The Liberation of Hydrogen Sulphide by X-Radiation from Cysteine and Glutathione

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In continuation of experiments on the effects of ionizing radiations on amino-acids, proteins, peptides, etc. (Dale & Davies, 1949; Dale, Davies & Gilbert, 1949*a*, *b*), we have found that hydrogen sulphide is split off, under certain conditions, from cysteine and glutathione. This may be a result of significance in view of the role attributed to the oxidation of thiol groups in glutathione and proteins for the inactivation of enzymes containing sulphydryl groups (Barron & Dickman, 1949; Barron, Dickman, Muntz & Singer, 1949), and the change of the other cell constituents. It shows in particular that the radiation effect does not consist solely of an oxidation of thiol groups to disulphides, and also shows that in the case of cysteine production of hydrogen sulphide takes place in preference to deamination.

This paper deals with the quantitative relationships obtaining for the liberation of hydrogen sulphide from cysteine and glutathione in aqueous solution, by the action of X-radiation under various conditions.

EXPERIMENTAL

Radiation was obtained from a 500 kV. continuously evacuated X-ray tube (half-value layer 5·2 mm. Cu, average $\lambda = 0{\cdot}056$ A.).

The treatment of glassware and the preparations of solutions, etc., followed the lines laid down by Dale, Gray & Meredith (1949).

Irradiation. The solutions were irradiated in sealed, cylindrical gas-washing bottles of soda glass (approximately 2·5 cm. diameter and 10 cm. high) with fused-in tubes. A measured volume of liquid was carefully introduced into the bottle through the inlet tube by means of a narrow-stemmed funnel drawn out from a test tube. If wetting of the inlet tube occurred a spill of filter paper was used to remove any trace of solution from the point where the glass was to be fused. The inlet and outlet tubes were then drawn out to a fine point, sealed, and the bottles irradiated.

Collection and estimation of hydrogen sulphide. The method employed for the determination of H_2S was, in principle, that of Almy (1925) suitably adapted for the present purpose. This method consists essentially of the removal of H_2S by a stream of CO_2, the H_2S being absorbed in zinc acetate solution and estimated colorimetrically as methylene blue. The method proved to be satisfactory, though slight modifications were applied.

After irradiation a scratch was made with a sharp glass knife 0·5 cm. from the sealed ends of each tube. The bottle was then connected to the bubbling train by close-fitting rubber tubing pushed well over the scratch mark, and the glass was broken at the file marks inside the rubber connexions by bending the rubber tubing sharply. The rubber tubing must be of good quality and of wall thickness about 1·5–2 mm. to avoid a puncture during breakage of the glass. It is important that the inlet tube be broken whilst under slight pressure from the CO_2 cylinder before breaking of the outlet tube. In this way the danger of sucking back the irradiated solution was avoided.

In those cases where the pH of the irradiated solution is 5 or greater, it is necessary to introduce acid into the solution in order to drive over the H_2S present. This was done by placing a T-piece in the train between the CO_2 cylinder and the gas-washing bottle. The side arm of the T-piece was connected to a small piece of narrow-bore (3 mm.) rubber tubing fitted with a screw clip. The nozzle of a calibrated all-glass syringe was inserted into the rubber tube, the clip opened, and a measured volume of HCl forced into the system while the stream of gas was already running. The injected fluid was thus swept into the washing bottle by the current of CO_2. The volume and strength of acid injected was adjusted so as to bring the mixture in the bottle to approximately pH 2. When this procedure was applied to the unirradiated solutions zero blanks were obtained.

During the preparation of the calibration curve it was found that the colour depth produced on direct addition of the reagents to a standard sulphide solution always slightly exceeded that obtained on driving over the H_2S by CO_2; i.e. there appeared to be some slight loss in the transfer process. However, this difference was only small and always strictly reproducible, so that no error was introduced in reading from a calibration curve constructed under the exact experimental conditions of transfer by bubbling.

We found that the standard H_2S solutions as recommended by Almy were not entirely satisfactory and, after some trials, adopted the following procedure; 15 ml. of a 20 % (w/v) zinc acetate solution was made up to approximately 430 ml. in a 500 ml. volumetric flask and sufficient aqueous H_2S added to bring the final concentration of ZnS to roughly 6 μg./ml. when made up to 500 ml. This solution was then accurately titrated by the iodate-iodide method. The concentration of ZnS must not greatly exceed 5–6 μg./ml. or slow precipitation will occur.

The amount of fluid irradiated in the bottle was 10 or 25 ml. depending on the yield of H_2S expected. The H_2S was trapped in two filter tubes in series, the first trap containing 10 ml. and the second 5 ml. of zinc acetate solution. At the con-

clusion of bubbling the trapping fluid was quantitatively transferred with 2 or 3 ml. of water into a 25 ml. flask, the reagents added, and the colour allowed to develop for the prescribed time before the volume was made up to the mark and read.

The zinc acetate absorbing solution was of 2% concentration, made by diluting a 20% solution ten times and adding 0·1 ml. N-acetic acid to every 100 ml.; the addition of the acetic acid clears any slight opalescence due to $Zn(OH)_2$.

Colorimetry. The methylene blue solutions were measured in a Hilger 'Spekker' absorptiometer using no. 608 red filters, and 4 cm. cells. We found that the most suitable colour intensities giving the greatest sensitivity for reading were those corresponding to H_2S concentrations in the range 0·05–0·20 μg./ml. After a little experience it can be seen at a glance whether the colour produced is in this range; if the colour was too intense it was suitably diluted with water containing the reagents in the requisite proportions.

RESULTS

Cysteine hydrochloride

The production of H_2S for any one concentration of cysteine hydrochloride is directly proportional to the dose up to 100,000 r. (Fig. 1) which was the highest dose applied.

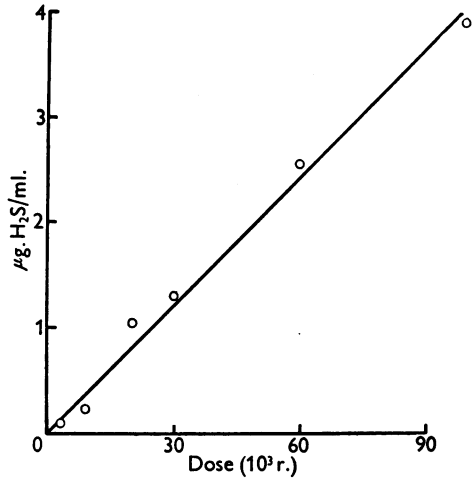

Fig. 1. The yield of H_2S in the X-radiation of cysteine as function of the radiation dose. Cysteine hydrochloride (concentration 7500 μg./ml., pH 2).

Effect of oxygen and of hydrogen peroxide. Solutions saturated with air yield the same amount of H_2S as those which have been highly evacuated; the process therefore seems to be independent of the oxygen content of the solution. Solutions of cysteine hydrochloride were made containing hydrogen peroxide in such concentrations (0·001 M) as would be produced by the X-ray dose used (Bonet-Maury & Lefort, 1948). With these solutions less than 2% of the H_2S yield was obtained in an equivalent time and even ten times the expected hydrogen peroxide concentration produced only one-fifth the yield of H_2S produced by irradiation.

Effect of concentration of cysteine hydrochloride. The yield of H_2S for a given X-ray dose rises with increasing concentration of cysteine hydrochloride solution in a way that is similar to, but not identical with, the production of ammonia from glycine and serine, a flat maximum yield being obtained in the concentration region 1–20% (Fig. 2). Cysteine

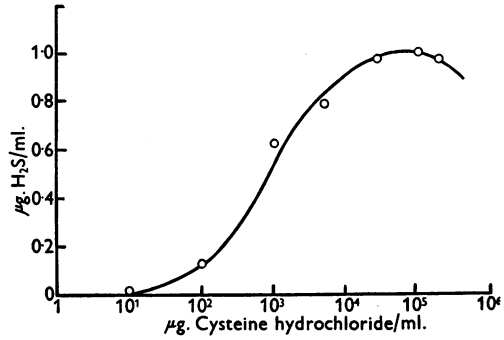

Fig. 2. The yield of H_2S in the X-radiation of cysteine as function of the concentration of cysteine hydrochloride (dose 33,000 r., pH 2).

hydrochloride is extremely soluble, and solutions of very high concentrations (maximum 51·8% at 18°) showed a pronounced falling off in the H_2S yield. The curve shows the total yield from direct as well as indirect effect of radiation. The efficiency of H_2S production is, in general, less than that of ammonia formation from amino-acids. The maximum ionic yield obtained in the concentration region 1–20% is approximately 0·3; the corresponding yield of ammonia from glycine varies from 1 to 3 according to the concentration of glycine (Dale *et al.* 1949*a*).

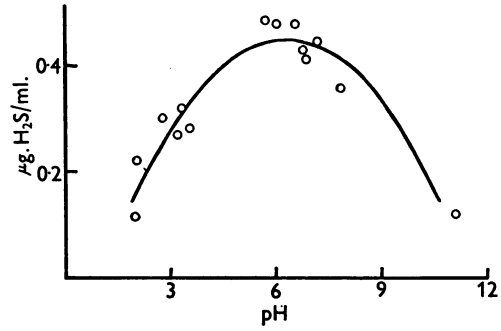

Fig. 3. The yield of H_2S in the X-radiation of cysteine as function of pH. Cysteine hydrochloride (concentration 5000 μg./ml., dose 9500 r.).

Effect of pH. The production of H_2S from cysteine by X-radiation is dependent on pH (Fig. 3) with a maximum at about pH 6. Cysteine, of course, is rapidly oxidized to cystine in alkaline solution, and

in order to study H_2S liberation from cysteine at a pH > 7, sodium pyrophosphate was added as an inhibitor of autoxidation (Warburg & Sakuma, 1923). Control experiments showed that sodium pyrophosphate in M/25 and M/60 concentration had no effect in the production of H_2S by radiation.

In an earlier communication (Dale *et al.* 1949*a*) on ammonia production by X-rays from a wide variety of amino-acids, etc., an effect of pH was noted and this was confirmed by Stein & Weiss (1949). In particular, it was observed that the ammonia yield from cysteine hydrochloride at pH 2 was nil or very small with doses of the order of 10^5 r. It was therefore important to know whether deamination follows the same course as liberation of H_2S over an extended range of pH. It was found that at pH 2, 4 and 6 no ammonia is produced whereas the H_2S production rises to a maximum at about pH 6·5.

Cystine

Cystine can only be studied in either strongly acid or alkaline solution because of its extreme insolubility at neutral pH. In acid solution no liberation of H_2S was observed with the doses used. There was, however, some evidence that in alkaline solution H_2S was produced in very small yield, although the accuracy of the result was lessened by the presence of a large blank; this blank could not be eliminated by recrystallization, and was small when estimated immediately after the solution was made up. It appears, therefore, to be due chiefly to the chemical action of the alkali. It is a well known fact, of course, that cystine loses sulphur, so-called 'labile sulphur', on treatment with alkali, even mild alkali such as sodium carbonate; see, for example, Andrews (1930) and Sheppard & Hudson (1930).

Commercial cysteine hydrochloride was used in the majority of these experiments, and some slight variation in H_2S yield was noted in different batches. Accordingly, attempts were made to purify the acid by recrystallization from concentrated hydrochloric acid (du Vigneaud, Audrieth & Loring, 1930). The crystalline form and appearance of the product appeared to be improved, but melting point and H_2S yield were not materially altered. It should be mentioned that the commercial product gave a zero blank value.

Glutathione

We have found that this biologically important tripeptide also yields H_2S on X-radiation, as might be expected on account of its relation to cysteine. For a solution containing 9500 μg./ml. of glutathione the ionic yield is about 0·25, almost the same as that for an equimolar solution of cysteine hydrochloride (5000 μg./ml.) at pH 2. Fig. 4 shows for reduced glutathione the relationship between pH and H_2S liberation by X-rays. In general, the yield of H_2S rises in the range pH 2–6·5, although an unexpected depression in yield is observed at about pH 5·5.

The oxidized form of glutathione in the same molar concentrations and treated with similar X-ray doses showed no liberation of H_2S, again showing analogous behaviour to the cysteine-cystine system.

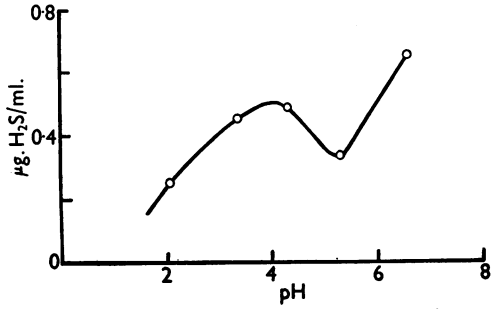

Fig. 4. The yield of H_2S in the X-radiation of reduced glutathione as function of pH. Reduced glutathione (concentration 9500 μg./ml., dose 12,800 r.).

DISCUSSION

It is becoming more and more apparent that sulphur in organic linkage, as well as in its elemental form and in the form of thiosulphate (Dale, 1947; Dale, Davies & Meredith, 1949; Barron & Dickman, 1949; Barron *et al.* 1949; Mole, Philpot & Hodges, 1950; Patt, Tyree, Straube & Smith, 1949; Chapman, Sipe, Eltzholtz, Cronkite & Chambers, 1949) plays an important part in radiation chemistry and radiation biology. Barron and his co-workers consider the oxidation of the thiol form of glutathione and proteins to the disulphide form (a reaction which can be reversed chemically) to be the biologically important step. Others have not investigated this first oxidation step, but rather the destruction of glutathione by X-rays, γ-rays, etc. (Hammett, 1932; Woodward, 1933; Kinsey, 1935), i.e. an irreversible change which may be an oxidation to a further stage or a complete unspecified breaking-up of the molecule. Our experiments have shown that, in addition to any reversible oxidation which may occur, there are irreversible changes leading to the liberation of hydrogen sulphide. We do not know as yet whether this is due to an oxidation or reduction, but experiments are in progress to attempt to solve this problem.

Because of the technique used in previous studies by Kinsey (1935), Hammett (1932) and Woodward (1933) the H_2S reaction escaped detection. These investigators were compelled to use low concentrations in order to be able to measure an at least 10% change in the original concentration with any degree of accuracy. They, therefore, only registered an overall change in concentration of thiol-reacting

material. If, however, reaction product is measured, as in the present investigation, a minute fraction of 1% of the original thiol groups can be determined with accuracy.

It is further of interest that although all the other amino-acids investigated (Dale *et al.* 1949*a*) show deamination by X-radiation in varying degree, cysteine was not deaminated. The radiation energy seems to be used, partly at least, for H_2S production and not for deamination.

The instability of glutathione, particularly its property of readily losing its sulphydryl group as H_2S was commented on by Hopkins (1929). He showed, for example, that during prolonged aeration of an aqueous solution of the reduced form at pH 7·6 (in the presence of a trace of iron) 80% of the thiol form was converted to the disulphide form. The remaining 20% was proved to have lost some of its nitrogen and sulphur. It should be noted that under the conditions of our work no H_2S was detected in the control experiments.

The majority of experiments was deliberately done with cysteine because of its greater stability and the easy suppression of autoxidation in this case. However, the experiments with glutathione prove the essentially similar response to radiation with the one peculiar exception of a depression in the pH curve near pH 5·5. One has to bear in mind, however, that at pH values near neutrality the stability of glutathione is greatly diminished.

It seems that the liberation of H_2S may be of importance from the biological point of view since its occurrence within the cell is likely to have a toxic effect.

SUMMARY

1. Experiments are described on the production of hydrogen sulphide by X-radiation from cysteine and glutathione.

2. The influence of pH, oxygen, hydrogen peroxide, concentration of solute, etc., on the process, has been studied.

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An L-Glutamic Acid Decarboxylase from Barley

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Okunuki (1937) discovered an enzyme in powders prepared from various plant tissues which was specific for the decarboxylation of glutamic acid. He reported that pollen from *Lilium auratum* was a particularly good source of the enzyme, whereas barley seedlings were included in a list of tissues which did not yield active preparations by his method. Schales, Mims & Schales (1946) have since shown that the enzyme can be extracted by water fairly readily from plant tissues and give a list of some thirty species from which it was obtained. Using aqueous extracts from carrots, Schales & Schales (1946*a*) have investigated the characteristics of the enzyme and the reaction kinetics. The present paper describes some experiments on a similar enzyme occurring in seedling barley roots.