REFERENCES

- Alexander, W. D. & Johnson, K. W. M. (1956). *Nature, Lond.*, **178**, 259.
- Arnold, K. H. (1951). *Tables to Facilitate Sequential t-tests. Appl. Math. Ser. U.S. Bur. Stand.* no. 7.
- Brody, T. M. (1956). *J. Pharmacol.* **117**, 39.
- Clark, A. J. (1937). *General Pharmacology*. In *Handbuch der Experimentellen Pharmakologie*, vol. 4. Ed. by Heffter, A. Berlin: Springer.
- Gaddum, J. H. (1948). *Pharmacology*, 3rd ed. London: Oxford.
- Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
- Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
- Peiss, C. N. & Field, J. (1952). *Arch. Biochem. Biophys.* **36**, 276.
- Simon, E. W. (1953). *Biol. Rev.* **28**, 453.
- Smith, A. H., Emmens, C. W. & Parkes, A. S. (1947). *J. Endocrin.* **5**, 186.
- Sproull, D. H. (1954a). *Brit. J. Pharmacol.* **9**, 121.
- Sproull, D. H. (1954b). *Brit. J. Pharmacol.* **9**, 262.
- Sproull, D. H. (1956). *Biochem. J.* **62**, 372.
- Wald, A. (1947). *Sequential Analysis*. New York: Wiley.

Studies on Sulphatases

18. PREPARATION OF CHONDROITINASE-FREE CHONDROSULPHATASE FROM EXTRACTS OF *PROTEUS VULGARIS**

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Dodgson, Lloyd & Spencer (1957a) have shown a strain of *Proteus vulgaris* (National Collection of Type Cultures, no. 4636) to be a particularly potent source of the enzyme chondrosulphatase. The enzyme was associated with a mucopolysaccharase (chondroitinase) system which was able to degrade the polysaccharide chain of chondroitin sulphate with release of reducing substances. The method used to extract chondrosulphatase from the acetone-dried cells of *Proteus* also liberated chondroitinase into solution, and the association of the two enzymes persisted during the subsequent purification procedures. The presence of both chondrosulphatase and chondroitinase has been noted in other bacteria by several workers, including Buehler, Katzman & Doisy (1951), Reggianini (1950a, b) and Konetza, Pelczar & Burnett (1954), and in the marine mollusc *Charonia lampas* (*Triton nodiferus*), by Soda & Egami (1938).

The extent to which the activities of the two enzyme systems are interdependent (i.e. whether chondroitinase action is dependent on preliminary chondrosulphatase attack, or vice versa) is not clear, although Konetza *et al.* (1954), working with viable cells of *P. vulgaris*, obtained evidence which suggested that chondroitinase action preceded that of chondrosulphatase. With the mollusc enzymes, chondroitinase action can proceed independently of the associated sulphatase (Soda & Egami, 1938).

The present communication describes a method by which the chondrosulphatase of *P. vulgaris* can be

separated from the associated chondroitinase. A preliminary account of the work has already been given (Dodgson & Lloyd, 1957).

MATERIALS AND METHODS

Potassium chondroitin sulphate. The method of Einbinder & Schubert (1950) was modified as described by Dodgson *et al.* (1957a).

Testicular hyaluronidase. Both commercial (L. Light and Co., Ltd.) and laboratory (Dorfman, 1955) preparations of the enzyme were used.

Degraded chondroitin sulphate. Potassium chondroitin sulphate (50 ml. of a 0.225% solution in 0.2M-sodium acetate, adjusted to pH 5.5 with acetic acid) was incubated with testicular hyaluronidase at 37.5°, toluene being added as a preservative. A total of 150 mg. of hyaluronidase, added in three 50 mg. portions over a period of 48 hr., was necessary before exhaustion of the release of reducing substance was achieved. At the end of the incubation period the pH of the mixture was re-adjusted to 7 with N-NaOH and the preparation was kept in the frozen state until required.

Calcium phosphate gel. This was prepared by the method of Keilin & Hartree (1938); the final volume of the suspension was adjusted with water to give a concentration of 8 mg. of calcium phosphate (dry wt.)/ml.

Assay of chondrosulphatase and chondroitinase activity. The methods used were described by Dodgson *et al.* (1957a).

Determination of nucleic acid and protein. The nucleic acid content of the enzyme preparation at various stages during the purification procedure was determined from the ratio of the spectrophotometric readings at 280 and 260 m μ (Warburg & Christian, 1941). Protein was determined by the method of Lowry, Roseborough, Farr & Randall (1951).

* Part 17: Dodgson, Rose, Spencer & Thomas (1957b).

A calibration curve was constructed from increasing concentrations of the *Proteus* stage-C enzyme preparation (see Dodgson *et al.* 1957*a*), and the spectrophotometric readings were made with the Unicam SP. 600 spectrophotometer at 750 m μ .

EXPERIMENTAL AND RESULTS

Preliminary concentration of chondrosulphatase and chondroitinase

With 40 g. of the acetone-dried cells of *Proteus* as starting material a preliminary concentration of the two enzymes as far as stage *B* (see Dodgson *et al.* 1957*a*) was made. The subsequent purification step, involving the removal of nucleic acid to give preparation *C* (Dodgson *et al.* 1957*a*), was modified slightly since the original procedure was not completely effective when applied to the bulk preparation of the enzyme concentrate, and the nucleic acid which remained (1–3 %) interfered markedly with the subsequent separation procedure. Complete removal of nucleic acid was achieved as follows. The procedure was identical with that described by Dodgson *et al.* (1957*a*) as far as the treatment with protamine sulphate. In the modified procedure the concentration of the aqueous protamine sulphate solution was increased to 2 % (w/v), the pH being adjusted to 6.7 with *N*-NaOH.

The protamine sulphate solution was added until the clear supernatant obtained by centrifuging at 18 000 *g* for 5 min. no longer gave a precipitate on adding further protamine sulphate solution. A slight excess of protamine sulphate solution (one-twentieth of the volume of the enzyme solution before the addition of protamine sulphate) was added and the whole dialysed for 48 hr. against several changes of water (40 vol. in all). Material which precipitated during dialysis was removed by centrifuging at 4000 *g* and 0° for 20 min. The supernatant solution was adjusted to pH 8 with *N*-NaOH, kept at 2° for 20 min. and clarified by centrifuging at 18 000 *g* and 0° for 15 min. The clear solution was freeze-dried and stored at 0°. Material prepared in this way (preparation *C*2) contained less than 1 % of nucleic acid (see Fig. 1).

Effect of inhibitors on the chondrosulphatase and chondroitinase activities of preparation C2. Some previous attempts had been made to determine the degree of interdependence of the sulphatase and chondroitinase systems of *Proteus* by comparing the time-activity curves for the two enzymes (Dodgson *et al.* 1957*a*). However, as no evidence of interdependence was obtained from these curves the effect on the two enzymes of some common inhibitors was tested. The inhibitor (0.3 ml.), in 0.2*M*-sodium acetate-acetic acid solution at pH 7, was added to 0.3 ml. of enzyme solution (preparation *C*2, 0.65 % solution in 0.2*M*-sodium acetate-acetic acid, pH 7) and the mixture was incubated for 20 min. at 37.5°. Potassium chondroitin sulphate (0.6 ml. of a 0.225 % solution in 0.2*M*-sodium acetate-acetic acid, pH 7, and previously warmed to 37.5°) was then added and incubation was continued for a further 1 hr. before determining the liberated sulphate and reducing substance. Suitable control determinations were made in which the inhibitor was omitted. It can be seen from Table 1 that phosphate and fluoride were powerful inhibitors of the sulphatase but were without effect on chondroitinase. It is clear that chondroitinase activity is not dependent on a preliminary hydrolysis of ester sulphate groupings. On the other hand, none of the compounds tested selectively inhibited chondroitinase activity and this finding is compatible with the possibility that some preliminary degradation of the polysaccharide chain is necessary before enzymic release of sulphate can occur. When the sulphatase-inhibition experiments were repeated with chondroitin sulphate which had previously been degraded by testicular hyaluronidase similar results were obtained, although there was an appreciable reduction in the extent of the inhibition by MgCl₂ which could not be attributed simply to the presence of extra protein (testicular hyaluronidase) in the incubation mixture.

Fig. 1. Ultraviolet-absorption spectra of aqueous solutions of the enzyme preparation at various stages during purification. Concentrations of the solutions were adjusted to give similar optical densities at 320 m μ . ●, Stage *B*; ○, stage *C*; ×, stage *C*2.

Table 1. *Effect of inhibitors on the chondrosulphatase and chondroitinase systems of Proteus vulgaris*

Inhibitors were allowed to act as described in the text and the residual enzyme activities were measured against a 0.225% solution of potassium chondroitin sulphate in the presence of 0.2M-sodium acetate-acetic acid solution, pH 7. Incubation was for 1 hr. at 37.5°. Chondrosulphatase activity was also measured with the same concentration of a solution of potassium chondroitin sulphate which had first been exhaustively degraded with testicular hyaluronidase.

Inhibitor	Concn. (M)	Inhibition (%)		
		Chondroitinase	Chondrosulphatase assayed with	
			Polymerized substrate	Degraded substrate
NaCl	0.25	0	0	—
MgCl ₂	0.10	84	77	36
	0.04	31	42	13
MnCl ₂	0.10	100*	100	100
	0.04	100*	100	100
CoCl ₂	0.10	100*	100	100
	0.04	100*	100	100
NaCN	0.04	0	0	—
NaF	0.01	0	87	—
Na ₃ PO ₄	0.01	0	100	—
NaHSO ₃	0.01	0	—†	—
Na ₂ SO ₃	0.01	0	—†	—
Hydroxylamine	0.01	0*	83	—
Phenylhydrazine	0.01	0*	52	—

* Inhibitors interfered with normal method of estimation and these results were obtained by the viscosity-reduction method of Swyer & Emmens (1947).

† Inhibitors interfered with method of estimation.

Preparation of chondroitinase-free chondrosulphatase

The close association of chondroitinase and chondrosulphatase was apparent when attempts were made to separate the two activities. Although some degree of purification of both enzymes was obtained by ammonium sulphate or acetone fractionation, paper electrophoresis, pH precipitation or pH inactivation, under a wide variety of experimental conditions, in no case was it possible to separate the two activities.

Some indication of the way in which the two enzymes might be separated was afforded by the work of Soda & Egami (1938). These workers noted in mollusc preparations that both enzymes were adsorbed on alumina gel. On eluting with sodium phosphate solution only chondroitinase activity could be recovered, and it was concluded that the sulphatase had been irreversibly inactivated by the phosphate ion. It was possible, of course, that the sulphatase had remained on the gel but was not noted owing to the inability to demonstrate the presence of the enzyme in the absence of chondroitinase, but this was not considered by these workers. The possibility of selectively adsorbing *Proteus* chondrosulphatase on calcium phosphate gel was investigated.

Adsorption of chondrosulphatase on calcium phosphate gel. For enzyme purification it is generally recommended that preparations of this gel should be allowed to age for varying times before use. However, when aged preparations were used to separate chondroitinase and chondrosulphatase, considerable inhibition of both enzymes occurred. The clear supernatant solution obtained by centrifuging aged gel preparations also inhibited both enzymes. No inhibition was observed, however, when the gel was washed in the following way immediately before use. The aged preparation was centrifuged at 2500 *g* and 0° for 10 min. and the sedimented material was suspended with a glass homogenizer in a volume of water equal to the original gel volume. This washing was repeated four times, and the final suspension was used immediately.

In preliminary separation experiments, portions (2.5 ml.) of an aqueous solution of preparation C2 (0.4%) were mixed with 2.5 ml. portions of gel suspension of varying concentrations, both components having previously been adjusted to pH 6.55 with acetic acid. Each mixture was kept at 2° for 20 min., and during this period was mixed by inversion at intervals of 5 min. The gel was separated by centrifuging at 2500 *g* and 0° for 10 min., and the amounts of chondrosulphatase, chondroitin-

ase and protein remaining in solution were measured. A control determination was made in which water was substituted for gel suspension. The results (see Fig. 2) showed that 50–60% of the sulphatase was adsorbed before adsorption of chondroitinase occurred. Strict control of all the experimental conditions was necessary in order to obtain reproducible results.

Elution of adsorbed chondrosulphatase. The conditions necessary for the elution of the sulphatase were determined as follows. Portions (2.5 ml.) of an aqueous solution of preparation C2 (containing 4 mg./ml.) were each treated with 2.5 ml. of gel suspension containing 6.4 mg. of calcium phosphate (dry wt.), as described in the previous section. These conditions are optimum for selective adsorption of chondrosulphatase (Fig. 2). After centrifuging at 2500 g and 0° for 10 min. the clear supernatant was assayed for chondroitinase, chondrosulphatase and protein; each gel deposit was washed by suspending it in 5 ml. of ice-cold water with a glass microhomogenizer and was kept for 5 min. before centrifuging at 2500 g and 0° for 10 min. The washing was repeated and the final washed deposits were each suspended in 5 ml. of ice-cold sodium acetate solution of varying concentration (0–2M, at pH 8) and were kept for 1 hr. at 2° with occasional mixing. The gel was subsequently removed by centrifuging as before and the clear supernatants were assayed for protein, sulphatase and chondroitinase.

Fig. 2. Adsorption of chondrosulphatase, chondroitinase and protein on calcium phosphate gel. The curves are plotted to show the amount of each component remaining in solution after gel treatment. ●, Chondrosulphatase; ○, chondroitinase; ×, protein.

At this point in the investigation it became obvious that, in the absence of chondroitinase, chondrosulphatase was virtually inactive towards the polymerized form of chondroitin sulphate, and the various eluates, although containing abundant protein, exhibited no sulphatase activity towards this substrate. However, when chondroitin sulphate which had been degraded with testicular hyaluronidase was used as assay substrate, considerable sulphatase activity was demonstrated in certain of the eluates (Table 2). Table 2 shows that maximum elution of sulphatase activity was obtained with 2M-sodium acetate. All the eluates were devoid of chondroitinase activity.

Large-scale preparation of chondroitinase-free chondrosulphatase

When the procedure described above was applied to the large-scale preparation of the sulphatase, about 10–15% of the chondroitinase activity was also adsorbed on to the gel and it was necessary to modify the elution procedure in order to remove this contaminant. The final procedure was as follows. An aqueous solution (50 ml.) of preparation C2 (containing 4 mg./ml.) was treated as described for the small-scale procedure with 16 ml. of washed gel (8 mg. dry wt./ml.) and 34 ml. of water, all the components having been adjusted to pH 6.55 with acetic acid. The gel-enzyme complex was separated by centrifuging as before and washed (×4) by centrifuging in 100 ml. portions of water and (×2) by centrifuging in 100 ml. portions of 0.005M-sodium acetate which had been adjusted to pH 7.2 with acetic acid. The washed gel sediment was suspended in 5 ml. of 2M-sodium acetate solution (pH 8) with a glass homogenizer and kept for 15 min. at 2° before centrifuging at 2500 g and 0° for 10 min. The clear supernatant was retained and the sedimented gel was treated with further 5 ml. portions of 2M-sodium acetate in the same way until no further protein could be eluted from the gel. The various eluates were assayed for chondrosulphatase, with degraded chondroitin

Table 2. *Elution of chondrosulphatase from calcium phosphate gel*

Chondrosulphatase was assayed in the presence of 0.2M-sodium acetate-acetic acid mixture, pH 7, against 0.225% solution of potassium chondroitin sulphate which had been exhaustively degraded by testicular hyaluronidase. Incubation was for 1 hr. at 37.5°. The amount of sulphatase eluted is expressed as a percentage of the activity of preparation C2 towards degraded substrate, before adsorption on the gel.

Concn. of sodium acetate soln. used as eluent (M)	Chondrosulphatase present in the eluates (%)
0	0
0.5	2
1.0	21
1.5	37
2.0	46
2.5	46

Fig. 3. Elution of chondrosulphatase, chondroitinase and protein from calcium phosphate gel. Each curve is plotted in terms of the percentage of the component present in the first eluate. The volume of each eluate was 5 ml. ●, Chondrosulphatase; ○, chondroitinase; x, protein.

sulphate as substrate; for chondroitinase, with polymerized chondroitin sulphate, and for protein. Fig. 3 shows the distribution of these three components in the eluates. The eluates containing sulphatase but no chondroitinase were combined, dialysed against several changes of distilled water (100 vol. in all) and freeze-dried (preparation *D*).

Incubation of preparation *D* (0.6 ml. of a 0.004% aqueous solution) at 37.5° with 0.6 ml. of a 0.225% solution of potassium chondroitin sulphate in 0.2M-sodium acetate-acetic acid, pH 7, resulted in the liberation of 5–6 $\mu\text{g.}$ of SO_4^{2-} ions, irrespective of whether incubation was for 6, 12, 24 or 48 hr. No chondroitinase activity could be detected over similar incubation periods. When degraded chondroitin sulphate was used as assay substrate under the same conditions, about 30 $\mu\text{g.}$ of SO_4^{2-} ions were liberated/hr.

DISCUSSION

The procedure outlined above has consistently yielded a preparation of chondrosulphatase which is devoid of chondroitinase activity. Although no attempt has been made to obtain a chondroitinase preparation which is free from chondrosulphatase, it seems probable that this could be achieved by a suitable modification of the procedure. In any case chondroitinase activity can be studied independently of sulphatase activity by taking advantage of the ability of phosphate ions to inhibit completely the latter enzyme.

There have been no indications during the present work that chondroitinase activity is facilitated in

any way by preliminary removal of ester sulphate groups. In this respect the behaviour of the enzyme towards chondroitin sulphate is similar to that of testicular hyaluronidase but differs from that of bacterial hyaluronidase (Meyer, Davidson, Linker & Hoffman, 1956). On the other hand, it is clear that chondrosulphatase action is greatly facilitated by preliminary degradation of the polysaccharide chain. Indeed, it seems highly probable that the enzyme is without action on polymerized chondroitin sulphate and that the small release of sulphate (5–6 $\mu\text{g.}$ of SO_4^{2-} ions) which was observed with the polymerized substrate merely reflects the presence of traces of sulphated oligosaccharide material in our substrate preparations. Support for this view comes from the fact that the amount of sulphate liberated from polymerized substrate was constant, irrespective of the length of the incubation period. Further support comes from the observation that polymerized substrate shows no marked ability to inhibit the activity of the sulphatase towards the degraded substrate. The implication of these findings is that chondroitin sulphate, as such, is not the true substrate for the enzyme. Certainly there is a strong possibility that the ester sulphate groups of the polymerized substrate would be 'spatially unavailable' to the enzyme, owing to the ability of the substrate molecule to exist in a highly coiled form. The factors governing the molecular shape of polyelectrolytes such as chondroitin sulphate have been discussed at length by Mathews (1953). Briefly, in dilute solution such molecules are considered to exist as extended spirals. The strongly dissociating ester sulphate groups play a considerable part in maintaining the integrity of such spirals by virtue of the forces of mutual repulsion which exist between adjacent sulphate groups. Because of the high negative environment there is a tendency for a molecule of this type to attract positive ions. These in turn tend to neutralize negative groups, such as sulphate, which are holding the molecule in an extended condition. Consequently, when the concentration of the polyelectrolyte is increased, there is an increased tendency for each molecule to collapse and coil. Addition of neutral salts to solutions of chondroitin sulphate decreases the viscosity of the solutions owing to the increased degree of coiling of the chondroitin sulphate molecules (see Table 3). Under the conditions used for the assay of chondrosulphatase in the present work there can be no doubt that the substrate molecules would be present in a highly coiled form. It is therefore interesting that no increased liberation of sulphate from chondroitin sulphate could be obtained by decreasing the concentration of neutral salt in the incubation mixture (Table 3). This may mean that,

Table 3. Effect of sodium acetate on the viscosity of a 0.225% solution of potassium chondroitin sulphate and on the release of sulphate from the solution by chondroitinase-free chondrosulphatase

Viscosity measurements were made as described by Dodgson *et al.* (1957*a*). The release of sulphate remained constant irrespective of the enzyme concentration and the length of the incubation period (6, 12, 24 or 48 hr.).

Concn. of sodium acetate (M)	Reduced viscosity (η_{sp}/C)	Sulphate liberated ($\mu\text{g.}$)
0.5	1.23	5.6
0.2	1.25	5.4
0.1	1.42	5.8
0.05	1.62	5.3
0.025	2.39	5.4
0.0125	2.75	5.5

at the concentration used for enzyme assay, potassium chondroitin sulphate, even in water solution, exists in a highly coiled condition. Under such conditions the spatial availability of the ester sulphate groups might be expected to be very low. The removal of sulphate groups as a result of sulphatase activity might be presumed to increase the degree of coiling of the substrate molecules, since adjacent sulphate groups are considered to be important in holding the molecule in an extended form. This in turn would decrease the availability to the enzyme of the remaining ester sulphate groups. If these postulates are correct then the action of the sulphatase would obviously be facilitated greatly by preliminary degradation of the highly coiled substrate molecule by chondroitinase.

The present work re-opens the question of the specificity of chondrosulphatase. Dodgson *et al.* (1957*a*), using crude *Proteus* concentrates, showed the enzyme to be inactive towards the sulphated polysaccharides agar, fucoidin, carrageenin, *Chondrus ocellatus* mucilage and sulphated laminarin. However, since chondroitinase activity was not followed during these experiments, the findings may simply reflect the inability of chondroitinase to degrade the polysaccharide moieties of these compounds. On the other hand, none of these substrates contains the sulphated acetylgalactosamine residues which are present in chondroitin sulphate.

Korn & Payza (1956) have shown that extracts of a flavobacterium are able to degrade heparin and suggest that a glycosidase and two sulphatases are involved. It will now be important to establish whether, in this case also, chain fragmentation must precede sulphatase action. It would also be interesting to re-investigate the enzyme glycosulphatase in the light of the present work. This enzyme occurs

in bacteria, molluscs and mammals (Dodgson & Spencer, 1957) and is able to hydrolyse glucose 6-sulphate and other simple sulphated carbohydrates. The natural substrates of the enzyme are not known, and it may be wondered whether, in nature, it is normally associated with a chondroitinase type of enzyme, both enzymes being collectively responsible for the degradation of some unknown polysaccharide sulphate. The *Proteus* sulphatase is not identical with glycosulphatase (Dodgson *et al.* 1957*a*) but, in view of the ability of the latter enzyme to liberate sulphate from acetylglucosamine sulphate (Soda & Egami, 1938), it may be identical with one of the two sulphatases which participate in the degradation of heparin (Korn & Payza, 1956).

The present findings may also throw a new light on the failure of certain workers to demonstrate chondrosulphatase activity in mammals (Neuberg & Cahill, 1936; Dohlman & Friedenwald, 1955). Unpublished results from this laboratory have shown that mammalian tissues, in general, do not exhibit sulphatase activity towards polymerized chondroitin sulphate. It will now be important to repeat this work with degraded chondroitin sulphate as substrate, particularly since recent investigations (Dr C. H. Dohlman, personal communication; Dzielatowski, 1956) have shown that slight hydrolysis of chondroitin sulphate can occur in the tissues of the intact rat.

SUMMARY

1. The chondrosulphatase of *Proteus vulgaris* N.C.T.C. no. 4636 has been separated from the associated chondroitinase by selective adsorption of the former on calcium phosphate gel under strictly controlled experimental conditions.

2. The chondrosulphatase which can subsequently be eluted from the gel shows negligible activity towards the polymerized form of chondroitin sulphate, but is very active towards chondroitin sulphate which has been exhaustively degraded by testicular hyaluronidase.

3. Chondroitinase activity is in no way dependent on a preliminary hydrolysis of ester sulphate groups by chondrosulphatase, since when preparations containing both enzymes are incubated with polymerized chondroitin sulphate in the presence of phosphate ions sulphatase activity is completely inhibited whereas chondroitinase activity is not affected.

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REFERENCES

- Buehler, H. J., Katzman, P. A. & Doisy, E. A. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 3.
- Dodgson, K. S. & Lloyd, A. G. (1957). *Biochem. J.* **65**, 4p.
- Dodgson, K. S., Lloyd, A. G. & Spencer, B. (1957a). *Biochem. J.* **65**, 131.
- Dodgson, K. S., Rose, F. A., Spencer, B. & Thomas, J. (1957b). *Biochem. J.* **66**, 363.
- Dodgson, K. S. & Spencer, B. (1957). *Meth. biochem. Anal.* **4**, 211.
- Dohlman, C. H. & Friedenwald, J. S. (1955). *J. Histochem. Cytochem.* **3**, 492.
- Dorfman, A. (1955). *Meth. Enzymol.* **1**, 166.
- Dziewiatkowski, D. D. (1956). *J. biol. Chem.* **223**, 239.
- Einbinder, J. & Schubert, M. (1950). *J. biol. Chem.* **185**, 725.
- Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc. B*, **124**, 397.
- Konetza, W. A., Pelczar, M. J. & Burnett, G. W. (1954). *Bact. Proc.* p. 37.
- Korn, E. D. & Payza, A. N. (1956). *Biochim. biophys. Acta*, **20**, 596.
- Lowry, O. H., Roseborough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mathews, M. B. (1953). *Arch. Biochem. Biophys.* **43**, 181.
- Meyer, K., Davidson, E., Linker, A. & Hoffman, P. (1956). *Biochim. biophys. Acta*, **21**, 506.
- Neuberg, C. & Cahill, W. L. (1936). *Enzymologia*, **1**, 82.
- Reggianini, O. (1950a). *Boll. Ist. sieroter., Milano*, **29**, 456.
- Reggianini, O. (1950b). *Boll. Soc. ital. Biol. sper.* **26**, 1117.
- Soda, T. & Egami, F. (1938). *J. chem. Soc., Japan*, **59**, 1202.
- Swyer, G. I. M. & Emmens, C. W. (1947). *Biochem. J.* **41**, 29.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.

Further Studies on the Reaction of Disulphides with Silver Nitrate

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The kinetics of the reaction of silver nitrate with cystine and oxidized glutathione (GSSG) was the subject of previous studies (Cecil, 1950, 1951). It was found that GSSG reacted more rapidly than cystine but, since the reaction showed a number of complexities, comparisons were only semiquantitative. An attempt has been made to investigate these differences of reactivity by a quantitative comparison of eight disulphides under conditions where the complexities are reduced to a minimum. There is still much that is unexplained but, as this work is not being continued, the results obtained so far are reported here.

There has been some disagreement as to the stoichiometry of the reaction of disulphides with silver and with other metals. Lavine (1937), Challenger & Rawlings (1937) and Cecil (1950) found that the reaction stopped at the sulphinic acid stage represented by equation (1):

However, Vickery & Leavenworth (1930) and Preisler & Preisler (1930, 1932) found that some sulphonic acid was formed. We have confirmed there is a definite end point represented by equation (1), but if the reaction is continued for long periods then further changes may occur.

Cecil (1950) assumed, on the basis of earlier work (Lavine, 1937; Challenger & Rawlings, 1937), that

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the initial process is a hydrolysis of the disulphide bond to give thiol and sulphenic acid [reaction (2)]. The thiol is taken up by the excess of Ag^+ ion present (4), causing reaction (2) to proceed to the right. Simultaneously the unstable sulphenic acid dismutates to give sulphinic acid and more thiol (3).

An alternative hypothesis is that the initial reaction is a direct attack by Ag^+ ion on the disulphide bond:

followed by reactions (6), (3) and (4)

In both cases the over-all reaction is given by equation (1). The most direct evidence for reaction (2) is the work of Schiller & Otto (1876) and Fromm (1908), who boiled diphenyl and di-*p*-toluyl disulphides with ethanolic potassium hydroxide. The corresponding thiophenols and sulphinic acids were obtained, but no sulphenic acids. However, the fact that an attack by OH^- ions occurs under these conditions does not necessarily mean that it does so under the milder conditions in this work.

There are several criteria by which the two possible reaction mechanisms can be distinguished: