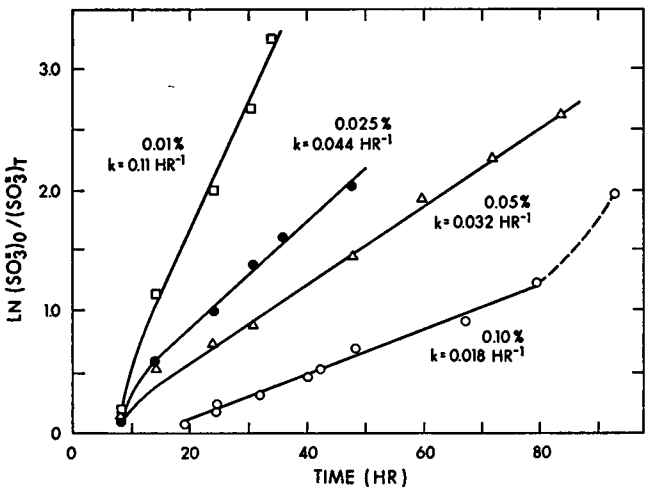

FIGURE 4 Plot of ln $[SO_3^=]_0/[SO_3^=]_t$ vs. time for initial concentrations of $Na_2SO_3 \geq 0.01\%$ Na_2SO_3 w/v. Growth conditions: as described in Fig. 3.

level of 0.01 % w/v whereas at higher concentrations, the rate is determined by a factor which is saturable. This saturation may represent an individual cell limitation and thus permit a measure of the maximum reduction rate of which a cell is capable under the experimental conditions. There is also the possibility that there is a toxicity phenomenon at these higher concentrations.

TABLE I

KINETICS AND SULFUR ISOTOPE FRACTIONATION DURING ANAEROBIC REDUCTION OF $SO_4^=$ BY GROWING CELLS OF *SALMONELLA HEIDELBERG**

Initial SO_4^- concentration (w/v)	Sample No.	Growth time	Column No. 1	2	3	4	5	6	7
			% Reaction	$\ln\frac{[SO_4^-]_0}{[SO_4^-]_t}$	δ_{34} (H_2S fraction)	δ_{34} (integrated product)	δ_{34} (reservoir)	k_1/k_2 (fraction)	k_1/k_2 (average)
		hr							
0.1%	1	24	20.04	0.2231	−27.4	−27.4	+6.9	1.032	1.032
	2	42	40.51	0.5105	−29.0	−28.2	+19.2	1.044	1.039
	3	48	49.85	0.6729	−20.0	−26.6	+26.5	1.044	1.039
	4	67	61.82	0.9163	−5.3	−22.5	+36.4	1.038	1.039
	5	79	73.04	1.2038	+11.7	−17.3	+46.9	1.031	1.037
	6	93	91.56	1.9952	+64.4	−0.8			
0.05%	1	8	3.0	0.029	−31.5	−31.5	+1.0	1.033	1.033
	2	14	37.8	0.474	−27.2	−27.5	+16.7	1.037	1.037
	3	24	47.8	0.649	−30.3	−28.1	+25.7	1.053	1.042
	4	31	55.0	0.798	−11.6	−26.0	+31.7	1.042	1.042
	5	48	77.3	1.484					
	6	60	86.3	1.990					
	7	72	91.1	2.410	+82.3				
	8	84	92.5	2.593					
0.025%	1	8	12.8	0.136	−32.1	−32.1	+4.7	1.036	1.036
	2	14	43.7	0.575	−26.6	−28.2	+21.9	1.041	1.040
	3	24	61.9	1.110					
	4	31	73.9	1.343	+0.7				(1.040)
	5	48	86.2	1.981	+96.0				(1.050)
0.01%	1	8	16.5	0.178					
	2	14	67.4	1.119	−9.9				(1.025)
	3	24	85.8	1.956	+22.1				(1.03)
	4	30	93.4	2.712					
0.0075%	1	12	52.2	0.738	−13.2	−13.2	+14.4	1.020	1.020
	2	24	70.3	1.214					
	3	31	78.1	1.518					
	4	36	84.2	1.845					
	5	48	90.0	2.303					
	6	72	97.0	3.506					
0.005%	1	12	43.1	0.563	−13.2	−13.2	+9.9	1.019	1.019
	2	24	63.7	1.013					
	3	31	72.4	1.285					
	4	36	83.0	1.766					
	5	48	90.2	2.394					
	6	82	100						
0.0025%	1	12	36.0	0.445					
	2	24	56.1	0.824					
	3	31	66.5	1.095					
	4	36	80.4	1.631					
	5	48	88.8	2.195					
	6	72	99.9	6.91					

* Growth medium: Trypticase Soy Broth, 150 μg/ml glucose added every 6 hr. Incubation temperature 37°C.

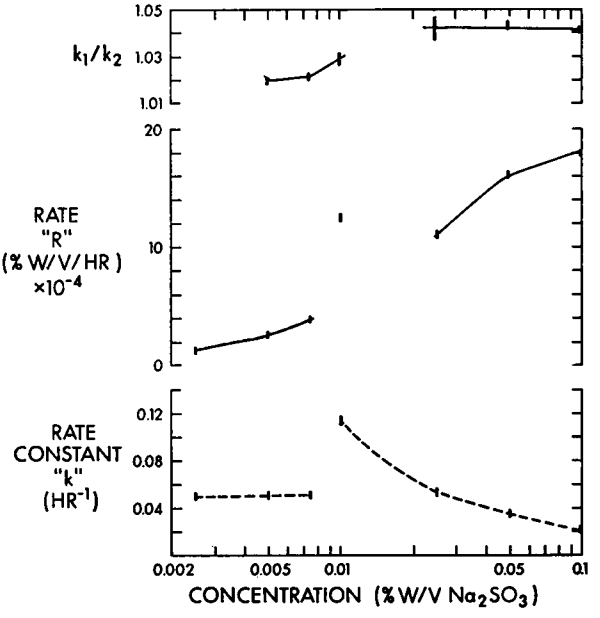

FIGURE 5 Effect of varying initial Na_2SO_3 concentrations on rate, rate constant, and k_1/k_2 values.

Columns 1, 2, 3, 4, and 5 of Table I are now considered and the isotope effects evaluated.

The δ_{34} (H_2S fractions) column, i.e. column 3, tabulates the isotopic composition of the H_2S collected over selected time intervals. For example, in the 0.1% w/v Na_2SO_3 study, $\delta = -29.0$ is the average composition of the H_2S collected over the time interval from 24–42 hr. This time interval is equivalent to the reaction interval 20.04 to 40.51%. It is seen that δ_{34} (H_2S fractions) (column 3) becomes increasingly positive as the reaction nears completion. This occurs because, as the reaction proceeds, the $SO_3^=$ reservoir becomes increasingly depleted in ^{32}S. This results from the spontaneous H_2S product being consistently enriched in ^{32}S over the reservoir $SO_3^=$ by the factor k_1/k_2.

δ_{34} (integrated product) (column 4) is the average isotopic composition of all the H_2S which has been formed at any point in the reaction. In terms of the δ–value of the H_2S fractions, it is given by the equation:

$$\delta_{34}(\text{integrated}) = \frac{\sum_i \delta_{34}(\text{fraction } "i") \times \text{mass } i}{\text{Total mass of product}} .$$

δ_{34} (integrated) becomes zero as the reaction nears completion; i.e., if the reaction is 100%, the total product must have the same isotopic composition as the initial reactant.

Columns 6 and 7 tabulate k_1/k_2 values which were calculated using I.B.M. computer programming of the exact expression for Equation 1 (see *Theory* section). Two sets of values were computed. One is based on δ_{34} (H_2S fractions) (column 3) and gives the average k_1/k_2 for the particular fraction collected. In column 7, the k_1/k_2 values are derived from δ_{34} (integrated product) (column 4). This gives the average k_1/k_2 of the total product at designated points in the reaction.

In the instances where the k_1/k_2 values are in brackets, insufficient data were obtained to permit precise calculations. However, Equation 1 was plotted for several k_1/k_2 values as a function of % reaction. Experimental values of δ_{34} (H_2S fractions) were geometrically fitted to the curves and k_1/k_2 estimated.

In many microbiological iostope fractionation studies, the term "fractionation factor" has been used to designate the relative isotopic compositions of the total product and the remaining reactant. This corresponds to $({}^{34}X/{}^{32}X)/({}^{34}A/{}^{32}A)$ as defined under *Theory*. Nakai and Jensen (7) have shown that for first order reactions,

$$\text{Fractionation factor} = \frac{{}^{34}X/{}^{32}X}{{}^{34}A/{}^{32}A} = \frac{F^{(k_2/k_1)-1} - F}{1 - F} \tag{3}$$

where $F = \dfrac{[SO_3^=]_0}{[SO_3^=]_t}$.

The fractionation factor corresponds to the isotopic difference between δ_{34} (integrated product) and δ_{34} (reservoir) of Table I. This factor has exceeded 1.3 in many of our experiments since the reactions have been carried very near to completion. As Equation 3 shows, the fractionation factor varies from k_1/k_2 at 0 reaction to ∞ at 100% reaction. Therefore, this quantity is not suitable for intercomparing laboratory reactions. In our studies, experimental values of this factor are available and Eq. 3 could have been used to evaluate k_1/k_2. This calculation is not independent of Equation 1, so that this procedure would give the same numerical results.

Data from one run, tabulated in Table I are plotted for illustrative purposes in Fig. 6.

Under anaerobic conditions, it was found that the initial H_2S fractions sampled possessed k_1/k_2 values which were lower than those for the bulk of the reduction. These fractions were produced while the cell population was going through the "peaking," i.e. becoming stabilized, as previously described. After population stabilization, the k_1/k_2 values were markedly constant over large portions of any one reaction. Therefore, it is obvious that the maximum k_1/k_2 is not realized in experiments where the medium is simply inoculated and the products collected. It is necessary to have a stabilized population to realize representative k_1/k_2 values.

It was also routinely found in the present study that k_1/k_2 values were in excess of those measured by Harrison and Thode (4) for the equivalent inorganic reduction. Kaplan and Rittenberg (8) previously reported similarly high isotope fractionations

in $SO_3^=$ reduction by *Saccharomyces cerevisiae*, and in $SO_4^=$ reduction by *Desulfovibrio desulfuricans*. Various proposals have been made to account for these higher fractionations (8, 9). If S—O bond rupture were the only isotopically dependent step, then k_1/k_2 should not exceed that of the inorganic reduction as discussed by Harrison and Thode (10). Therefore, it is clear that there are other isotopically dependent steps in the process. The following model can adequately describe the observations of the present study.

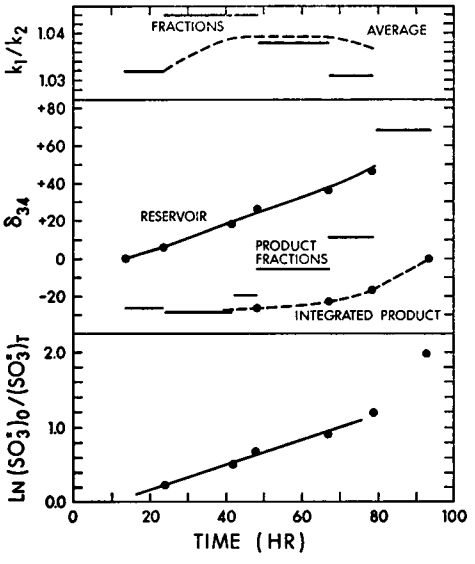

FIGURE 6 Kinetics and isotope effects under anaerobic conditions. Growth conditions as described in Fig. 2, initial $SO_3^=$ concentration = 0.1% w/v.

$$\text{SUBSTRATE} \xrightarrow{k_{1A}} \text{INTERMEDIATE RESERVOIR} \xrightarrow{k_{1B}} \text{PRODUCT}$$
$$\text{SUBSTRATE} \xrightarrow{k_{2A}} \text{INTERMEDIATE RESERVOIR} \xrightarrow{k_{2B}} \text{PRODUCT}$$

Step "A" (Isotopically dependent)

Step "B" (Isotopically dependent and saturable)

Both *Steps A* and *B* are isotopically dependent. *A* may be $SO_3^=$ concentration dependent while *B* is physically limited to produce saturation effects. At low concentrations, *B* can pace *A* so that no reservoir forms. At higher concentrations *Step B* cannot keep up with *Step A* and a reservoir forms.

The extent to which this model accounts for the present results will be considered.

1. The behavior of the kinetics as a function of concentration. At low concentrations, *Step* A is rate controlling and the system obeys first order kinetics. With increase of concentration, the saturable *Step B* causes the kinetics to fall below those of first order.

2. The behavior of k_1/k_2 as a function of concentration. At low concentrations, where *B* paces *A*, the net k_1/k_2 would be equal to k_{1A}/k_{2A}. At higher concentrations where *Step B* cannot keep up with *Step A*, a reservoir forms and the net k_1/k_2[1] may have the upper limit equal $(k_{1A}/k_{2A}) \times (k_{1B}/k_{2B})$.

3. The change of k_1/k_2 throughout any one run. The k_1/k_2 values near the beginning of any one reduction were lower than for the bulk of the conversion. These low k_1/k_2 values also corresponded to the population peaking region. Therefore it would seem that initially the reservoir was not present. It then achieved significant size as a function of time and decrease in cell population. As a result, k_1/k_2 steadily increased.

4. Deviations of the isotopic composition from predicted values. Although both the conventional kinetic data and k_1/k_2 behavior produce linear plots for most of the experiments, deviations appear as the reactions approach completion. Some H_2S fractions (e.g. No. 6 of 0.1% and No. 7 of 0.05% concentrations) are isotopically heavier than predicted by Equation 1. This effect may be described as follows: As the reduction proceeds and the $SO_3^=$ concentration lowers, *Step B* is again able to pace *Step A* and the reservoir starts to deplete. During the course of the reaction, the precursor of *Step A* has become very enriched in ^{34}S. The reservoir is also very enriched in ^{34}S because of the isotopic selectivity of *Step B*. The resultant product will reflect both these enrichments.

If the reservoir concept is correct, it is interesting to postulate its character as well as those of *Steps A* and *B*. Two points are evident.

1. In the case of higher $SO_3^=$ concentrations, lower k_1/k_2 values resulted near the beginning of the reaction when the cell population was much higher than for the remainder of the experiment.

2. Lower k_1/k_2 values resulted from reductions at lower $SO_3^=$ concentrations.

Both these observations suggest that the reservoir is a property of the individual cell. This may also partially explain the differences in isotope fractionation realized with different species in the prior study (1). The cells of each species have characteristic limitations with respect to $SO_3^=$ reduction and these are dependent on *Steps A* and *B*, and reservoir capacity.

Two consecutive S—O bond ruptures would be consistent with the observed k_1/k_2 values. At lower concentrations, $k_1/k_2 = k_{1A}/k_{2A} = 1.020$ while at higher concentrations k_1/k_2 is $(1.020)^2$. Presently accepted mechanisms for $SO_4^=$ and $SO_3^=$ reduction, however, picture the steps after the initial S—O bond breakage as being comparatively rapid. Therefore the double S—O isotope effect postulation is unreasonable physically.

Another interesting consideration is that either *Step A* or *B* may be a physical process. For example, *Step A* might correspond to diffusion while *B* corresponds to bond rupture. The lighter isotope would diffuse faster through a membrane.

[1] Many workers prefer to reserve the term "ratio of isotopic rate constants" for simple one step processes. Therefore, at higher concentrations, it might be desirable to replace k_1/k_2 by a quantity termed "the instantaneous isotopic fractionation factor" defined as the ratio of the $^{32}S/^{34}S$ composition of the H_2S produced at any instant to the $^{32}S/^{34}S$ composition of the reservoir at that instant.

On the other hand, it is probable that the net result of more than one biological (i.e., enzymatic) process is in fact being measured in these experiments with whole cells. The data from studies in $SeO_3^=$ reduction by *Salmonella* show that reduction occurs intracellularly (11). If, as appears likely, this is also true for $SO_3^=$ reduction, then at least two steps are essential at which isotope fractionation could occur: (*i*) transport of $SO_3^=$ across the membrane and (*ii*) intracellular reduction to H_2S. Studies now underway, of isotope effects using the sulfite reductase isolated from these organisms and comparing the results with those from whole cells should permit an estimation of the contribution of the transport mechanism to the over-all isotope effect.

It is obvious that further studies are needed to clarify the processes and to evaluate the suggested model for $SO_3^=$ reduction. One requirement is an extensive computer analysis to investigate the behavior of k_1/k_2 as a function of reservoir size and various rates for *Steps A* and *B*. ^{35}S tracer experiments would also be useful in checking the physical validity of this model.

Kinetics and Isotope Fractionation during an Aerobic Reduction

Under aerobic conditions, the stabilized population was a factor of roughly 50 times higher than under anaerobic conditions. The H_2S production rate, however, was much lower in these aerobic experiments (Table II, Fig. 7). The largest contributing factor seems to be that O_2 is used by this organism and in fact is preferred to $SO_3^=$. Other possible reasons for the decrease in H_2S production are:

1. Some of the product H_2S may have been reoxidized by the oxygen.
2. Some of the $SO_3^=$ may have been oxidized to $SO_4^=$. Previous studies revealed that $SO_4^=$ is not reduced to H_2S by this organism.

If the $SO_3^=$ concentration were decreased sufficiently by oxidation to $SO_4^=$, then lower k_1/k_2 valus should arise as discussed above. Consistent with this concept is the observation that k_1/k_2 decreased with time in aerobic studies (Fig. 7). Unfortunately, the extent of $SO_3^=$ oxidation was not determined as a function of time in order to verify this possibility. It can only be concluded that oxygen plays a role in altering the k_1/k_2 ratio in the system. Studies for exact interpretations will require extensive time because of the slow H_2S production rate.

Relationship between k_1/k_2 and Reduction Rate

In studies involving $SO_4^=$ reduction, several workers (8–10, 12) have demonstrated that k_1/k_2 varies inversely as the rate of reduction. In these studies, the relationship appeared to be independent of the method used for rate alteration.

In our studies, over 100 k_1/k_2 values were plotted against the $SO_3^=$ reduction rate per unit cell and no consistent pattern was found. Certainly, rate alterations due to varying the $SO_3^=$ concentrations or O_2 pressure do not reveal the simple inverse relationship.

TABLE II

KINETICS AND SULFUR ISOTOPE FRACTIONATION DURING AEROBIC REDUCTION OF $SO_3^=$ BY GROWING CELLS OF *SALMONELLA HEIDELBERG**

Sample No.	Growth time	Column No. 1	2	3	4	5	6	7
		% Reaction	$\ln \frac{[SO_3^=]_0}{SO_3^=]_t}$	δ_{34} (H_2S fraction)	δ_{34} (integrated product)	δ_{34} (reservoir)	k_1/k_2 (fraction)	k_1/k_2 (average)
	hr							
1	10	3.61	0.0382	−38.7	−38.7	+1.4	1.041	1.041
2	18	4.91	0.0507	−34.5	−37.6	+1.9	1.036	1.040
3	24	5.31	0.0535	−31.2	−37.1	+2.1	1.033	1.040
4	44	8.97	0.0944	−34.9	−31.7	+3.1	1.025	1.035
5	68	10.42	0.1096	−12.2	−29.0	+3.4	1.013	1.032

* Growth medium: Trypticase Soy Broth, 150 μg/ml glucose added every 6 hr. Incubation temperature 37°C, initial $SO_3^=$ concentration 0.1% w/v Na_2SO_3 .

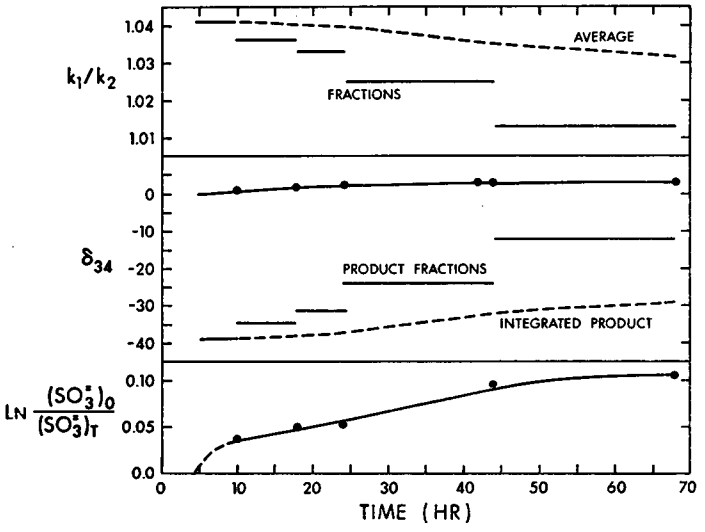

FIGURE 7 Kinetics and isotope effects under aerobic conditions. Growth conditions as described in Fig. 3, initial $SO_3^=$ concentration = 0.1% w/v.

In more specific cases, however, this inverse relationship can be demonstrated as shown in Fig. 8 and Table III. It is seen that at the point where the reduction rate was lowered (by decreasing the temperature), k_1/k_2 increased to 1.042 from its previous value of 1.035.

It does not seem reasonable that the relationship $k_1/k_2 \alpha 1/R$ should be a general one. The rate R depends on a number of factors, of which some are $SO_3^=$ dependent

TABLE III

KINETICS AND SULFUR ISOTOPE FRACTIONATION DURING ANAEROBIC REDUCTION OF $SO_3^=$ BY GROWING CELLS OF *SALMONELLA HEIDELBERG** WHERE REDUCTION RATE WAS THERMALLY ALTERED

Sample No.	Growth time	Column No. 1	2	3	4	5	6	7
		% Reaction	$\ln \frac{[SO_3^=]_0}{[SO_3^=]_t}$	δ_{34} (H_2S fraction)	δ_{34} (integrated product)	δ_{34} (reservoir)	k_1/k_2 (fraction)	k_1/k_2 (average)
	hr							
1	19	6.61	0.0677	−22.9	−22.9	+ 1.6	1.024	1.024
2	24	12.22	0.1301	−26.8	−24.7	+ 3.4	1.029	1.027
3	32	28.25	0.3308	−26.8	−25.9	+10.2	1.035	1.032
4	40	37.29	0.4650	−21.0	−24.7	+14.7	1.035	1.033
5	51	41.13	0.5297	−24.3	−24.7	+17.2	1.042	1.034
6	64	46.92	0.6329	−19.3	−24.0	+21.2	1.040	1.035
7	72	48.52	0.6637	−13.9	−23.5	+21.8	1.037	1.035
8	88	53.52	0.7613	−13.0	−22.6	+26.0	1.038	1.035
9	96	55.47	0.8087	− 5.0	−21.9	+27.3	1.033	1.035
10	114	58.87	0.8883	−6.2	−21.0	+30.1	1.036	1.035

* Growth medium: Trypticase Soy Broth, 150 μg/ml glucose added every 6 hr. Initial $SO_3^=$ concentration 0.1% w/v Na_2SO_3. Initial temperature 37°C. After Sample 4 was taken, incubation temperature was dropped to 26°C.

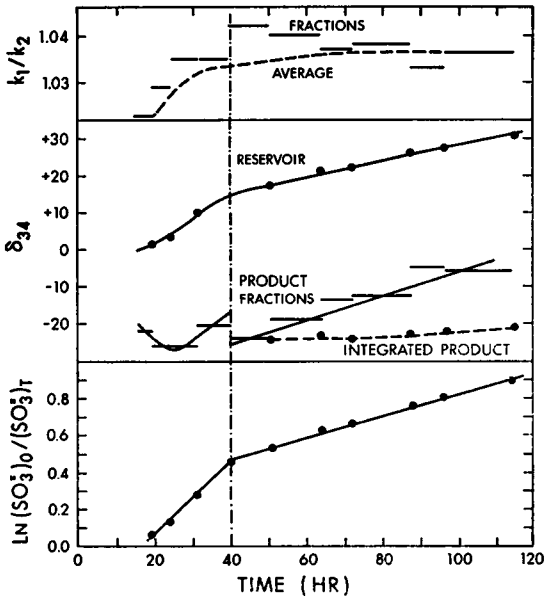

FIGURE 8 Kinetics and isotope fractionation in an experiment where the reduction rate was thermally altered. Growth conditions as described in Fig. 2, initial $SO_3^=$ concentration = 0.1% w/v Na_2SO_3, initial temperature 37°C, dropped to 26°C at 40 hr.

whereas others depend on the medium or the cells' physiological structure. A simple relationship which would encompass all of these variables seems unlikely.

SUMMARY

By pulse feeding glucose to *Salmonella heidelberg* during $SO_3^=$ reduction, large percentages of $SO_3^=$ conversion were possible while maintaining a reasonably stable cell population. These conditions permitted an effective combination of the data from sulfur isotope fractionation and conventional kinetic studies. As a result, the behavior of the system could be better assessed than from studies where only small percentages of $SO_3^=$ conversion resulted or the population was not stabilized. The The system was analyzed in terms of first order kinetics with respect to $SO_3^=$ concentration for two reasons, (*i*) at lower $SO_3^=$ concentrations, first order kinetics appeared to be strictly obeyed, (*ii*) the equations involving the isotope effects are relatively complex for kinetics other than first order.

It is quite probable that as techniques are further developed and the system is studied under more varied conditions, the kinetics can be more exactly defined and some of the interpretations in this paper will require revision.

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