Some Properties of the Rhodanese System of *Thiobacillus denitrificans*

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1. Rhodanese has been extracted from *Thiobacillus denitrificans* by ultrasonic disintegration of the cells. 2. Studies with Sephadex columns have shown that the enzyme aggregates, forming a tetramer. 3. The molecular weights of the monomer and of an enzymically active sub-unit one-quarter this size have been determined by gel filtration. 4. Higher-molecular-weight forms of rhodanese are broken down by mercaptoethanol to enzymically active fragments of mol.wt. 7000 and 2000 respectively. 5. It is suggested that these fragments are linked *in vivo* via disulphide bridges to form the monomer, which can then aggregate via further disulphide links. 6. The fragment of mol.wt. 7000 has been obtained in a substantially pure state. 7. Both disulphide and thiol groups are necessary for enzyme activity. 8. Similarities and differences existing between bacterial rhodanese, mammalian rhodanese and β -mercaptopyruvate sulphurtransferase are discussed.

Rhodanese (thiosulphate-cyanide sulphurtransferase, EC 2.8.1.1) catalyses the reaction:

It was discovered by Lang (1933), and has been crystallized from ox liver by Sörbo (1953*a,b*) and from kidney by Westley & Green (1959). The enzyme is widely distributed in Nature. In animals the largest amounts are found in liver and kidney cortex, the smallest in skeletal and smooth muscle, bone marrow and spleen (Sörbo, 1953*c*). Its occurrence in the roots of plants has been reported (Castella Bertran, 1954). In bacteria, rhodanese has been shown to be present in *Escherichia coli* (Stearns, 1953), *Thiobacillus thiooxydans* (McChesney, 1957) and *T. denitrificans* (Woolley, 1961).

The formation of CNS⁻ from colloidal sulphur and CN⁻ is catalysed by a different enzyme, rhodanese S, present in blood serum (Sörbo, 1955).

The biological role of rhodanese is not clear, although its possible function in cyanide detoxification has been repeatedly stressed. Rhodanese and thiosulphate added to cyanide-inhibited cytochrome *c* oxidase give a rapid reactivation of the latter enzyme (Sörbo, 1957). The inhibition of cytochrome *c* oxidase is the chief reason for the high toxicity of cyanide, since the cyanide complex can no longer act as an electron acceptor in the chain (West & Todd, 1961).

No differences were observed in a comparison of rhodanese from liver and kidney (Westley, 1959).

The reasons for the present work are twofold: (i) to compare the properties of *Thiobacillus rho-*

danese with those of the mammalian enzyme to see whether any species differences exist; (ii) to add to knowledge of the thiobacilli, whose metabolism is very imperfectly understood. Rhodanese is a sulphurtransferase, and sulphur is central to the metabolism of these organisms. Moreover, one of the substrates of rhodanese is thiosulphate, the energy source of *T. denitrificans* used in these studies.

A preliminary report on rhodanese from *T. denitrificans* has been published (Bowen, Butler & Happold, 1965).

MATERIALS AND METHODS

Organism and culture. The organism used was *Thiobacillus denitrificans*, Oslo strain, originally obtained from Dr K. S. Baalsrud, and maintained in this Department.

The growth medium was that of Baalsrud & Baalsrud (1954) and contained (per l.): Na₂S₂O₃·5H₂O, 5g.; KNO₃, 2g.; NH₄Cl, 0.5g.; MgCl₂·6H₂O, 0.5g.; KH₂PO₄, 2g.; NaHCO₃, 1g.; FeSO₄·7H₂O, 0.01g. The organism was maintained in 30 ml. screw-capped bottles, completely filled with the above medium, and stored at 0–5°. Subcultures were made at monthly intervals by using a 1 ml. inoculum. During incubation for 3 days at 30° a bubble of gas formed and the medium became turbid owing to precipitation of sulphur and an increase in bacterial population.

Crops of *T. denitrificans* were grown in 91. batches contained in 10l. flasks, by using a 90 ml. inoculum. After incubation, cells were harvested in a de Laval centrifugal separator, then centrifuged at 9000g at 0° for 30 min., giving a red-brown layer of cells overlying a cream layer of sulphur. The cell layer was removed, suspended in 0.1M-phosphate buffer, pH 7.0, and recentrifuged. The yield of cells after washing was about 1g. wet wt./91. of culture.

This low yield of cells hindered work in the earlier stages, but, subsequently, large crops of the organism were grown for us at the Microbiological Research Establishment, Porton, Wilts.

Assay of enzyme. The method used was a modification of that of Bowler (1944): a mixture of 1.5 ml. of 0.067-M-phosphate buffer, pH 8.1, 0.5 ml. of 0.1-M- $\text{Na}_2\text{S}_2\text{O}_3$ and 0.5 ml. of enzyme solution was preincubated for 10 min. at 30°, and then 0.5 ml. of 0.1-M-KCN was added. After a further 10 min. the reaction was stopped by adding 0.25 ml. of formalin. Then 2.75 ml. of 16% (w/v) $\text{Fe}(\text{NO}_3)_3$ in N-HNO_3 was added, and the extinction at 470 m μ was determined in a Unicam SP.600 spectrophotometer, reading against a blank consisting of the complete system but to which the formalin had been added before the cyanide. A calibration curve was prepared by using known amounts of KCNS: a linear relationship was found up to extinction 1.0, corresponding to 1304 m μ moles of KCNS.

Protein estimation. Protein was estimated either by the biuret method of Robinson & Hogden (1940) or by the Lowry modification of the Folin-Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951).

Chemicals. Chemicals were obtained from the following suppliers: insulin was from Boots Pure Drug Co. Ltd., Nottingham; $(\text{NH}_4)_2\text{SO}_4$ (enzyme grade, low in heavy metals), iodoacetamide, GSH and tris were from British Drug Houses Ltd., Poole, Dorset; Aquacide I was from Calbiochem, Lucerne, Switzerland; cytochrome c, deoxyribonuclease (ex bovine pancreas), lipoic acid, 2-mercaptoethanol, *N*-ethylmaleimide, ribonuclease and sodium dodecyl sulphate were from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; glucagon was from Eli Lilly and Co. Ltd., Basingstoke, Hants.; Blue Dextran 2000 and Sephadex were from Pharmacia, Uppsala, Sweden; *p*-chloromercuribenzoate (sodium salt) and mammalian rhodanese (bovine, type I) were from Sigma Chemical Co., London, S.W.6. The lipoic acid was neutralized with NaOH; NaBH_4 was used to obtain reduced lipoate from the sodium salt. Tris was purified before use by recrystallization from ethanol-water (4:1, v/v) containing 0.05% of EDTA. All other chemicals were A.R. grade.

RESULTS

Induction of the enzyme. The addition of potassium cyanide at 40 μM to a 48 hr. culture increased rhodanese activity threefold after a further 24 hr. compared with a culture to which no cyanide was added. An increase in potassium cyanide concentration to 400 μM gave no further increase in rhodanese activity.

Preparation of extracts. Extracts were made by subjecting a suspension composed of 3 g. of cells and 30 ml. of 0.1-M-phosphate buffer, pH 7.0, to ultrasonic disintegration in MSE apparatus (Measuring and Scientific Equipment Ltd., London, S.W. 1). After 25 min. the rhodanese activity of the supernatant (after centrifuging at 15000 *g* for 30 min. at 0°) reached 47% of that of the whole cell suspension; continued treatment resulted in a fall of activity. Simultaneously, the concentration of

protein in the supernatant reached a maximum, and thereafter remained constant.

Attempts to prepare extracts by using the Hughes press and extraction of acetone-dried cells, either alone or in conjunction with the butanol procedures of Morton (1955), gave less active extracts, 20–39% of the activity being recovered.

Preliminary treatment of extracts. Small amounts of deoxyribonuclease and ribonuclease were added to break down nucleic acids. The enzyme was then precipitated by the addition of ammonium sulphate to 2 M, at pH 7.8. After centrifuging, the precipitated protein was dissolved in 0.01-M-tris-hydrochloric acid buffer, pH 7.2, containing sodium thiosulphate (0.01 M) and dialysed overnight at 0° against this buffer. Thiosulphate was incorporated because Sörbo (1953*a,b*) found it had a stabilizing effect on mammalian rhodanese. Although fractionation at pH 7.8 gave a broad precipitation of activity, a 60% recovery was obtained. Precipitation at pH 3.8 or 5.8 gave somewhat sharper fractionation of activity, but a much lower recovery of total activity.

Behaviour on Sephadex. The mol.wt. of rhodanese, 37100 from mammalian sources (Sörbo, 1953*a,b*), would appear to render it suitable for purification on Sephadex G-75 (nominal exclusion limit 50000). Fig. 1(*a*) shows the elution of 5 ml. of extract from a column (2 cm. $\times$ 25 cm.) of Sephadex G-75 equilibrated with 0.01-M-tris-hydrochloric acid buffer, pH 7.2, containing sodium thiosulphate (0.01 M): most of the activity was excluded from the gel. In subsequent experiments with Sephadex, 0.1-M-tris-hydrochloric acid buffer, pH 7.2, containing sodium thiosulphate (0.01 M) was used unless otherwise stated. Fig. 1(*b*) shows the elution of 5 ml. of extract from a column (2.3 cm. $\times$ 30 cm.) of Sephadex G-100 (nominal exclusion limit 100000); elution from a column of Sephadex G-200 (nominal exclusion limit 200000) is shown in Fig. 1(*c*). In both experiments the buffer concentration was 0.01 M.

In an effort to break down these rhodanese aggregates and obtain the enzyme in the monomeric form, the experiment with Sephadex G-75 was repeated, with the following modifications, which have been successful in dissociating aggregates of other proteins (Reithel, 1963): (i) buffer pH changed to 3.8 and sodium thiosulphate omitted; (ii) buffer pH changed to 5.8 and sodium thiosulphate omitted; (iii) buffer pH changed to 10.0; (iv) extract submitted to further ultrasonic treatment (15 min.) before application to the column; (v) M-NaCl in 0.01-M-buffer; (vi) 8 M-urea in 0.01-M-buffer; (vii) 1% (w/v) sodium dodecyl sulphate in buffer; (viii) 1% (w/v) 2-mercaptoethanol in buffer; (ix) 3% (w/v) 2-mercaptoethanol in buffer. In experiments (i)–(vii) the elution profile was as Fig. 1(*a*).

Fig. 1. Elution of bacterial rhodanase from columns of: (a) Sephadex G-75; (b) Sephadex G-100; (c) Sephadex G-200. Rhodanase activity is expressed as μ moles of KCNS produced/min. by 0.5 ml. of effluent under the conditions described in the text. The protein recovered in the fractions was in each case 100%. The rhodanase activity eluted compared with the activity applied to the column was: (a) 109%; (b) 145%; (c) 112%.

Figs. 2(a) and 2(b) show the effect of mercaptoethanol at 1% and 3% respectively.

The fraction containing rhodanase of low molecular weight from experiment (ix) was dialysed to remove mercaptoethanol and then concentrated by using Aquacide I (concentration in the presence of mercaptoethanol gave precipitation of protein and loss of activity). Mercaptoethanol was then replaced (final concn. 3%) and the preparation examined in a Beckman-Spinco model E ultracentrifuge (Spinco Division, Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) at 59780 rev./min. No sedimentation peak was observed, and activity was found to be still present in the supernatant. When submitted to disk electrophoresis (Ornstein & Davis, 1962) on polyacrylamide gel, the supernatant was found to contain rhodanase in an essentially pure state: one deeply coloured band was observed after staining,

Fig. 2. Elution of bacterial rhodanase from a column of Sephadex G-75: (a) in the presence of 1% mercaptoethanol; (b) in the presence of 3% mercaptoethanol. Rhodanase activity is expressed as μ moles of KCNS produced/min. by 0.5 ml. of effluent under the conditions described in the text. The protein recovered in the fractions was: (a) 100%; (b) 93%. The rhodanase activity eluted compared with the activity applied to the column was: (a) 100%; (b) 136%.

along with three very faint ones, visible only under optimum conditions of illumination and contrast.

Size of the mercaptoethanol-produced fragment. Ultracentrifuge data suggested the mol.wt. was much less than 37100, and this was confirmed when extract containing 3% of mercaptoethanol was applied to a Sephadex G-50 column (nominal exclusion limit 10000), a considerable amount of activity entering the gel particles (Fig. 3a). A cytochrome *c* marker was incorporated into the sample. Under identical conditions, no activity was eluted after a cytochrome *c* marker when mammalian rhodanase was applied to the Sephadex G-50 column in the presence or absence of mercaptoethanol (Fig. 3b).

Although the mercaptoethanol-produced fragment is small enough to enter a Sephadex G-50 gel, it is totally excluded from Sephadex G-25 (nominal exclusion limit 5000) (see Fig. 3c).

The method of Andrews (1964) was used to determine the molecular weight of the mercaptoethanol-produced fragment, and of the other rhodanase-active species observed. A column (2.3 cm. $\times$ 55 cm.) of Sephadex G-75 was equilibrated with 0.05 M-tris-hydrochloric acid buffer, pH 7.2, containing sodium chloride (0.1 M). Column packing was checked by running 2 ml. of a 0.2% solution of Blue Dextran 2000 through the column.

Fig. 3. Elution of rhodanese from Sephadex columns: (a) bacterial rhodanese (plus cytochrome *c*) from Sephadex G-50 in the presence of 3% mercaptoethanol; (b) mammalian rhodanese (plus cytochrome *c*) from Sephadex G-50 in the presence of 1% mercaptoethanol; (c) bacterial rhodanese from Sephadex G-25 in the presence of 1% mercaptoethanol. Rhodanese activity (O) is expressed as μ moles of KCNS produced/min. by 0.5 ml. of effluent under the conditions described in the text. Cytochrome *c* (●) is expressed as extinction units at 407 $m\mu$. For (a), the recovery of protein was 85% and of rhodanese activity 161%; the recoveries for (b) and (c) are not available.

Glucagon, insulin, cytochrome *c* and mammalian rhodanese were used to obtain the relationship between $\log(\text{mol.wt.})$ and elution volume from the column (Fig. 4). In the experiment with glucagon, the buffer pH was changed to 8.0 to facilitate solution of the glucagon. Glucagon was detected by extinction at 215 $m\mu$, insulin at 280 $m\mu$, cytochrome *c* at 407 $m\mu$, and both bacterial and mammalian rhodanese by using the enzyme assay. When the weight of protein applied to the column was less than 5 mg., sucrose (5 mg.) was added to the sample to increase its density. From this graph, it was found that, within the accuracy of the method: (i) the mol.wt. of bacterial rhodanese monomer is 38 000; (ii) a sub-unit of mol.wt. 9000 also exists in

Fig. 4. Molecular weights of rhodanese species as a function of their elution volumes from a column of Sephadex G-75. O, Proteins of known molecular weight: 1, glucagon; 2, insulin dimer; 3, cytochrome *c*; 4, mammalian rhodanese. ●, Rhodanese-active species: A, bacterial rhodanese fragment; B, bacterial rhodanese fragment; C, bacterial rhodanese sub-unit; D, bacterial rhodanese monomer.

small amount; (iii) the mercaptoethanol-produced fragment is smaller than this, the mol.wt. being 7000; (iv) a second fragment, of mol.wt. 2000, is also produced by mercaptoethanol. This last fragment showed very weak activity, only 0.67% of that of the larger fragment. If we assume that the fragments of mol.wt. 7000 and 2000 are produced in equal numbers by mercaptoethanol (by fission of the sub-unit of mol.wt. 9000, then the turnover numbers of the two fragments are in the ratio 150:1.

In Fig. 1(c), if the third and fourth peaks are taken to be monomer and sub-unit respectively (identified by eluting mammalian rhodanese and cytochrome *c* from the same column), the second peak corresponds from its elution volume to an aggregate of mol.wt. about 140 000–150 000, i.e. a tetramer.

Other properties. A rhodanese preparation obtained from a Sephadex G-75 column (plus mercaptoethanol) and previously shown to be substantially pure was used.

(a) *Dialysis.* On dialysis, considerable activity was lost. This could be partially restored by the addition of mercaptoethanol. Reapplication of the dialysed preparation to Sephadex G-50 did not show the presence of monomeric or aggregated forms of the enzyme.

The dialysed preparation was used in the following experiments.

Table 1. *Effect of various inhibitors on bacterial rhodanese*

Reaction systems contained 0.25 ml. of enzyme solution, 0.5 ml. of 0.1 M-Na₂S₂O₃, 1.5 ml. of 0.067 M-phosphate buffer, pH 8.1, and 0.25 ml. of inhibitor solution of concentration 12× that shown in the Table. After incubation for 10 min. at 30°, 0.5 ml. of 0.1 M-KCN was added; the remainder of the assay was performed as described in the text.

Inhibitor	Concn. (mm)	Inhibition (%)
Iodoacetamide	5.0	100
N-Ethylmaleimide	1.0	20
Sodium sulphite	10	35
p-Chloromercuribenzoate	0.001-0.1	0
Sodium arsenite	0.01-1.0	0

(b) Active centre. The effects of various inhibitors on the enzyme are shown in Table 1. Mercaptoethanol enhanced the activity of rhodanese 1.9-2.4-fold over a concentration range 0.1-3.0% (w/v). This corresponded to final concentrations of 2.1-64.0 mM in the completed reaction systems. A 2.2-fold increase in activity was found with reduced lipoate at 10 mM, and GSH at 1 mM gave a 1.4-fold increase.

(c) pH optimum. This was determined by using tris-hydrochloric acid buffers, pH 7.2-9.0. A broad optimum between pH 8.0 and 9.0 was obtained.

(d) Copper. Copper was not present in the preparation either in the free or bound form (dithione method of Sandell, 1959).

(e) Sulphurtransferase activity. The preparation showed no sulphurtransferase activity with mercaptoethanol as substrate, CNS⁻ being produced only when S₂O₃²⁻ was present.

DISCUSSION

Aggregates. In view of the specificity of mercaptoethanol in breaking down rhodanese aggregates it seems likely that these are linked via disulphide bridges. A mammalian rhodanese polymer has been postulated by Sörbo (1953a,b). He found that insoluble material was sometimes produced on ammonium sulphate precipitation. This was soluble in 0.1 M-sodium thiosulphate, however, to give a highly active preparation, from which crystalline rhodanese could be obtained, and he suggested that this thiosulphate-soluble rhodanese represented a polymer in which enzyme molecules were held together with disulphide bridges. Other proteins dissociated by mercaptoethanol are β -mercaptopyruvate sulphurtransferase (EC 2.8.1.2) and serum globulins (Reithel, 1963).

The mol.wt. of rhodanese 'monomer' has been shown to be 38 000 (36 000 calculated from 4 × sub-

unit mol.wt.), the same as that of the mammalian enzyme. As well as the sub-unit of mol.wt. about 9000, i.e. one-quarter the size of the monomer, a tetramer (mol.wt. about 150 000) has been detected, but no dimer or trimer. A pattern is apparent, with molecular weights of rhodanese-active species occurring in multiples of 4. Some activity is also excluded from Sephadex G-200 gel (nominal exclusion limit 200 000, but probably about 500 000 for proteins). Hence a larger aggregate of mol.wt. 600 000 may exist, though this activity excluded from Sephadex G-200 gel may be caused by particulate enzyme, i.e. enzyme attached to minute particles of cell debris, not sedimented at 15000g during preparation of the extract.

In view of the existence of several active species of rhodanese the lack of sharpness of precipitation of activity by ammonium sulphate is not surprising, as different species would be expected to be precipitated at different ammonium sulphate concentrations.

Fragments of lower molecular weight. The larger rhodanese fragment produced by mercaptoethanol was excluded from Sephadex G-25, though not from Sephadex G-50, suggesting that its mol.wt. was 5000-10 000; a determination by using gel filtration gave a value of 7000 with another fragment of mol.wt. 2000.

The action of mercaptoethanol on bacterial rhodanese is similar to its action on β -mercaptopyruvate sulphurtransferase (Fanshier & Kun, 1962). This enzyme is related to rhodanese, catalysing the transfer of sulphur from mercaptopyruvate to cyanide and producing pyruvate and thiocyanate. The enzyme has mol.wt. 40 000 and is split into four copper-containing sub-units of mol.wt. 10 000 by mercaptoethanol. Both bacterial rhodanese and mercaptopyruvate sulphurtransferase can exist as a highly-active unstable fragment and a less-active stable monomer. Bacterial rhodanese differs from β -mercaptopyruvate sulphurtransferase in that it can be further dissociated by mercaptoethanol into fragments of mol.wt. 7000 and 2000, of which the larger is highly active and does not contain copper.

The further dissociation of bacterial rhodanese to particles of mol.wt. 7000 and 2000 by mercaptoethanol suggests a disulphide bridge between the two pieces in the intact sub-unit, which does not exist in the mercaptopyruvate sulphurtransferase sub-unit. Dialysis of the mercaptoethanol-produced fragment of bacterial rhodanese led to a loss of activity, which can be explained either by the removal of mercaptoethanol needed to stabilize the fragment, or by removal of the piece of mol.wt. 2000. No recombination of the bacterial rhodanese fragment to active higher-molecular-weight forms could be detected, as had also been shown for mercaptopyruvate sulphurtransferase.

In contrast with bacterial rhodanese, mammalian rhodanese does not break down into smaller fragments with mercaptoethanol.

Active centre. Inhibition of the enzyme by iodoacetamide and *N*-ethylmaleimide (both thiol-group alkylators) suggests that thiol groups are necessary for enzyme activity. The mercaptide formers *p*-chloromercuribenzoate and arsenite did not inhibit the enzyme: it is probable that chelation of the mercurial by the cyanide present as substrate protected the active centre. The activating effect of mercaptoethanol on the enzyme supports the idea that thiol groups are necessary for activity.

Inhibition by sulphite points to disulphide groups also being involved, and this conclusion was supported by the deactivating effect of mercaptoethanol at higher concentrations. Although sulphite is an enzyme product, the reaction producing SO_3^{2-} and CNS^- is virtually irreversible, so that SO_3^{2-} cannot act by displacing the equilibrium.

It was found for mercaptopyruvate sulphurtransferase that a balance between thiol and disulphide groups was needed for optimum activity, and the same appears to be the case here. Mammalian rhodanese is also inhibited by SO_3^{2-} and by reagents for thiol groups (Sörbo, 1953*a,b*). Copper was found in the mercaptopyruvate sulphurtransferase sub-unit, but is not present in the mercaptoethanol-produced fragment of bacterial rhodanese. For mercaptopyruvate sulphurtransferase, mercaptoethanol also increased activity up to a certain concentration, then decreased it.

A possible mechanism for bacterial rhodanese is shown in Scheme 1. The intermediate complex, containing the trisulphide group, may react with one step or in two to regenerate the enzyme. A similar complex with the trisulphide group has been postulated as an intermediate for mercaptopyruvate sulphurtransferase by Fanshier & Kun (1962). Villarejo & Westley (1963) suggest that (for mammalian rhodanese) sulphur is removed from 'Enzyme-S' by dihydrolipoate to give lipoate persulphide, which then reacts with cyanide to regen-

erate the dihydrolipoate and produce CNS^- . No inactivation of our enzyme was found on dialysis (unless mercaptoethanol was present initially), and, as dihydrolipoate is present in cells bound to protein (Reed, 1960), would not be expected if dihydrolipoate is a cofactor. The participation of dihydrolipoate in the bacterial rhodanese system therefore cannot be ruled out.

The pH optimum of bacterial rhodanese is the same (pH 8-9) as that of the mammalian enzyme (pH 8.6).

To conclude, bacterial rhodanese resembles the mammalian enzyme in its use of $\text{S}_2\text{O}_3^{2-}$ as substrate, non-participation of copper in the active centre, formation of aggregates and pH optimum. But in its sensitivity to mercaptoethanol, with the formation of a highly-active unstable fragment, it resembles β -mercaptopyruvate sulphurtransferase. Thus bacterial rhodanese is a distinct enzyme, with a unique combination of properties and occupying a transitional position among sulphurtransferases. The discovery of an enzymically active form of rhodanese of mol.wt. approx. 7000 opens up the possibility of structural determinations that could throw further light on the mechanism of action of the enzyme.

The very weak rhodanese activity of the fragment of mol.wt. 2000 is of a very much lower order than that of the fragment of mol.wt. 7000. The low activity shown may be connected with the existence in it of thiol groups formed by the action of mercaptoethanol on the sub-unit of mol.wt. 9000. Disulphide bridges could also be present, possibly by mild reoxidation. This may simulate, inefficiently, the active centre of rhodanese.

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Scheme 1.

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