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Sulfur Reduction by Human Erythrocytes

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ABSTRACT Washed human erythrocytes incubated with glucose and S_8 and purged with N_2 produced H_2S at a nearly constant rate of 170 μmol (L cells)$^{-1}$ min^{-1}, which continued for several hours. In sealed vials up to 25 mM HS^- accumulated. Glucose caused the fastest H_2S production, although either lactate or glycerol could support slower rates. When glucose was added without S_8, anoxic H_2S production nonetheless occurred at about 1.5% of the maximum rate, after 24 hr totaling 0.5 mmol H_2S (L cells)$^{-1}$, suggesting the presence of endogenous reducible sulfur. Anaerobic conditions were not required, since oxygenated cells produced H_2S from S_8 at 80% of the anoxic rate. Using cell lysates, production of H_2S occurred after addition of either glutathione, NADH, or NADPH. The observations suggest possible physiological roles for H_2S as an electron carrier, and are consistent with an evolutionary relationship between eukaryotic cytoplasm and sulfur-reducing Archaea. *J. Exp. Zool. 282:310–322, 1998.*

The "Universal Tree of Life" based upon rRNA sequences shows a high proportion of thermophilic, sulfur-respiring organisms nears its base, particularly in the Archaea (Fischer et al., '83; Pace, '97). These archaebacteria are the most closely related of all prokaryotes to the eukaryotic nucleocytoplasm, and in some ways may resemble the ancestral eukaryotic phenotype (see below). Nonetheless, modern eukaryotic cells are not thermophilic (Brock, '85), and sulfur reduction has attracted little attention. We undertook the present investigation in order to test whether animal cells might be able to respire upon elemental sulfur (S^0).[1]

Although systematic surveys have not been published, S^0 reduction is probably widespread among prokaryotes (Postgate and Kelly, '82; Le Faou et al., '90; Widdel and Hansen, '92). Thus, it can not be taken as evidence of a putative eukaryotic-Archaea relationship. However, absence of S^0-reduction in eukaryotes could weigh against such a relationship.

Eukaryotic genomes contain sequences of disparate evolutionary origins, suggesting origin from a fusion of archaebacterial and eubacterial cells (Gupta and Golding, '96; Margulis, '96). The archaebacterial component encompasses the genetic apparatus from chromosome structure to protein synthesis and most functions in between (Lake et al., '84; Pühler et al., '89; Keeling et al., '94; Starich et al., '96; Clayton et al., '97). In addition, several metabolic enzymes clearly resemble those in archaebacteria, such as the vacuolar H^+-translocating ATPase (Gogarten et al., '89). Thus, the Archaeal contribution to eukaryotic cells is substantial and might include also traits such as S^0 reduction.

Sulfur reduction must be distinguished from SO_4^{2-} reduction. Neither animal nor plant cytoplasms commonly reduce SO_4^{2-} (Huovinen and Gustafsson, '67; Bray and Till, '75), nor do archaebacteria such as *Thermoplasma acidophilum* (Segerer et al., '88; Searcy and Hixon, '91). Plants reduce and assimilate SO_4^{2-}, but it occurs in the plastids (Brunold and Suter, '89) and is not a genuinely cytoplasmic trait. Exceptions are certain fungi that can reduce SO_4^{2-} (Roy and Trudinger, '70), where it may have evolved secondarily. Thus, SO_4^{2-} reduction is typically a eubacterial trait. In contrast, S^0 reduction may be more widespread.

In the 19th century there were several published references to S_8 reduction, first appearing in connection with use of S_8 as a fungicide (reviewed in McCallan and Wilcoxon, '31). When dusted with S_8, either fungi or plant leaves were

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[1]Conventions: S^0 refers to "sulfane" sulfur, i.e., to sulfur that is covalently bound to only other sulfur atoms, including elemental sulfur (S_8), polysulfides ($^-S\text{-}S_n\text{-}S^-$), and the central sulfur in polythionates ($^-SO_3\text{-}S_n\text{-}SO_3^-$; Roy and Trudinger, '70; Westley et al., '83; Le Faou et al., '90). Aqueous sulfide is typically a mixture of H_2S + HS^-, since pK_a = 7.0 (National Research Council, '79). For brevity, [HS^-] shall refer to total sulfide concentrations in neutral, aqueous solutions.

reported to produce H_2S. Several confirmatory reports have appeared since then (Rankine, '64; Tweedy and Turner, '66; Legris-Delaporte et al., '87; Sorokin, '93), but little is known of the mechanism.

There was also an early report that animal cell extracts can reduce S_8 to H_2S (de Rey-Pailhade, 1888). Subsequently this was explained as nonenzymic reaction between S_8 and reduced glutathione (GSH) (Sluiter, '30; Roy and Trudinger, '70). Since then the phenomenon has been ignored.

Certain medical observations published early in the 20th century suggested to us that S^0 reduction might occur in humans. Patients with rheumatoid arthritis were found to be deficient in sulfur, suggesting that supplements of S_8 might be possible treatments (reviewed in Comroe, '39). Ointments containing S_8 were applied to the skin, and sometimes caused symptoms of H_2S poisoning (Basch, '26). In a different study, a man was injected intravenously with colloidal sulfur and within seconds H_2S was detected on his breath (Monaghan and Garai, '24). Since it happened too quickly to involve intestinal bacteria, the observation suggested to us that S_8 reduction can occur in human tissues.

In a preliminary test we added S_8 to a sample of bovine whole blood and immediately the odor of H_2S was evident. That observation encouraged us to do the experiments described here, which primarily concern whole cells. Biochemical studies are expected to follow.

Human erythrocytes were used for these experiments because they were readily available. These cells have lost nuclei, organelles, and other intracellular membranes (Denstedt, '53; Giggenbach, '72), so that they can be regarded as a simplified eukaryotic cell consisting only of soluble cytoplasm and a surrounding membrane. Nonetheless, erythrocytes are highly loaded with hemoglobin, which can cause unexpected pitfalls. (See later text.)

Several forms of S^0 are potential substrates for reduction, including colloidal sulfurs, polysulfides ($^-S\text{-}S_n\text{-}S^-$), polythionates ($^-SO_3\text{-}S_n\text{-}SO_3^-$), and S_8. For this initial study colloids were avoided because they are unstable and may have undefined surface ionic groups, or may be contaminated with organic solvents depending on how they are made. Polysulfides and polythionates can be similarly unstable, and often are mixtures of different length polymers (Giggenbach, '72). Thus, because of its unambiguous, simple identity, S_8 was used for most of the experiments described here. Preliminary tests showed that S_8 was indeed a good substrate for HS^- production, the optimum concentration being about 0.5 g-atoms L^{-1}. Although that concentration may seem high, it might be explained by the unwettable and insoluble nature of S_8.

When positive results were obtained during initial studies, one possibility was that H_2S production might somehow be "accidental." Although all possible reactions could not be tested, one of particular concern was spontaneous reaction between S_8 and GSH because GSH is abundant in human erythrocytes (Beutler, '84). Thus, a soluble cell lysate was incubated with dithiothreitol in order to reduce disulfide bonds that can covalently bind GSH to proteins (Cleland, '64), and then purified free of GSH by gel-exclusion chromatography. Finally, GSH, NADH, and NADPH were added back separately or in combination, testing each for a role in H_2S production.

Transition metals such as Fe catalyze HS^- oxidation (Krebs, '29), but that effect is nearly eliminated by the chelator diethylenetriaminepentaacetate (DTPA) (Buettner et al., '83). Thus, DTPA was included in some of the buffers described below.

MATERIALS AND METHODS

Erythrocytes

Venous blood was drawn from the authors, heparinized, chilled in ice, and diluted with several volumes of 0.15 M NaCl, 5 mM potassium phosphate, pH 7.4 (PBS). After centrifugation at 4,000*g* for 5 min, the plasma and upper buffy coat were removed by aspiration. The erythrocytes were washed by centrifugation 3 times more with 10 vol. PBS and finally resuspended using 0.25 vol. PBS.

In one experiment the blood was collected without anticoagulant, immediately defibrinated by agitation with crushed glass, and the white cells removed by passage through cellulose powder (Beutler, '84). At this point no white cells or platelets were visible by phase microscopy.

Sulfur

Elemental sulfur (sublimed powder; S_8) was ground in a mortar and suspended in PBS (1 g-atom S L^{-1}). Na_2S_3 and Na_2S_5 were prepared by mixing S_8 and Na_2S in the required proportions, sealing under N_2, and heating until dissolved.

Bubbling technique

Glass tubes (5 mL) with conical bottoms were coated over their upper $^2/_3$; inner surface with silicone oil (Antifoam A, Sigma Chemical Co.) and

rinsed with a forceful stream of H_2O. In a typical experiment each tube contained 0.5 mL 40% (v/v) washed erythrocytes, 0.5 g-atoms S^0 L^{-1} (as S_8), and 10 mM glucose, all in PBS. The tubes were sealed with rubber septa and connected by polyethylene tubing (0.76 mm ID) as follows: purified N_2 or Ar (about 25 mL/min) was passed first through an O_2-scavenging cartridge, bubbled through water at 37°C, through the cell suspension at 37°C, and finally through 5 mL of 5 mM $ZnCl_2$, 10 mM NaOH. Specifically, entering N_2 gas was conducted by one polyethylene tube to the bottom of the sample, and the gas emerging above the sample was collected by a second tube and carried to the $ZnCl_2$-NaOH solution. At intervals the $ZnCl_2$-NaOH solutions were exchanged and assayed for S^{2-}. For standardization, following each experiment some vials were spiked with standards of 100 nmol Na_2S while others were used as blanks. All were bubbled for an additional 5 min, while the H_2S was collected into fresh $ZnCl_2$-NaOH solutions. The yield of sulfide in the $ZnCl_2$-NaOH was about 50%. (See Results.)

Sealed vial experiments

In a typical experiment, a sample (0.5 mL) contained 40% (v/v) erythrocytes, 0.5 g-atom S L^{-1}, and 10 mM glucose in PBS buffer. Each sample was placed in a 2 mL tube, sealed with a rubber septum, evacuated for 4 min using a hypodermic needle attached to a vacuum manifold, and then refilled with purified Ar. Incubation was at 37°C with continuous rolling (120 rpm). At the end of incubation 0.5 mL of 20% trichloroacetic acid was injected through each stopper, mixed, and the tubes centrifuged at 4°C for 2 min at 100*g*. Finally the tubes were opened and the supernatant solutions immediately assayed for H_2S. Standards were prepared by injecting Na_2S solution into vials containing only PBS, which showed that 74% of injected sulfide remained in solution while the rest was volatilized into the gas space as predicted by Henry's Law.

Cell lysates and removal of GSH

Freshly collected and washed erythrocytes were resuspended in 0.25 vol. 10 mM potassium phosphate, 1 mM DTPA, pH 7.40 (KPD buffer). Phenylmethylsulfonylfluoride (10 mM final conc.) was added with rapid mixing. The cell suspension was purged with N_2 for several minutes, sonicated under N_2 for 10 sec, cooled in ice while being flushed with N_2, and sonicated again. Cell lysis was complete. Dithiothreitol was added to 10 mM, the lysate incubated at 4°C for 30 min, and centrifuged at 10,000*g* for 10 min, all under N_2.

The supernatant was applied to a Sephadex G25 "spin column" that had been prepared as follows: Sephadex G25 (fine, 10 mL) was packed into a 10 mL syringe barrel and equilibrated with KPD buffer that included freshly added 1 mM phenylmethylsulfonylfluoride. The column was centrifuged at 200*g* (1,000 rpm) for 3 min and then flushed with N_2, which passed easily through the column. The lysate (2 mL) was applied to the top of the column, and after centrifugation at 200*g* for 1 min the excluded enzyme-containing fraction was collected from the bottom.

Assays

Sulfide was measured using the methylene blue technique (Greenberg et al., '81). For example, after sulfide was collected in 5 mL of $ZnCl_2$-NaOH solution (see above), it was combined with 0.25 mL DPDO reagent (=0.675 g dimethyl-*p*-phenylenediamine oxalate in 100 mL 9 M H_2SO_4) and then with 0.2 mL 3 M $FeCl_3$. After 20 min at room temperature the absorbance was measured at 670 nm.

Glucose, lactate, and pyruvate were measured using enzymatic assays linked to NAD^+ reduction (Beutler, '84). For GSH analysis, the purified red cell lysates were deproteinized using 5% trichloroacetic acid, and the acid was then removed by extraction with diethyl ether (Beutler, '84). These extracts were assayed for GSH using an enzymatic procedure (Griffith, '85). Organic acids were analyzed using gas chromatography on a Supelco "Carbopack" column at 175°C, with He carrier gas, and a flame-ionization detector. Also, methyl esters were prepared as described by Bricknell et al. ('79), and similarly analyzed by gas chromatography. Carbon dioxide was measured by acidifying an incubated cell suspension with H_2SO_4, and then flushing it with Ar to transfer the CO_2 into 5 mL 10 mM NaOH. At the Environmental Analysis Lab., Univ. of Mass., the NaOH solutions were acidified and gaseous CO_2 measured by infrared absorbance. Standards were put through the same procedures of gas-transfer and measurement. Standards spiked with Na_2S showed that H_2S did not interfere with CO_2 measurement.

Inhibitors

The following stock solutions were used: 6 M NaF; 0.26 M iodoacetamide (freshly dissolved); 1 M KCN; and 1 M phenylmethylsulfonylfluoride in dimethylsulfoxide. Carbon monoxide treatment was by bubbling CO gas through a cell suspen-

sion for 1 min and then sealing it into a vial filled with CO.

RESULTS

Bubbling technique

To characterize the bubbling technique, preliminary measurements were made using PBS buffer spiked with Na_2S. The stream of N_2 gas transferred H_2S from the sample to the $ZnCl_2$-NaOH solution with first-order kinetics ($t_{1/2}$ = 40 sec) and overall yield 50% to 70%. The unaccounted H_2S passed through the $ZnCl_2$ solution, as shown by analyzing the exhaust gas with a second $ZnCl_2$ solution. Variation occurred apparently because of differences in bubble dispersion and in the gas-flow rates, which sometimes was reduced in order to avoid foaming in the sample. At the end of each experiment Na_2S standards were added to the samples, so that transfer efficiency was automatically taken into account.

Washed erythrocytes incubated with S_8 and glucose produced H_2S (Fig. 1). If glucose was omitted there was an initial burst of H_2S production that was not sustained. When S_8 was omitted H_2S production was about 1.5% of the rate when S_8 was present.

To confirm that red cells accounted for H_2S production, white cells and platelets were removed by defibrination and passage through cellulose powder (see Materials and Methods). The purified erythrocytes produced H_2S at rates similar to those prepared by the usual centrifugation technique.

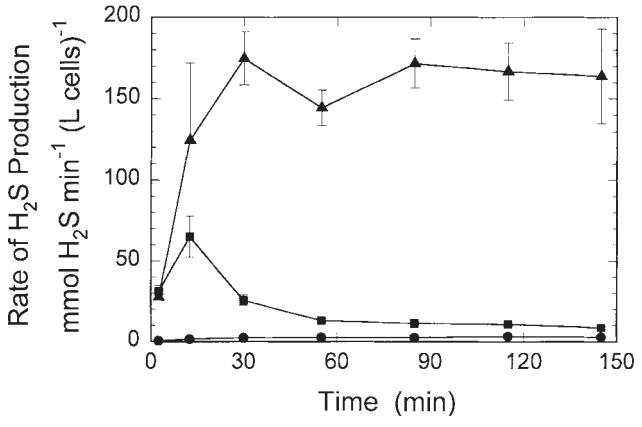

Fig. 1. Rates of H_2S production measured in cell suspensions flushed with N_2. Washed erythrocytes were suspended to 40% (v/v) in PBS buffer containing 0.5 g-atoms S_8 L^{-1} and 10 mM glucose. Aliquots (0.5 mL) were placed in 5 mL glass test tubes and bubbled with N_2. The excurrent gas was bubbled through 5 mL of 5 mM $ZnCl_2$, 10 mM NaOH, which at intervals was exchanged and assayed for sulfide using the methylene blue technique. (▲) complete mix; (■) no glucose; (●) no sulfur.

Additional experiments and controls are shown in Table 1. Data were collected between 20 min and 40 min after the start of incubation in order to be in a period of approximately steady H_2S production and to increase dependence on exogenous substrates.

Also included in Table 1 are certain compounds typically identified with mitochondrial metabolism, such as succinate, because the corresponding enzymes are reportedly present in erythrocytes (Denstedt, '53; Pennell, '74).

Glucose stimulated greatest H_2S production, while either glycerol, gluconate, or ethanol stimulated less. Succinate, lactate, or citrate tended to support S^0 reduction that might have become statistically significant with more replications.

*TABLE 1. Production of H_2S by erythrocytes while purged with N_2**

Sample	Rate of H_2S production μmol (L cells)$^{-1}$ min^{-1} mean ± SE (n)
Buffer + S_8 + D-glucose	0.0 ± 0.0 (2)
Erythrocytes	2.4 ± 1.1 (2)
+ 10 mM D-glucose	2.4 ± 0.2 (4)
+ 0.5 g-atoms S_8 L^{-1}	30.7 ± 3.2 (20)
+ S_8 + D-glucose	144.7 ± 9.8 (14)
+ S_8 + D-glucose + 60 mM NaF	105.7 ± 10.7 (4)
+ S_8 + D-glucose + 2.6 mM iodoacetamide	1.2 ± 0.5 (3)
+ 0.5 mM Na_2S_5	14.8 ± 4.3 (4)
+ 0.5 mM Na_2S_5 + D-glucose	133.6 ± 15.3 (4)
+ 10 mM $Na_2S_2O_3$	0.7 ± 0.2 (3)
+ 10 mM $Na_2S_2O_3$ + D-glucose	1.4 ± 0.3 (4)
+ 10 mM $Na_2S_4O_6$	0.0 ± 0.2 (3)
+ 10 mM $Na_2S_4O_6$ + D-glucose	0.4 ± 0.6 (3)
Boiled cells + S_8 + D-glucose	0.5 ± 0.1 (2)
Erythrocytes + 0.5 g-atoms S_8 L^{-1}	32.8 ± 3.8 (16)
+ 10 mM sodium L-lactate	36.3 ± 3.9 (5)
+ 10 mM sodium L-malate	29.8 ± 4.6 (3)
+ 10 mM sodium pyruvate	29.3 ± 5.9 (4)
+ 10 mM sodium pyruvate + 10 mM sodium L-malate	39.4 ± 4.4 (8)
+ 10 mM sodium succinate	38.5 ± 6.4 (9)
+ 10 mM sodium succinate + 10 mM sodium malonate	29.0 ± 0.1 (2)
+ 10 mM sodium citrate	34.9 ± 7.2 (5)
+ 10 mM sodium DL-isocitrate	31.4 ± 5.8 (4)
+ 10 mM sodium D-gluconate	45.5 ± 3.6 (2)
+ 10 mM glycerol	46.2 ± 4.5 (4)
+ 10 mM ethanol	38.3 ± 2.1 (4)
+ 10 mM acetaldehyde	32.9 ± 2.3 (4)

*Washed erythrocytes (40% v/v) were suspended in 0.15 M NaCl, 5 mM potassium phosphate (pH 7.40) plus the substrates shown in the table. Each sample (0.5 mL) was bubbled with N_2 at 37°C, and H_2S was measured in the excurrent gas over the time interval 20 min to 40 min after start of incubation (see text). Replicates used separate batches of blood, from only 2 donors.

Other substrates were unexpectedly ineffective, such as isocitrate, possibly because they did not enter the cells.

Iodoacetamide and NaF are both classical inhibitors of glycolysis, and were used to confirm that H_2S production depends upon glucose metabolism. Since iodoacetamide reacts with -SH groups, potentially it might consume HS^-, and result in an underestimate of true H_2S production. Thus, buffer containing 2.6 mM iodoacetamide was spiked with 0.2 mM HS^- and analyzed using the bubbling technique; the yield of H_2S was 70% compared to a sample without iodoacetamide. Thus, reaction of H_2S with iodoacetamide can not explain the nearly complete inhibition shown in Table 1, and probably the conventional explanation is valid—that iodoacetamide inhibits glucose metabolism by inactivating the enzyme glyceraldehyde-3-phosphate dehydrogenase.

When various forms of elemental sulfur were tested, thiosulfate ($S_2O_3^{2-}$) and tetrathionate ($S_4O_6^{2-}$) were poor substrates for H_2S production. In contrast, polysulfide (0.5 mM S_5^{2-}) was a good substrate, supporting H_2S production during the first 5 min at a rate 5 times the maximum rate from S_8. After 5 min H_2S production from S_5^{2-} quickly declined, apparently because the polysulfide was all consumed. Adding higher initial concentrations of S_5^{2-} were inhibitory.

When no glucose was added there was an initial burst of H_2S production from S_5^{2-}, presumably from endogenous cellular metabolites but also perhaps from enzymic polysulfide disproportionation (see Discussion).

Sealed vial experiments

The "bubbling technique" was designed to prevent accumulations of HS^- in the samples, which otherwise might inhibit cellular metabolism. To test the limit of HS^- accumulation, sealed vials were used. The first step in measuring HS^- was to remove hemoglobin and other proteins by trichloroacetic acid precipitation and centrifugation (Beutler, '84).

When [HS^-] was measured in the incubated sealed vials, it began to increase after a lag of about 4 hr and eventually reached 25 mM (Fig. 2). As observed with the bubbling technique, HS^- production depended on the simultaneous availability of S^0 and glucose. Additional experiments and controls are shown in Table 2. Blood plasma with S_8 and glucose produced no detectable HS^-, which is something that could not tested using the bubbling technique because it foamed too much.

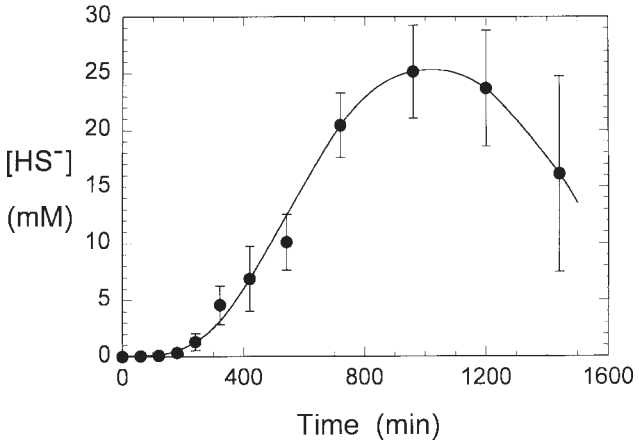

Fig. 2. Sulfide accumulation in sealed vials. Erythrocytes were washed and resuspended (40% v/v) in 0.15 M NaCl, 0.01 M potassium phosphate (pH 7.40) containing 10 mM glucose and 0.5 g-atoms S_8 L^{-1}. Aliquots (0.5 mL) were placed into 2.0 mL vials, evacuated for 5 min, refilled with argon, and incubated at 37°C with continuous rolling (120 rpm). When incubations were complete, 0.5 mL of 20% trichloroacetic acid was injected into each vial, mixed, and centrifuged at 1,000*g* for 2 min. The supernatant solutions were assayed immediately for H_2S using the methylene blue technique. Error bars = SD, 4–10 replicates.

Differences in HS^- production shown in Table 2 are magnified compared to those in Table 1 because in the sealed vials there was a time-lag before HS^- began to accumulate. This lag is explained by a saturable sink for HS^-, as explained below.

During the first 4 hr the concentration of HS^- in the sealed vials was barely detectable. Nonetheless, Figure 1 shows that HS^- was rapidly produced during this time interval. Thus, we speculated that a secondary reaction might consume the HS^- at the start of experiment, but eventually the secondary reaction is exhausted and HS^- begins to accumulate. To test this, washed erythrocytes were sealed into vials and deoxygenated, and varying amounts of Na_2S injected through the stoppers. After incubation for 10 min, trichloroacetic acid was injected through the stoppers, the sealed vials centrifuged, and the clear supernatant solutions assayed for H_2S. Only when Na_2S had been injected in excess of 8 mM did significant H_2S appear in the supernatant, consistent with a saturable sink. Since a suspension of 40% v/v erythrocytes contains about 8 mM hemoglobin monomer (Beutler, '84), hemoglobin was implicated as the sink.

When cells were first treated with CO there was no initial lag in HS^- accumulation, and final accumulations of HS^- were greater (Table 2). Since

TABLE 2. Accumulation of HS^- in sealed vials incubated for 8 hr[1]

Description	Relative accumulation of HS^-
Complete mix	100%
S_8 omitted	1.0
Glucose omitted	0.1
Erythrocytes omitted	<0.1
Erythrocytes boiled 10 min	<0.1
Blood plasma instead of erythrocytes	0.1
Complete + 2.6 mM iodoacetamide	0.0
Complete + 60 mM NaF	0.1
Complete + 10 mM HCN	110
Complete + CO	170
10 mM $Na_2S_2O_3$ instead of S_8	0.3
10 mM S_3^{2-} instead of S_8	21
10 mM S_5^{2-} instead of S_8	24

[1]The "Complete mix" was 0.5 mL 40% (v/v) erythrocytes, 0.01 M glucose, and 0.5 g-atoms S_8 L^{-1} in 0.15 M NaCl, 5 mM potassium phosphate buffer (pH 7.4). Incubation was at 37°C with rolling at 120 rpm. Blank samples (not shown) were incubated for 10 min to allow rapid disproportionation reactions to occur, and have been subtracted from the data. These blanks were significant only for S_3^{2-} and S_5^{2-}, where they amounted to 3% of the maximum H_2S accumulation after 8 hr. The total accumulation of HS^- in the "Complete mix" was 7.4 mM ± 1.2 mM (SE, n = 4). To reduce day-to-day variation, each day's set of data was normalized so that the "Complete mix" was 100%.

hemoglobin is specifically bound and inactivated by CO, it again was implicated as the HS^- sink.

The "sealed vial procedure" uses trichloroacetic acid to remove hemoglobin before assaying HS^-, which was unavoidable because some hemolysis occurred after several hours incubation. However, after 10 min incubation hemolysis was insignificant, and supernatant solutions free of hemoglobin could be prepared by centrifugation of the cells without use of trichloroacetic acid. When Na_2S was added to deoxygenated cells, incubated 10 min, and centrifuged, the supernatant contained the expected concentration of HS^- with no evidence of loss. Thus, the lag in HS^- accumulation in sealed vials was apparently an artifact caused by reaction between hemoglobin and HS^- in the presence of trichloroacetic acid (see Discussion).

Optimal pH

Figure 3 shows that with intact erythrocytes H_2S production occurred optimally near pH 7.5. Two time intervals were tested, with similar pH maxima.

Stoichiometry and excretory products

Conventionally, erythrocytes ferment glucose to lactate by a metabolic pathway that is redox-balanced. In order to produce electrons for S^0 reduction, glucose must be metabolized to a product more oxidized than lactate.

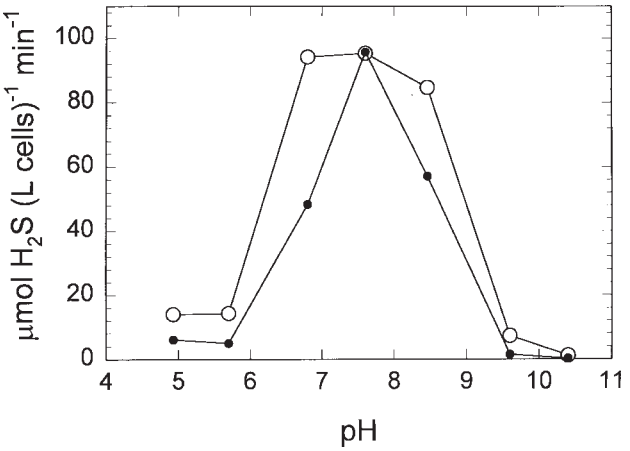

Fig. 3. Effect of pH on the rate of H_2S production by intact cells. Washed red cells (0.2 v/v) in PBS with 0.5 gram-atom S_8 L^{-1} and 10 mM glucose were adjusted to the pH's shown. The rates of H_2S production were measured using the bubbling technique, as in Figure 1. Time intervals: 0–20 min (●); 20–40 min (○).

An indication of the glucose oxidation required can be obtained from the ratio of glucose consumed to H_2S produced. Using the bubbling technique, paired samples (1 mL each) of red cell suspensions were incubated with S_8, with or without 0.5 mM glucose. Incubation continued 140 min, after which no detectable glucose remained and H_2S production essentially stopped. The samples with 0.5 μmol glucose produced 1.4 μmol H_2S in excess over those with no added glucose, suggesting a stoichiometry near 1:3.

Using higher concentrations of glucose a similar ratio was measured. When erythrocytes were suspended in 5 mL 75 mM NaCl, 100 mM sodium 3-(N-morpholino)propane-sulfonate (MOPS buffer, pH 7.4), 10 mM glucose, and 2500 μg-atoms S_8, after 23 hr incubation at 37°C in sealed vials the glucose (50 μmol) was 99% consumed, including in samples with no added S_8. In the absence of S_8, 89 μmol lactate and 0.5 μmol pyruvate accumulated. When S_8 was present, 75 μmol lactate and 7 μmol pyruvate accumulated. Thus, S^0 reduction correlated with increased pyruvate production, but not by enough to account for the HS^- production. Also, the fate of the glucose was not entirely accounted.

Additional organic acids were sought by gas chromatography, particularly acetate and succinate, but none was detected (see Materials and Methods).

In separate experiments CO_2 production was measured, and found to increase significantly during H_2S production. In conditions equivalent to the experiment above, 42 μmol CO_2 accumulated in the absence of S_8 and 91 μmol accumulated in its presence. This accounts for all the glucose carbon atoms plus about 10% more (see Discussion).

S^0 reduction by cell lysates

A soluble cell lysate was prepared by sonication and centrifugation. When S_8 and NADPH were added, H_2S was produced (Table 3). The centrifugal pellet including membrane fragments produced no detectable H_2S.

In order to examine the role of GSH in H_2S production, endogenous GSH and other molecules <10,000 M_r were removed from the lysate by gel-exclusion chromatography. Assay of the enzyme-containing fraction indicated that no detectable GSH remained. As a control the unpurified lysate was tested and found to contain 2 mM GSH, which is the expected concentration (Beutler, '84). We estimate the limit of detection to be about 10 μM GSH, so at least 99% of the endogenous GSH had been removed.

When the chromatographically purified enzyme fraction was combined with S_8 plus either GSH, NADPH, or NADH, then H_2S was produced (Table 3). Combinations of GSH and NADH or NADPH were synergistic, nearly restoring the original rate of H_2S production. The rate of H_2S production was approximately constant for 15 min, but by 30 min decreased to about half. Exceptions to this were samples with no added organic substrate, which stopped producing H_2S after about 5 min.

TABLE 3. H_2S production in cell lysates[1]

Sample (each with 0.5 g-atom S_8 L^{-1})	H_2S production (μmol L^{-1} min^{-1})
Buffer + 1 mM GSH + 1 mM NADPH	6
Centrifuged lysate + 1 mM GSH + 1 mM NADPH	87
Buffer + 1 mM NADPH	0
Membrane pellet + 1 mM NADPH	0
Membrane pellet + 1 mM GSH + 1 mM NADPH	5
Sephadex G-25 excluded fraction + S_8	5
+ 1 mM GSH	25
+ 1 mM NADPH	17
+ 1 mM GSH + 1 mM NADPH	78
+ 1 mM NADH	15
+ 1 mM GSH + 1 mM NADH	65
+ 1 mM GSSG	6
+ 1 mM GSSG + 1 mM NADPH	31

[1]Each sample (0.5 mL) contained 0.25 mL lysate and 0.5 g-atom S_8 in KPD buffer, plus the substrates shown. Production of H_2S was measured for 30 min using the bubbling technique. Data are means of 3–5 replicates and shown in terms of diluted lystate (0.5 mL). When the S_8 was omitted H_2S production was undetectable.

H_2S production can also occur by spontaneous reaction between GSH and S_8 (Sluiter, '30). As a control, we measured that reaction in protein-free PBS buffer using the bubbling technique. In solutions containing 0.5 g-atoms S_8 L^{-1} and 1.0 mM to 3.0 mM GSH, rates of H_2S production were proportional to GSH concentration with an apparent pseudo-first-order rate constant of 0.002 min^{-1}, although H_2S production is not a simple, one-step reaction. Thus, non-enzymic reaction between S_8 and GSH may account for about 1% of the H_2S production observed with red cells.

Endogenous S^0 in erythrocytes

To measure the extent to which H_2S production might occur from endogenous S^0, washed erythrocytes were incubated with glucose but without added S^0. Using the bubbling technique, production of H_2S started at 3 μmol (L cells)$^{-1}$ min^{-1}, and then slowly declined. When glucose was omitted the initial rate of H_2S production was only 0.8 μmol (L cells)$^{-1}$ min^{-1}, suggesting that starvation-induced catabolism of sulfur-containing molecules was not the source, but rather that it was mostly linked to glucose metabolism. With glucose present but no S_8, over a 24 hr period H_2S production was 0.5 mmol (L cells)$^{-1}$.

Cell contact with S_8

Since S^0 has little solubility in water, contact between the cells and S^0 could be important. To examine this, a dialysis bag was partially filled with wetted S_8, collapsed to provide large surface area, sealed, and inserted into a suspension of red cells plus glucose. Production of HS^- was measured using the bubbling technique, and occurred at 5% of the rate when S^0 and cells were intimately mixed. Thus, contact between cells and S_8 may be helpful, but is not essential.

Stored cells lose activity

Using cells that had been stored at 4°C 1 day, H_2S production was about half that with fresh cells. This rate of decline approximately parallels the loss of glycolytic activity (Denstedt, '53). When 10 mM dithiothreitol or 10 mM phenylmethylsulfonylfluoride were added during storage, H_2S production declined less rapidly, suggesting that oxidation and enzymic hydrolysis each accounted for some of the loss.

Aerobic H_2S production

When air was substituted for N_2 in the bubbling technique, but otherwise using the same conditions as in Figure 1 (40% v/v cell suspension), HS^- was collected at 23 μmol L^{-1} min^{-1}, which continued at a nearly constant rate for 3 hr. At that time half the samples were spiked with 200 nmol Na_2S, and during the next 10 min an excess of 81 nmol H_2S was collected in the $ZnCl_2$–NaOH solution. Thus, the efficiency of H_2S transfer using air was 41% and the corrected rate of aerobic H_2S production was 138 μmol (L cells)$^{-1}$ min^{-1}.

DISCUSSION

The data indicate that erythrocytes can reduce S_8 to HS^- using reducing equivalents obtained from glucose oxidation. Since the terminal electron acceptor is inorganic, this is "anaerobic respiration" as the term is defined by some microbiologists (Brock et al., '84), with no implication that an electron transport chain and energy conservation are necessarily involved.

Although glucose best supported H_2S production, certain other substrates also were effective. It is doubtful that all would be linked directly to S^0 reduction, suggesting that electron carriers such as NADH and NADPH are likely to be intermediates. Consistent with such a possibility, NADH, NADPH, and GSH each stimulated H_2S production in cellular lysates.

The rate of glucose consumption in the presence of S^0 was about 60 μmol (L cells)$^{-1}$ min^{-1}, similar to that in the absence of S^0 (Brewer, '74). When S_8 was added, H_2S production was about 170 μmol HS^- L^{-1} min^{-1}. Thus, the ratio of glucose consumption to H_2S production rates is near 1:3, in agreement with total yields measured in experiments where glucose consumption was taken to completion.

Conventional red cell metabolism is mainly via the Embden-Meyerhof pathway, and lactate is excreted (Beutler, '84). Overall, the pathway is redox balanced and can provide no reducing equivalents for H_2S production. However, if pyruvate is excreted instead of lactate, 2 HS^- can be produced per each glucose consumed. In order to produce 3 HS^-, as observed, there must be an excretory product more oxidized than lactate. Apparently this is CO_2, produced in the phosphogluconate pathway.

Phosphogluconate pathway

In normal resting erythrocytes the phosphogluconate pathway (=pentose phosphate pathway, or hexose monophosphate shunt) accounts for 5%–10% of glucose metabolism, with glycolysis accounting for the rest (Yunis and Yasmineh, '69). When cells are subjected to oxidative stress the phosphogluconate pathway can become more active, producing NADPH that is used in various antioxidative functions (Denstedt, '53; Yunis and Yasmineh, '69). We observed that either gluconate or NADPH supported S^0 reduction, consistent with an important role for the phosphogluconate pathway during H_2S production.

In erythrocytes the fraction of each glucose molecule that is oxidized to CO_2 varies according to oxidative stress (Yunis and Yasmineh, '69). Assuming that lactate is the main product of glucose metabolism, our data approximate the following reaction:

$$2\ C_6H_{12}O_6 + 6\ S^0 + 3\ H_2O \rightarrow 3\ C_3H_6O_3 + 6\ H_2S + 3\ CO_2. \quad (1)$$

Experimentally, we found that for each 2 molecules of glucose consumed there were produced 6 H_2S and 2 CO_2. Thus, less CO_2 was produced than predicted by Reaction 1, possibly correlating with pyruvate excretion.

Inhibitors

Iodoacetamide and NaF are classical inhibitors of glucose metabolism. Iodoacetamide completely inhibits glucose oxidation, including both glycolysis and the phosphogluconate pathway (de Loecker and Prankerd, '61). We found that it completely eliminated HS^- production. In contrast, F^- only partially inhibited glucose metabolism, and likewise inhibited HS^- production. Although it is possible that these unrelated inhibitors might each affect sulfur-reducing enzymes directly, the conventional interpretation is more likely: that they inhibited H_2S production by interfering with glucose metabolism.

Optimal pH

The optimum $[H^+]$ for cellular HS^- production was near pH 7.5, typical of a biological process. In intact erythrocytes the intracellular pH does not necessarily match the extracellular pH (Hladky and Rink, '77), and several enzymes are probably involved between glucose oxidation and S^0 reduction. Thus, this should not be interpreted as a simple enzymic pH optimum, but more likely is the global optimum for erythrocyte metabolism.

One concern is that pH might affect the efficiency of the bubbling technique, which is dependent on gas-phase transfer of H_2S to the $ZnCl_2$ solution. At higher pH a greater fraction of H_2S

is ionized and not volatile, resulting in slower transfer rate. In preliminary experiments (not shown) we verified that pH did affect the transfer rate in the expected manner. Nonetheless, if gas-transfer is taken to completion the rate of transfer should not affect the final yield. Figure 3 shows that there is efficient transfer up to pH 8.5 at least, suggesting that the observed optimum between pH 7 and pH 7.5 is not an artifact.

Nonenzymic H_2S production by GSH

Reduced glutathione can spontaneously react with S_8 to produce H_2S (Sluiter, '30), and so we included nonenzymic controls with our experiments. Using constant 0.5 g-atoms S_8 L^{-1}, production of H_2S followed pseudo-first-order kinetics ($k = 0.002$ min^{-1}). Human erythrocytes contain 2.2 mM GSH (Beutler, '84), and since we used a 40% v/v cell suspension we calculate nonenzymic H_2S production to be 1.8 μmol L^{-1} min^{-1}, which is about 1% of the observed cellular rate. With intact erythrocytes this nonenzymic reaction is likely to be even slower because the GSH is inside the cells and S_8 is outside. Thus, nonenzymic reaction with GSH can not account for the H_2S production observed.

Nonetheless, GSH can be a substrate for enzymic H_2S production, as observed when GSH and S_8 were added to purified cellular lysate (Table 3). A second component of H_2S production appears to be coupled directly to NAD(P)H, for which there is precedent in prokaryotic S^0 reductases that have been previously studied (Childers and Noll, '94; Ma et al., '94). Since dissimilar substrates such as GSH and NADPH each supported H_2S production, erythrocytes may contain more than one type of S^0 reductase.

Combinations of GSH and NAD(P)H were synergistic. One explanation may be that GSH is directly responsible for most H_2S production, but the product (oxidized glutathione, GSSG) is reduced by NAD(P)H and used again. A second explanation may be that GSH reacts with S_8 to produce polysulfides (S_n^{2-} or GS_n^-), which are soluble and presumably more accessible for enzymic reduction. A third possibility is that GSH and NAD(P)H work together to remove enzymic disulfide bonds and persulfide groups, thereby causing enzyme activation (Valentine et al., '87).

Thioredoxin

Purified lysates still have some capacity for H_2S production, even after GSH, NADH, NADPH and other low molecular weight reductants have been removed (Table 3). This might be explained by protein -SH groups (Wood, '82). "Thioredoxins" are particular candidates in this regard; these are small proteins that contain oxidizable -SH groups plus catalytic activity for the reduction of other molecules (Mieyal et al., '95). Apparently none has been tested with S_8.

Interaction of HS^- with hemoglobin

It has been previously reported that deoxygenated hemoglobin at neutral pH does not bind HS^- (Lemberg and Legge, '49; Arp and Childress, '83), which we confirm. Nonetheless, erythrocytes in the presence of trichloroacetic acid absorbed an amount of HS^- that was equal to their hemoglobin content. An hypothetical explanation may be that acidic conditions exposed the heme in such a way that the iron could bind HS^-.

Sulfur accessibility

Rapid enzymic reactions typically use soluble substrates, in contrast to the insoluble form of sulfur used here. Sulfur (S_8) can be solubilized by serum albumin (Westley et al., '83). When we used cells that had been centrifuged once only, and not carefully washed, rapid HS^- production started immediately and was 50% faster (250 μmol HS^- L $cells^{-1}$ min^{-1}) than with washed cells. That observation is consistent with a carrier role for serum albumin. However, it does not explain how the cells were able to access S_8 through a dialysis membrane.

With bacteria, soluble polysulfides can be important substrates for H_2S production (Schauder and Müller, '93). The same is true for purified enzymes (Cammack et al., '84). However, in most of our experiments we added only S_8, so that polysulfides were not present initially. About 20 nM S_8 dissolves in water (Boulegue, '78; Steudel, '96), and might provide the substrate for initial HS^- production. Once present, HS^- can spontaneously react with S_8 to produce polysulfides, such as by the following reaction (Roy and Trudinger, '70; Giggenbach, '72; Steudel, '96):

$$2\ HS^- + S_8 \leftrightarrow 2\ S_5^{2-} + 2\ H^+. \qquad (2)$$

Figure 1 shows that H_2S production started slowly, then increased about 5-fold during the first 30 min of incubation, consistent with such a scheme.

Nonetheless, soluble polysulfides occur significantly only at pH > 8 (Keller et al., '95), and we used pH 7.4. Alternative types of soluble S^0 are known, such as organic polysulfides ($R\text{-}S_n\text{-}S^-$; Zöphel et al., '88). Future studies should iden-

tify which is the true enzymic substrate for H_2S production.

H_2S production from S_5^{2-}

When S_5^{2-} but no organic substrate was added, there was a burst of H_2S production that was over in about 5 min. At least 2 explanations can be suggested: (1) H_2S was produced from endogenous electron donors that were quickly exhausted, such as metabolic intermediates or protein –SH groups; (2) H_2S can be produced by polysulfide disproportionation, the reverse of Reaction 2.

Late decline in [HS^-]

After about 18 hr, the concentration of HS^- in sealed vials declined (Fig. 2). Experiments showed that this was not due to diffusion of HS^- out through the septum, nor inward diffusion of O_2, since it occurred only when S^0 was present. It might be explained by polysulfide formation, such as Reaction 2. In control experiments (not shown) we confirmed that polysulfide formation can indeed correlate with HS^- disappearance when measured by the methylene blue technique.

Endogenous S^0 in erythrocytes

When no S_8 was added to the washed erythrocytes, HS^- production occurred at about 1.5% of the maximum rate. During the first 24 hr a total of 0.5 mmol HS^- (L cells)$^{-1}$ was produced. This HS^- production was mostly glucose-dependent and can not be explained by starvation-induced catabolism of sulfur-containing molecules. Instead, there appears to be a supply of intracellular reducible sulfur.

"Neutral Sulfur" (~1 mg-atoms L^{-1}) has been reported previously in human blood (Reed and Denis, '27; Schneider and Westley, '69; Buzaleh et al., '90; Westley and Westley, '91). It was mostly associated with erythrocytes and not covalently bound to proteins (Reed and Denis, '27). A more recent measurement on mouse blood found 3 mM S^0 (Buzaleh et al., '90).

The endogenous S^0 has been variously reported to be S_8, polysulfides, polythionates, or any of those bound to an organic molecule (Schneider and Westley, '69; Roy and Trudinger, '70; Wood, '82; Westley et al., '83; Westley and Westley, '91). Thus, nearly every possible form of S^0 has been postulated to occur in animal tissues. Resolving which is important is a goal for future research.

Aerobic S^0 reduction

There is a generalization in biology that when several electron acceptors are available only the most electropositive one is reduced first. When it is exhausted the next is used, and so on in a hierarchical manner (Stumm and Morgan, '81). One does not expect S^0 reduction to occur in the presence of O_2. Nonetheless, S^0 reduction was rapid in aerated erythrocytes. In this specialized case it might be explained by the lack mitochondria in mammalian erythrocytes. Nonetheless, aerobic H_2S production has been reported also in yeast and plant cells, which do have mitochondria (McCallan and Wilcoxon, '31; Sorokin, '93). Thus, anoxic conditions may be not essential for eukaryotic H_2S production.

HS^- toxicity

H_2S is about equally as poisonous as HCN (National Research Council, '79), and explained by inhibition of cytochrome oxidase ($K_i \approx 0.2$ μM; Nicholls and Kim, '81). Nonetheless, mitochondria are reported to respire normally in concentrations up to 30 μM HS^- or more (Bartholomew et al., '80; Nicholls and Kim, '81). Possibly, mitochondria protect themselves by rapidly oxidizing any HS^- that penetrates (Baxter et al., '58; Bartholomew et al., '80; Bagarinao and Vetter, '90). In one remarkable demonstration of the rate of detoxification, a dog was injected with 5 times the lethal dose of Na_2S gradually over a period of 20 min, and at the end of the experiment was "none the worse" (Haggard, '21). Thus, although HS^- may be produced, in physiological situations it does not necessarily accumulate nor become toxic.

Physiological function

There may be no function for H_2S production, such as suggested by the term "incidental S^0 reduction" (Widdel and Hansen, '92). Alternatively, one might conjecture any of several possible functional roles, as follows.

HS^- might help to salvage *met*hemoglobin, which is oxidized hemoglobin that is nonfunctional for O_2 transport. Conventionally, *met*hemoglobin is reduced back to functional hemoglobin by an enzyme that uses NADH (Beutler, '84). However, it also can be reduced by reaction with HS^- (Lemberg and Legge, '49; Arp et al., '87).

"Facilitated fermentation" is a phenomenon that occurs when a supplemental e^- acceptor enhances the rate of fermentation (Widdel and Dische, '46; Hansen, '92). For example this might occur when all the cell's NAD^+ and $NADP^+$ are reduced, so that no oxidized cofactors are available for the initial steps of glucose metabolism. Fermentation to lactate in certain erythrocytes is reported to in-

crease 3-fold in the presence of O_2 (Dische, '46; Denstedt, '53), and sulfur reduction could have a similar effect in regenerating NAD^+ and $NADP^+$.

Finally, H_2S production can be a mechanism for exporting reducing equivalents from the erythrocyte, eliminating the requirement for completely balanced fermentation. The HS^- might then be oxidized in other tissues by their mitochondria (Baxter et al., '58; Bartholomew et al., '80). Potentially, mitochondrial HS^- oxidation can be coupled to ATP synthesis, which has been verified in a few animals (Powell and Somero, '86; Bagarinao and Vetter, '90). If the sulfur product is of a form that can be returned to the erythrocyte and used again, sulfur would act as a shuttle carrier for electrons much like NAD^+, except that sulfur compounds can exchange between cells.

The hypothetical sulfur cycle described above occurs between cells. However, cells that have mitochondria can produce HS^- in the cytoplasm and oxidize it in the mitochondria, all within the same cell. Such an intracellular sulfur cycle might account for the symbiotic origin of mitochondria (Searcy, '92). Hypothetically, as mitochondria evolved and the symbiosis became increasingly intimate, smaller concentrations of sulfur could still maintain a significant sulfur exchange. Thus, in modern eukaryotic cells a sulfur-cycle may yet persist, but has been undetected because of the small amount of sulfur involved and the rapidity with which it cycles. That hypothesis remains to be tested.

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