Skin tests on human subjects showed no significant difference between the vesicant power of 'deutero-*H*' and that of ordinary *H*.

SUMMARY

1. The preparation, by two methods, of mustard gas containing radioactive sulphur (S^{35}) is described.

2. Methods are described by which the radio-sulphur in S^{35} -containing mustard gas and related compounds, and in biological materials treated with these substances, can be determined.

3. The possible effect on living tissue of the radiation from the S^{35} -containing compounds used in these investigations is briefly discussed.

4. A synthesis of mustard gas with practically all its hydrogen replaced by deuterium is described.

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Studies on Mustard Gas ($\beta\beta'$ -Dichlorodiethyl Sulphide) and some Related Compounds

4. THEIR ACTION ON PROTEINS (STUDIED WITH THE AID OF RADIOACTIVE SULPHUR)†

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Treatment of serum proteins with mustard gas (*H*), under physiological conditions of pH and temperature, effects a very marked alteration in their immunological properties (Berenblum & Wormald, 1939). There is also evidence that several other proteins undergo distinct changes, including an increase in the S content, as a result of the action of *H*; nucleoprotein, for example, is precipitated (Berenblum, 1940); keratine reacts with *H*, in part at least by virtue of its SH groups (Peters & Wakelin, 1941) and collagen is also attacked (Pirie, 1941).

These observations suggest a definite combination of *H* with groups which are present in a variety of

proteins, and since the reaction takes place fairly rapidly at 37° and at pH 7.5, it is not improbable that similar combination occurs when *H* comes into contact with the proteins of the skin and neighbouring tissues [Berenblum (1940), for example, noted that no nucleoprotein could be extracted from *H*-treated rabbit skin]. It was decided, therefore, that a further investigation of this reaction should be made with the aid of *H**, i.e. mustard gas containing radioactive sulphur. (In this paper an asterisk is used to denote the presence of the radioactive S^{35} in a substance, e.g. *H**, *S**, *TG**, *HO** etc.)

This method has the advantage that the amount of *S** present can readily be determined, however small it is, and, within certain limits, however much

† The investigations described here were the subject of reports to the Medical Research Council in 1940-2 and the Ministry of Supply 1941-5.

ordinary sulphur is present in the original protein, and the determinations are not affected by changes in the ordinary sulphur content of the protein during the experiment. Also, since no radio-compound other than the H^* is used, whatever radioactivity is found in the product can be correlated directly with the amount of H^* which has undergone combination. (The possibility of interference by the natural radioactivity of potassium, due to K^{40} , can be excluded, for this activity is very slight, being less than one disintegration/min./mg. of ordinary K, and, in any case, the K present in biological material does not contaminate the $BaSO_4$ spread for S^* determinations.) The sensitivity of this method is, of course, very much greater than that of other methods not employing an isotopic indicator, provided the 'specific activity' of the available S^* is sufficiently high. Even with specimens of relatively low activity, however, the method still possesses a high degree of sensitivity.

Several widely differing proteins have been used in the investigations described in this paper, and all the H^* -treated proteins and similar protein derivatives have been exhaustively extracted with various solvents to remove loosely attached H^* and other S^* -containing compounds. It is believed that the values recorded here for the S^* contents of the various preparations give a measure of the amount of H^* , etc., which has combined firmly with the protein. For many of the experiments we have used human-serum proteins, or the separated globulins or albumins, but several other proteins have also been tested. All the experiments have been carried out under physiological conditions, i.e. at about pH 7.5–8 and at 30–38°.

These investigations with H^* have been made to study (a) the capacity of different proteins to combine with H^* , (b) the speed and extent of the reaction with varying amounts of H^* , and (c) the nature of the linkage involved. In connexion with the last-named problem, particular attention has been paid to the SH groups of the protein, in view of the observation of Peters & Wakelin (1941) that H reacts with these groups. We have made determinations, by iodine titration, of the 'masked' plus free SH in ovalbumin, a protein of high SH content, and also in serum proteins, both before and after treatment with H^* , with the object of comparing the gain in S^* with the decrease in SH. In other experiments the free amino-groups of serum globulin have been blocked by treatment with phenyl isocyanate in order to determine the effect of this treatment on the capacity of the protein to react with H .

The vesicant action of H is destroyed by the action of water, and it was therefore decided to find out whether this loss of vesicant activity is accompanied by loss of the capacity to react with various proteins. For a similar reason, we have studied the

reaction between proteins and thiodiglycol* (TG^*), one of the chief products of hydrolysis of H^* .

The possibility that H might be partly or wholly converted into some other derivative in the tissues cannot yet be excluded, and a few experiments have therefore been carried out to determine the combining powers of $\beta\beta'$ -dichlorodiethyl sulphoxide* (HO^*) and $\beta\beta'$ -dichlorodiethyl sulphone* (HO_2^*), two oxidation products of H^* , towards the serum and other proteins. It is, of course, not suggested that H^* undergoes oxidation before it combines with proteins in the *in vitro* experiments described here.

Many other experiments have been carried out with various proteins, using ordinary H and its derivatives, in an effort to determine which protein groups are affected by these reagents. A few of these experiments are described in this paper and others are dealt with in a paper dealing with the immunological aspects of this work (Bournsnel, Francis & Wormall, 1946*d*).

MATERIALS

H^* , HO^* , HO_2^* and TG^* . The following radioactive sulphur (S^{35})-containing compounds were prepared as described elsewhere (Bournsnel *et al.* 1946*b*): mustard gas (H^*), $\beta\beta'$ -dichlorodiethyl sulphoxide (HO^*), $\beta\beta'$ -dichlorodiethyl sulphone (HO_2^*) and thiodiglycol (TG^*). The TG^* was distilled *in vacuo* before use.

Thymus nucleoprotein. Preparation (i). About 400 g. of fresh calf thymus were extracted several times with a considerable volume of 5.8% NaCl solution (Mirsky & Pollister, 1942). The dilute solution was strained through muslin, filtered, and the nucleoprotein precipitated by adding a little 2*N*-acetic acid. The centrifuged precipitate was suspended in water and dissolved by adding a minimum of 0.25 *N*-NaOH. A small amount of undissolved matter was removed by centrifuging, and the precipitation and re-solution were repeated twice more. The final solution contained about 2.8% protein.

Preparation (ii). The nucleoprotein was extracted from the thymus partly with 5.8% NaCl and partly with 0.01 *N*-NaOH. The combined extracts were purified by precipitation and re-solution as for preparation (i). The final solution contained about 3% protein.

Collagen (ox corneal). This was kindly supplied by Mrs A. Pirie.

Kerateine. This was a sample prepared from wool and kindly supplied by Prof. R. A. Peters. Nitroprusside tests and iodine titrations carried out at a later date suggested that there may have been considerable reoxidation of SH at the time that our experiments were made, and this may explain why the protein combined with less H in our experiments than was observed by Peters & Wakelin (1941).

Ovalbumin. A sample of crystalline chicken ovalbumin kindly supplied by Dr J. F. Danielli.

Human serum. We are greatly indebted to Dr G. A. Harrison for many samples of pooled human serum.

Human-serum globulin and phenylcarbamido-globulin. The globulins were precipitated from human serum by half-saturation with $(NH_4)_2SO_4$, separated by centrifuging and

redissolved by dialysis against 0.9% NaCl. Precipitation and re-solution were repeated twice more, yielding finally an NH_3 -free solution containing 0.47% N.

Phenylcarbamido-globulin was prepared from 165 ml. of this globulin solution by the method described by Hopkins & Wormald (1933), 5.8 ml. of phenyl isocyanate being used. The phenylcarbamido-globulin was finally dissolved in 55 ml. of 0.9% NaCl and the pH adjusted to 7.5; this solution contained 0.41% N. Since the amino-N content of serum globulin is under 1%, the small amount of phenyl isocyanate reacting with the globulin does not appreciably alter its total-N content. To obtain comparable conditions the globulin solution was therefore diluted with 0.9% NaCl to give a N content of 0.41%.

$\text{NaHCO}_3\text{-H}_2\text{CO}_3$ buffer. 0.2–0.75 M- NaHCO_3 saturated with CO_2 to a pH of 7.5.

METHODS

Determination of radioactive sulphur (S^) in H^* -treated proteins, etc.* The material was oxidized by a mixture of HNO_3 , NaClO_3 and Cu wire, and the S^* precipitated as BaSO_4^* ; the radioactivity of the precipitate was measured and compared with standards as described elsewhere (Bournsnel *et al.* 1946*b*), using the Geiger-Müller counter system described by Banks (1946).

Various methods were used to precipitate the treated proteins from the mixtures, but the usual procedure involved addition of the sample to an excess of ethanol or acetone. The precipitates were washed with a number of different solvents and exhaustively Soxhlet-extracted with solvents (ether and acetone) to remove uncombined H^* , HO_2^* , etc. The resulting products were dried *in vacuo* over P_2O_5 .

S^* and total N (Kjeldahl) determinations were made, and the S^* contents are recorded in this paper as percentage S^* in the dried product and sometimes in terms of mmol. of $\text{S}^*/16$ g. of protein N; the latter method enables one to obtain some idea of the number of H^* groups or residues which have entered the protein molecule, for a S^* content of 22 mmol./16 g. of protein N is equivalent to the combination of 22 mol. of H^* with each molecule of protein, assuming a mean mol.wt. of 100,000 and a N content of 16% for the protein.

Determination of 'masked' plus free SH (iodine titration). These determinations were based on the methods described by Lavine (1935) and Balls & Lineweaver (1939). 4 g. of urea, 0.5 ml. of 5 N-KI, 1.0 ml. of 0.03 N- KIO_3 and 0.5 ml. of 5 N-HCl were added, in that order, to 2 ml. of the protein solution. The residual iodine was titrated with 0.01 N-thiosulphate (with 1% soluble starch as indicator) at any time up to 30 min. after the addition of the HCl. External cooling was unnecessary under these conditions, and no difference in the 'blank' titration (with 2 ml. of water) was observed over this interval. The end-point with ovalbumin was poor, owing to partial precipitation of the protein by the HCl, but satisfactory results were obtained with 1 ml. of the protein solution plus 1 ml. of water.

In some cases, where the SH content of the protein solution was very low, a modification of the above procedure was adopted. 5 ml. of protein solution were treated with 7 g. of urea, 830 mg. of KI, 1.0 ml. of 0.01 N- KIO_3 , and 1 ml. of 5 N-HCl, and titrated with 0.005 N-thio-sulphate.

RESULTS

The action of mustard gas (H) on proteins

The S^ content of various H^* -treated proteins.* Guinea-pig and rabbit sera, kerateine and gelatin solutions, and a collagen suspension, were stirred at pH 7.5 and 37–38° (collagen at 30°) with H^* . In two cases (kerateine and collagen) the H^* was dissolved in a small quantity of ethanol to assist dispersion. Stirring was continued for varying periods (2½–12 hr.), and drops of N-NaOH or Na_2CO_3 solution were added when necessary to maintain the pH at about 7.5. At the end of the experiment, except where otherwise stated, the product was precipitated by adding the mixture to 5–6 vol. of ethanol or acetone, and the precipitate was thoroughly extracted with solvents, and dried *in vacuo* over P_2O_5 . The details and results of these experiments are given in Table 1.

The action of H^ on thymus nucleoprotein.* (a) 15 ml. of nucleoprotein solution (i) was stirred in an atmosphere of N_2 at 37° for 4½ hr., a solution of 43 mg. of H^* in absolute ethanol being added at intervals during the first 2½ hr. The pH remained constant throughout at about 8.3. The resulting slightly turbid solution was poured into 6 vol. ethanol and the precipitated protein was centrifuged, washed with ethanol and acetone (7 ml. each time), then Soxhlet-extracted four times, for 3 hr. each time, with ether and acetone alternately, and dried *in vacuo* with fine grinding. The product contained 14.2% N and 0.46% S^* (16.2 mmol. $\text{S}^*/16$ g. of protein-N or/100 g. of dry protein, assuming a nitrogen content of 16% for the protein).

(b) In a second experiment a relatively larger quantity of H^* was used (c. 1.5 mmol./g. of protein). 20 ml. of nucleoprotein solution (ii) + 2 ml. of 0.75 M- NaHCO_3 buffer (pH 7.5) were stirred as before with 144 mg. of H^* dissolved in ethanol, the whole of the H^* being added at the start. A heavy precipitate formed within a short time (cf. Berenblum, 1940). Samples were withdrawn after 1 and 5 hr., and the proteins, precipitated and extracted as in preparation (a), gave the following analyses: 1 hr. sample: 15.8% N; 0.77% S^* (24 mmol. $\text{S}^*/16$ g. of protein-N). 5 hr. sample: 16.0% N; 1.0% S^* (31 mmol. $\text{S}^*/16$ g. of protein-N). These S^* contents are appreciably higher than the corresponding values for other H^* -treated proteins (cf. Tables 1, 2), even when the latter were obtained in experiments with appreciably more $\text{H}^*/\text{g.}$ of original protein (cf. Table 2, preparation E).

The action of varying amounts of H^ on human-serum proteins.* Experiments have been carried out to determine the extent of the reaction between H^* and human-serum proteins, and also the time required for the reaction to go to completion under

Table 1. *S** contents of a number of proteins after treatment with *H** at pH 7.5 and subsequent solvent extraction

Protein	Experimental conditions			<i>S*</i> in dry product† (%)
	Conc. of protein	mg. of <i>H*</i> /g. protein	Duration of stirring (hr.)	
Guinea-pig serum	Assumed 7%	77	5½	0.27
Rabbit serum	Assumed 7%	78	7	0.64
Kerateine	2%	340	2½	0.40
Collagen suspension	c. 1%	645	5	0.30
Gelatin	4%	29	12	0.06

† Owing to the low radioactivity of the *H** used in this particular series of experiments and the low sensitivity of our earlier Geiger-Müller counters, the figures in this column are not as reliable as those in subsequent tables. The 'probable error', calculated on the statistical error in counting, was about 10% for most of the *S** values in Table 1. With the more active supplies of radioactive *S* used in the later experiments, the statistical error was about 2-3%. In view of the variation of the ratio of *H**/protein used in these experiments very little can be inferred from a comparison of the figures in column five.

Experimental details*

H-serum proteins.* Fresh undiluted guinea-pig serum and rabbit serum were used. The former was not stirred so vigorously as the latter, as this treatment tends to inactivate complement, and it was hoped to correlate these observations with the results of investigations on the inactivation of guinea-pig serum complement by *H* (Bournsnel *et al.* 1946*e*). The acetone-precipitated *H*-rabbit* serum proteins were Soxhlet-extracted 4 times, with ether and acetone alternately for 3 hr. each time, and the *H*-guinea-pig* serum proteins extracted with acetone only (2 days with cold acetone).

H-keratine.* This preparation was carried out in 0.5 M-phosphate buffer (pH 7.4) in an atmosphere of N_2 , and the product was precipitated by the addition of acetic acid to pH 4.8, filtered, washed with 0.01 N-acetate buffer at pH 4.8, and with acetone until free from 'hydrolyzable chlorine'. It was then suspended in ice-cold water, dissolved by cautious addition of N-NaOH, reprecipitated as before, and washed twice with ice-cold acetone as described by Peters & Wakelin (1941); it was finally Soxhlet-extracted with ether (3 hr.) and acetone (3 hr.) and dried.

H-collagen.* 0.2 g. of ox-corneal collagen suspended in 20 ml. of 0.9% NaCl, was stirred with 129 mg. of *H** in an atmosphere of N_2 . The product was washed with water until free from chloride, then with ether, and kept under acetone for 14 days before being dried.

H-gelatin.* Leaf gelatin in 0.1 M-phosphate buffer was used, and the product was precipitated with ethanol, washed with ethanol and acetone, and then with ice-cold water to remove inorganic salts; it was dried, and Soxhlet-extracted for four 3 hr. periods with ether and acetone alternately.

the conditions of our experiments. Fresh human serum was stirred at 37-38° with varying amounts of *H** dissolved in ethanol, with bicarbonate buffer of pH 7.5 added when relatively large amounts of *H** were used. Samples were taken from the mixtures at intervals, and the proteins were precipitated with ethanol, Soxhlet-extracted and dried, as described for *H*-serum* proteins in Table 1.

The results of these experiments (Table 2) show that the time required for completion of the reaction between *H** and serum proteins varied with the amount of *H** used/ml. of serum. In this series of experiments, 6-9 hr. was required with the larger amounts of *H**, but the reaction was apparently complete in 15 min. with the smallest amount of *H**. It must be remembered, however, that *H* undergoes fairly rapid hydrolysis in bicarbonate buffer at pH 7.5, and perhaps it is only to be expected that no further increase in the *S** content of the proteins would be detectable after the first 15 min. or so in experiments with very small amounts of *H*, as in Exp. A; after this period of time there would probably be very little *H* which had escaped reaction with the protein or with water. On the other hand, when relatively large amounts of *H* are used, there

is still much undissolved *H* left after several hours' stirring.

Fig. 1 shows the relationship between (a) the *S** content of the *H*-treated* serum proteins, and (b) the relative amounts of *H** and serum used in the preparation. There were slight differences between the conditions of the experiments (e.g. addition of bicarbonate buffer in some experiments; and sometimes the *H** was added in more than one portion), but it is believed that these do not seriously affect the shape of the curve. From Fig. 1 and Table 2 it will be seen that the maximum *S** content of the serum proteins (about 22 mmol./16 g. of protein N) was obtained, under the conditions of our experiments, when 0.13 mmol. of *H** was used/ml. of serum.

Comparison of the amounts of H combining with serum proteins and gelatin.* Immunological investigations showed that whereas HO_2 -treated gelatin readily reacted with antisera to HO_2 -serum proteins, there was no precipitin reaction between *H*-gelatin and antisera to *H*-treated serum proteins (Bournsnel *et al.* 1946*d*). One possible explanation is that *H*-gelatin contains too few determinant groups to give precipitation, and to test this explanation an experi-

ment has been carried out to determine the relative amounts of H^* which combine with serum proteins and with gelatin under comparable conditions. For

Table 2. S^* contents of human-serum proteins treated with H^*

Exp.	Preparation		Product		mmol. $S^*/16$ g. protein-N
	mmol. $H^*/$ ml. serum	Duration of stirring (hr.)	N (%)	S^* (%)	
A	0.00067	$\frac{1}{2}$	12.6	0.013	0.5
		2	13.5†	0.013†	0.5
B	0.00227	2	14.8	0.030	1.01
		6	14.5	0.036	1.24
C	0.0136	2	15.1	0.12	4.0
		$3\frac{1}{2}$	14.4	0.16	5.6
		6	14.3	0.17	6.0
D	0.0661	5	13.5†	0.42†	15.6
		9	14.0	0.52	18.6
E	0.132	9	13.7†	0.61†	22.3

† Average for two samples.

‡ Average for three samples.

Experimental details

Exp.	Human serum (ml.)	H^* (mg.)	0.75 M- $\text{NaHCO}_3\text{-H}_2\text{CO}_3$ (ml.)	H_2O (ml.)
A	26	2.78	—	1.0
B	40	14.4	—	—
C	40	86.4	—	—
D	20	210	3.5	—
E	10	210	3.5	—

Fig. 1. S^* content of human-serum proteins after treatment with H^* .

† Assuming an average mol.wt. of 100,000 and a N content of 16% for the serum proteins.

this purpose diluted serum containing about the same percentage of total N as the gelatin solution was used. 40 ml. of (a) 1% gelatin in 0.2M- NaHCO_3 buffer (pH 7.5) and (b) diluted human serum (1 vol.

of serum plus 7 vol. of the bicarbonate buffer), were each stirred with 245 mg. of H^* at 38° for 12 hr. Samples of each mixture were then treated with ethanol, and the precipitated proteins exhaustively extracted as described above (cf. Table 1). Analysis of the dried products gave the following results (averages of triplicate determinations):

	S^* %	mmol. of $S^*/$ 16 g. of N
H^* -serum proteins	0.41	16.6
H^* -gelatin	0.152	5.3

The effect of H^* on the sulphhydryl groups of proteins. The 'free' SH groups of proteins (those detectable by the nitroprusside reaction or titratable with porphyrindin) and the 'masked' SH groups (detectable by the nitroprusside reaction in proteins denatured by heat, urea, or guanidine hydrochloride), may both be determined by titration of the native protein with iodine in approximately N-HI solution under carefully controlled conditions. In many cases a concentration of urea of approximately 1 g./ml. of solution must, however, be maintained in order to prevent precipitation of the protein by the acid. The technique described earlier in this paper has been used to determine the change in the SH content of proteins resulting from treatment with H^* .

Diminution of the SH content of ovalbumin on treatment with H^* . 30 ml. of a solution of crystalline chicken ovalbumin containing about 7% protein were mixed with 10 ml. of 0.75 M- NaHCO_3 buffer (pH 7.5) and stirred with 144 mg. of H^* (added in four approximately equal amounts after 0, 15, 60 and 110 min.) in an atmosphere of N_2 at 37°.

Iodine titrations were carried out initially and after 8 hr., and a 31% reduction in the SH content of the protein was observed in this time. (A control solution of ovalbumin showed no reduction from the initial figure of 17.2 mg. of SH/100 ml. of solution.) Nitroprusside tests (two drops of the protein solution saturated with urea on a white tile, and treated with 1 drop of dilute sodium nitroprusside and 1 drop of a 1:5 solution of 0.880 ammonia) gave results of a confirmatory nature. Unfortunately, S^* determinations could not be carried out, as the H^* ovalbumin preparation was lost by fire.

S^* and SH determinations on human-serum proteins treated with H^* . 40 ml. portions of fresh human serum were stirred with (i) 14.4 mg. of H^* , and (ii) 86.4 mg. of H^* , at 37° in an atmosphere of N_2 . The H^* was added in ethanol solution in four portions at 15 min. intervals. Two 5 ml. samples were withdrawn from each mixture at 0, 2, $3\frac{1}{2}$, and 6 hr. after the addition of the first portions of H^* . One of these samples was used for SH determination as described earlier in this paper, and the other for S^* determination on the dried extracted proteins. Total-N

determinations were also carried out on the reacting solutions and on the dried products in order to correlate the S* and SH content with the protein content in each case.

The results of this experiment (cf. Table 3) show that part of the SH was abolished within 2 hr., and that no further decrease occurred in a further 4 hr., although there was a further increase in the S* content of the protein during this second period. The S* fixed in the protein molecule was much more than equivalent to the SH diminution even at 2 hr.

Table 3. *Diminution in the titratable SH and the 'fixed' S* content of H*-treated human serum*

mg. H*/ml. serum	Duration of stirring (hr.)	mmol. of SH or S*/16 g. of protein-N		
		Titratable SH	Diminution in SH	S* fixed
0.00	6	1.2	—	—
0.36	2	0.7	0.5	1.01
	3½	0.6	0.6	—
	6	0.7	0.5	1.24
2.16	2	0.5	0.7	4.0
	3½	0.6	0.6	5.6
	6	0.6	0.6	6.0

The action of H on human-serum globulin and on phenylcarbamido-globulin.* This experiment was carried out to determine whether or not the blocking of the free amino-groups of globulin by the action of phenyl isocyanate (Hopkins & Wormall, 1933) would reduce the capacity of the protein to react with H*. 20 ml. of a solution of human serum globulin plus 3.5 ml. of 0.75 M-NaHCO₃ buffer (pH 7.5) were stirred with 200 mg. of H*, dissolved in a small volume of ethanol, in an atmosphere of N₂ for 5 hr., and a parallel experiment was carried out with a phenylcarbamido-globulin solution containing approximately the same amount of protein as the globulin solution. Samples of the two mixtures were taken before the addition of H*, and at

the end of the reaction, for formal titrations carried out as described previously (Bournsnel *et al.* 1946a). Other samples taken at the end of the reaction were treated with ethanol and the precipitated proteins were Soxhlet-extracted and dried in the usual manner for total N and S* determinations. The results of this experiment are recorded in Table 4.

The action of hydrolysis products of H
on proteins*

In view of the loss of vesicant power when H is hydrolyzed, it is of interest to know whether there is a similar loss of ability to combine or react with proteins. Experiments have therefore been carried out in which H* was hydrolyzed by prolonged shaking with a bicarbonate buffer at pH 7.5 and the resulting mixture allowed to react with human-serum proteins and with a nucleoprotein solution; the proteins were then separated and S* determinations were made in the usual manner.

When H is hydrolyzed, one of the main products is thiodiglycol, S(CH₂CH₂OH)₂. Experiments have therefore been made to determine (a) whether or not this compound (TG*), prepared synthetically and thus free from the other hydrolysis products present in hydrolyzed H*, reacts with a protein (serum albumin), and (b) whether or not treatment with TG* impairs the ability of the protein to react with H*.

Hydrolyzed H and serum proteins.* 65.8 mg. of H* were hydrolyzed by shaking with 1.5 ml. of 0.75 M-NaHCO₃-buffer (pH 7.5) for 9½ hr. at 37°; at the end of this period 1 drop of this solution had no visible vesicant action detectable in 24 hr. on the skin of a human subject. This solution and a small volume of ethanol were added to 30.5 ml. of fresh human serum, and the mixture stirred at 37°. Samples were taken after 1 and 6 hr. and the proteins precipitated and extracted as described previously (cf. Table 1);

Table 4. *S* content and 'alkali-titratable' groups in (a) H*-treated human-serum globulin, and (b) H*-treated phenylcarbamido-globulin*

(All results expressed as mmol./16 g. protein-N.)

	Globulin	H*-globulin	Phenyl- carbamido- globulin	H*-phenyl- carbamido- globulin
S*	—	14.0	—	26.0
No. of titratable groups:				
pH 7-9	20.1	47.3	7.5	39.5
pH 9 after HCHO	46.5	34.1	3.1	2.9
Total	66.6	81.4	10.6	42.4
Increase in no. of titratable groups:				
Globulin to H*-globulin		+14.8		
Globulin to phenylcarbamido-globulin			-56.0	
Phenylcarbamido-globulin to H*-phenylcarbamido-globulin				+31.8

the products contained 0.8 mmol. of S^* and 1.2 mmol. of $S^*/16$ g. of protein-N respectively. In a previous experiment with a comparable amount of unhydrolyzed H^* (Table 2, Exp. C) a product containing 6 mmol. of $S^*/16$ g. of protein-N was obtained.

Hydrolyzed H^ and nucleoprotein.* 72 mg. of H^* , dissolved in a little ethanol, was shaken vigorously with 1 ml. of the bicarbonate buffer until some considerable time after a homogeneous solution had been obtained (about 7 hr. in all). 20 ml. of nucleoprotein solution (preparation ii, above) were then added and the mixture stirred at 37° in an atmosphere of N_2 for 5 hr. The precipitated extracted protein product contained 6.0 mmol. of $S^*/16$ g. of total N.

Thiodiglycol (TG^*) and serum albumin.* The albumin was prepared from fresh human serum by saturation with $(NH_4)_2SO_4$, after the globulins had been removed by half-saturation. The $(NH_4)_2SO_4$ -albumin paste was dialyzed against distilled water until it was free from NH_3 , and the resulting solution contained about 3.5% protein.

Samples of this albumin solution were stirred for 5 hr. with H^* or TG^* (dissolved in each case in a small volume of ethanol), and at the end of this period, part of the TG^* -treated albumin solution was stirred with H^* for a further 5 hr. The proteins in the various mixtures were precipitated by ethanol, extracted and dried, and S^* and total-N determinations were carried out. The results, given in Table 5, show (a) that the amount of TG^* combining under these conditions is very small indeed and possibly

negligible, and (b) that TG^* -treated albumin is capable of combining with as much H^* as does untreated albumin.

The action of oxidation products of H^
on proteins*

Action of HO^ on (a) serum proteins and (b) ovalbumin.* This experiment was carried out in connexion with an investigation of the distribution of S^* in the tissues of a rabbit injected with HO^* , to determine whether the retention of S^* in the tissues might be attributed to a general reaction of the HO^* with the tissue proteins. Bailey & Webb (1944) have found that there is no measurable increase in the S content of fibrinogen or ovalbumin following treatment with HO , but it was thought desirable that further observations should be made with other proteins and with the aid of the radio-S technique. The results of our experiments, described below, confirm the observations of Bailey & Webb.

12.1 mg. of HO^* , dissolved in 0.2 ml. of ethanol, were stirred for 4 hr. at 37° with either (a) 6.0 ml. of human serum or (b) 6.0 ml. of approx. 3.5% ovalbumin solution. The proteins, precipitated by ethanol and Soxhlet-extracted in the usual way, were found to contain very small amounts only of S^* , the HO^* -treated ovalbumin containing less than 1 μ g. of $S^*/g.$ and the HO^* -treated serum proteins about 8 μ g. of $S^*/g.$; these values correspond respectively to 0.004 mmol. (or less) of S^* and 0.03 mmol. of $S^*/16$ g. of protein-N, and the higher figure is only equivalent to 0.03 mol. of S^* or $HO^*/mol.$ of protein, assuming an average mol.wt. of 100,000 and a N content of 16% for human-serum proteins.

Comparison of the action of HO_2^ with that of H^* on rabbit plasma proteins.* An experiment has been carried out to determine the rates of action of H^* and HO_2^* on plasma proteins and to determine the maximum amount of each reagent combining with the proteins when equivalent small amounts of H^* and HO_2^* are used.

27 ml. samples of freshly prepared heparinized rabbit plasma were stirred, at 37° and under parallel conditions, with (a) 2.7 mg. of H^* dissolved in 0.5 ml. of triacetin, or (b) 3.24 mg. of HO_2^* dissolved in 0.5 ml. of triacetin. Samples were taken from each mixture after 5, 10, 20, 30, 45, 60, 90 and 150 min., and the proteins were precipitated with ethanol, Soxhlet-extracted, dried and incinerated for S^* determinations.

The results of this experiment are given in Fig. 2. The curves show a rapid increase in S^* content of the precipitated proteins with a maximum at about 30 min. for the H^* -treated plasma and 60 min. for the HO_2^* -treated plasma. In each case a slight decrease occurred with further stirring, but this may have been due to the progressive removal of fibrin,

Table 5. *The action of H^* and thiodiglycol* (TG^*) on human-serum albumin, and of H^* on albumin previously treated with TG^**

Reagent	Acting on	Product		
		N (%)	S^* (%)	$S^*/16$ g. protein-N (mmol.)
H^*	Albumin	14.0	0.96	34
TG^*	Albumin	13.5	0.03†	1
H^*	TG^* -albumin	13.8	0.94	34

† This figure corresponds to about 0.8 mol. $S^*/mol.$ of protein, assuming a mol.wt. of 69,000 and 16% N for human-serum albumin.

Experimental details

Exp.	Albumin solution (ml.)	0.75 M- $NaHCO_3$ buffer (ml.)	H_2O (ml.)	H^* or TG^* (mg.)
A	10	2.0	—	125 (H^*)
B	32	—	6.4	306 (TG^*)

A and B were stirred in N_2 for 5 hr. at 37° . 12 ml. of B were then treated with 2.0 ml. of bicarbonate buffer and 126 mg. of H^* , and the mixture stirred in N_2 for a further 5 hr.

which was noticed to have attached itself to the glass stirrers during the later stages of the experiment; if this is the true explanation, it would mean that the H^* -fibrin would have a S^* content at least three times that of the serum proteins, but other experiments have shown that fibrin does not differ very appreciably from the other plasma proteins in its affinity for H^* . It is of interest to note that the maximum S^* content of the H^* -treated plasma proteins (0.0135%) was in very close agreement with the value obtained in a much earlier experiment in which human serum was treated with a similar amount of H^* (Table 2, Exp. A).

Fig. 2. S^* content of rabbit-plasma proteins after treatment with H^* or HO_2^* (0.00063 mmol. of H^* or HO_2^* /ml. of plasma).

The reaction of HO_2 and divinyl sulphone with the amino-groups of gelatin. 100 ml. portions of a 1% solution of leaf gelatin in 0.33 M- $NaHCO_3$ (pH 8.5) were stirred at 30° with one or other of the two sulphones. 0.5 g. of HO_2 was added at the start and a further 0.5 g. after 5 hr., and the stirring was continued for 12½ hr. in all. One addition of 0.39 g. of divinyl sulphone was made at the start, and the mixture was stirred for 10 hr.

Amino-N determinations (formol method) were carried out as described previously (Boursnell *et al.* 1946a) on 5 ml. samples of the two mixtures and on control solutions (no sulphone) at the end of the reaction periods. The results showed that about 97% of the free amino-groups of the gelatin had reacted with HO_2 , and about 99% with divinyl sulphone. Neither of the protein products, which, from the immunological evidence presented in a later paper (Boursnell *et al.* 1946d) and by analogy with the corresponding amino-acid derivatives, appear to be identical, was precipitable by acetic acid; in this respect they differ from the sulphone-serum-protein derivatives.

DISCUSSION

These investigations have fully confirmed the view that H combines with a variety of proteins to form compounds or complexes from which the H or H -residue cannot be extracted by very exhaustive extraction with ethanol, acetone and ether. By using radioactive S as a tracer, a quantitative measure has been obtained of the amount of H which reacts with the protein under varying conditions; the reaction may, of course, concern more than one type of group in the protein, and there will in many cases be elimination of HCl . This tracer method cannot differentiate between the S^* which has entered the protein molecule as a result of the action of H^* and that introduced by the action of hydrolysis products of H^* . However, hydrolyzed H^* reacts to a slight extent only with proteins, and in any case, it is, from some points of view, immaterial whether the combination of H^* with proteins is due to the action of unchanged H^* or of its hydrolysis products. Hydrolysis of H^* similar to that occurring in these *in vitro* experiments will undoubtedly occur *in vivo*.

A variety of widely differing proteins has been used in these investigations. Most experiments were made with human serum proteins, for it was our intention to correlate the results with the immunological studies of H -treated serum proteins by Berenblum & Wormall (1939), but several experiments were made with nucleoprotein in view of the observation of Berenblum (1940) that H reacts with and precipitates this protein. Amongst the other proteins we have used were keratine and ovalbumin, which are rich in free and 'masked' SH respectively, and the scleroprotein collagen. No attempt was made to make a strict comparison of the amounts of H^* combining with these different proteins, but it is of interest that H^* -nucleoprotein contained significantly more S^* than did H^* -serum proteins prepared under similar conditions, i.e. with the same amount of H^* /g. of the original protein. The maximum S^* contents of the various H^* -treated proteins were 22 mmol. for the serum proteins, 34 mmol. for human-serum albumin and 31 mmol. for nucleoprotein/16 g. of protein-N, but it is not suggested that these figures represent the maximum combining capacities of these proteins for H^* . The values for the serum-protein derivatives correspond to the introduction of 22 and 23 S^* atoms or H^* residues/mol. of protein, assuming mol.wt. of 100,000 and 69,000 for the mixed serum proteins and serum albumin respectively and N contents of 16%.

The experiments involving the treatment of human-serum proteins with varying amounts of H^* showed that, with small amounts of H^* , combination with the proteins was complete in a short time, possibly in ¼ hr. or so, and that about half, or slightly more, of the added H^* had become firmly attached

to the protein; the remainder had presumably been hydrolyzed to thiodiglycol* and other products which do not combine with proteins. With increasing amounts of H^* there is a greater loss by hydrolysis, and with the highest amount of H^* used in this series of experiments only about one-ninth became firmly attached to the proteins. As expected from earlier observations, the reaction between the serum proteins and H^* was not complete, when these larger amounts of H^* were used, until the mixture had been stirred for several hours (possibly 9 hr. or longer). When the results of these experiments are shown graphically, as in Fig. 1, an approximate measure can be obtained of the amount of H^* which combines with the serum proteins with different initial H^* /protein ratios. The curve suggests that the maximum amount of S^* which can be introduced into the serum proteins in this way is about 22 or 23 mmol./16 g. of protein-N, but because of our limited supplies of S^* we were unable to test this hypothesis further by the use of still larger quantities of H^* . Although the experiment is not strictly comparable with those described in Table 2, because of the different protein concentrations employed, it is interesting to note that a later experiment, involving the use of diluted human serum and an H^* /protein ratio higher than that in Exp. E, the amount of S^* introduced into the protein was about 17 mmol./16 g. of N. The amount of H^* or H^* -residue introduced into the proteins in Exps. D and E (of the order of 18–22 mol./mol. of protein) was certainly sufficient to account for the marked change in immunological properties which occurs when serum proteins are treated with H , and for the new specificity which is acquired.

The comparison between the amounts of H^* combining with (a) serum proteins and (b) gelatin showed that, with the same concentration of protein and the same H^* /protein ratio at the beginning, the resulting H^* -serum proteins contained about three times as much S^* as did the H^* -gelatin. This difference is undoubtedly very significant, and it might easily explain why H^* -gelatin, in contrast to the H -serum proteins, will not give a precipitate with antisera to H^* -proteins (Bournsnel *et al.* 1946*d*). No comparable figure is available for the S^* content of HO_2^* -gelatin, but from the diminution of amino-N resulting from the action of HO_2 on gelatin it can be calculated that gelatin combines with HO_2 to at least 8 times the extent that it does with H , and it is not surprising to find that HO_2 -gelatin is readily precipitated by antisera to HO_2 -proteins.

The question as to which protein groups are affected by H is one of considerable importance, particularly in view of the action of this substance on enzymes. The investigations described here have not solved this problem, but the results allow certain general conclusions to be reached. The action of H ,

unlike that of HO_2 , is not primarily concerned with the free amino-groups of the protein, and it affects groups which are more common in the molecules of the serum proteins and nucleoprotein than in those of gelatin. Peters & Wakelin (1941) have shown that H diminishes the free SH of keratine. Our results with H^* -treated ovalbumin and H^* -treated serum proteins are in conformity with this finding, but the amount of S^* in H^* -serum proteins may be 10 times the equivalent of the reduction in free plus 'masked' serum SH caused by the H^* . Even at an early stage in the reaction, when the maximum reduction in the titratable serum SH has been effected, the amount of firmly combined S^* in the H^* -protein may be the equivalent of 2–5 times this reduction. Also it should be noted that the maximum S^* content of the H^* -serum proteins (Table 2) is the equivalent of nearly 20 times the total titratable SH of the serum. The reaction with SH can, therefore, account for a small fraction only of the H^* combining with the serum proteins.

The evidence against a reaction between H^* and the free amino-groups of proteins is partly indirect but it is fairly conclusive. (1) There is no reduction in the amino-N (formol titration) when simple amino-acids (e.g. alanine) or proteins are subjected to the action of H . (2) The H -treated serum proteins are not precipitated by acetic acid, as are the HO_2 -, phenylcarbamido- and carbobenzyloxy-serum proteins; the introduction of a large group into the NH_2 groups of the serum and certain other proteins appears to lead to acid-precipitability. (3) The blocking of the free NH_2 groups of a protein (serum globulin) by phenylisocyanate does not reduce its capacity to combine with H^* ; actually, the phenylcarbamido-globulin combined with considerably more H^* than did the untreated globulin.

Information about the alkali-titratable groups of globulin and phenylcarbamido-globulin and their H^* -derivatives was obtained by determinations of (a) the amount of NaOH required to change the pH of the solution from 7 to 9 (phenolphthalein) and (b) the further NaOH required to restore the colour of the phenolphthalein after the addition of neutral formaldehyde. It may possibly be significant that the increase in the total number of alkali-titratable groups, i.e. (a) plus (b), resulting from the action of H^* , was approximately equivalent to the amount of H^* which had combined with the protein, with both globulin and phenylcarbamido-globulin; the conversion of globulin into H^* -globulin, for example, led to an increased requirement of 14.8 mmol. of alkali/16 g. of protein-N for the complete titration, and to the combination of 14.0 mmol. of H^* /16 g. of protein-N.

The action of H^* on globulin or phenylcarbamido-globulin is accompanied by a marked increase in the amount of NaOH required for the titration from

pH 7 to 9. This is in agreement with the observation of Northrop (1942) that, after treatment with *H*, pepsin shows an increased titration in this range, and is presumably to be accounted for by a change in the pK values of some group or groups in the protein molecule such that they become partly or completely titratable over the pH range 7–9 (for a discussion of the significance of these titrations, cf. Richardson, 1934–5).

A change of this nature might be effected in several ways, e.g. (a) combination of *H* with a protein group in such a way as to alter its pK value, or (b) an increase in the basicity of basic groups due to esterification of COOH groups. Another possibility is that certain groups of the protein molecule might be converted to other groups, of different pK values, without any corresponding combination with *H* (e.g. the conversion of guanidino- to amino-groups by hydrolytic fission, though there is no direct evidence that such a change actually occurs). In connexion with (b) it is of interest to note that Northrop (1942) and Bergmann (1942) have demonstrated that the action of *H* on certain proteins and polypeptides results in esterification of the COOH groups. Moritz (1942) and du Vigneaud (1942) showed that a reaction occurs with iminazole groups, and Ball (1943), from the titration curves of oxyhaemoglobin and *H*-oxyhaemoglobin, deduced that most of the reaction between HbO_2 and *H* occurs at the COOH and iminazole groups, with a less extensive reaction with groups titrating between pH 9 and 11.5, i.e. those of lysine, arginine and tyrosine.

Our experiments with H^* which has been hydrolyzed in a slightly alkaline medium have shown that there is some retention of the power to combine with proteins even when normal vesicant activity has completely disappeared, but hydrolyzed H^* possesses weak protein-combining capacity compared with unhydrolyzed H^* . In one experiment, hydrolyzed H^* gave with human-serum proteins a product containing 1.2 mmol. of $S^*/16$ g. of protein-N, whereas an equal amount of unhydrolyzed H^* gave a product containing 5 times this amount of S^* . Somewhat similar results were obtained with nucleoprotein, for an S^* content of 6 mmol. was obtained with hydrolyzed H^* , whereas a relatively smaller amount of unhydrolyzed H^* gave a product containing 16 mmol. of $S^*/16$ g. of protein-N. Thiodiglycol* (TG^*), one of the hydrolysis products of H^* , does not combine with proteins to any significant extent, nor does it reduce the capacity of the protein to react with H^* . It may be concluded that hydrolyzed *H* contains compounds, perhaps sulphonium salts of the *H-TG* or *H-2TG* type (Davies & Oxford, 1931; cf. also Bergmann, 1942; Rydon, 1943), which, although not normally vesicant, are capable of combining with proteins.

Of the two oxidation products of *H* studied in these investigations, one, $\beta\beta'$ -dichlorodiethyl sulphone (HO_2), quickly reacts with proteins (Berenblum & Wormall, 1939) by a reaction with the free amino-groups similar to its action on amino-acids, whereas the other, $\beta\beta'$ -dichlorodiethyl sulphoxide (*HO*), does not combine with proteins at pH 7.5 and at 37°. All our experiments with HO_2^* and HO^* have confirmed these observations.

In a later paper (Boursnell *et al.* 1946c) evidence is produced that intravenous injection into different rabbits of approximately equivalent amounts of H^* , HO^* and HO_2^* results in similar distributions of 'fixed' or firmly combined S^* in the tissues 1 hr. after the injections. The failure of HO^* to combine with proteins in the *in vitro* experiments seems to exclude the possibility that all three injected substances become attached in a similar manner to the tissue proteins, unless, of course, it can be shown that HO^* is converted in the tissues to HO_2^* or some other reactive compound, a possibility which we hope to study at a later date. In connexion with these injection experiments we have made a comparison of the relative rates of action of H^* and HO_2^* on the plasma proteins, using relatively small amounts of H^* and HO_2^* in order to obtain conditions similar to those which would occur in the tissues of the injected rabbits. The results of the experiment (Fig. 2) show that, as measured by the amount of 'fixed' S^* in the H^* - or HO_2^* -proteins produced in a given time, the action of HO_2^* is somewhat more rapid than that of H^* , but, under the conditions of our experiment, the reaction with H^* was completed in about 30 min., whereas that with HO_2^* required about twice as long. This difference between the time required for completion of the reaction may, however, simply be due to the fact that, because of the rapid hydrolysis of dissolved H^* , the reaction of H^* on the proteins will necessarily cease in a relatively short time when small amounts of H^* are used (cf. also Table 2, Exp. A). With larger amounts of H^* , i.e. when undissolved H^* is present in the mixture for some appreciable time, the reaction between H^* and proteins continues for several hours, and the curve under these conditions would probably show a closer resemblance to that for HO_2^* in Fig. 2. It is interesting to note that, at the points of maximum reaction in Fig. 2, over 85% of the added HO_2^* and about 55% of the H^* had combined with the plasma proteins; the figure for H^* agrees very well with the results of an earlier experiment with human serum and a small amount of H^* (Table 2, Exp. A). The results of this experiment are not inconsistent with the view that the 'fixed' S^* in the tissues of rabbits 1 hr. after the injection of H^* or HO_2^* may be attributable to a direct reaction between the H^* or HO_2^* and the tissue proteins.

Other experiments, not described here, have been carried out to study the action of HO_2 (and divinyl sulphone) on proteins, and all have tended to show that the main changes involve reaction with the amino- and the SH-groups of the protein; the use of HO_2^* would make it possible to decide whether or not these are the only reactions concerned, but insufficient S^* was available for this purpose. The action of H on proteins appears to be of quite a different nature, although here again, combination with SH accounts for part of the reaction.

SUMMARY

1. The action of mustard gas (H) and related compounds on proteins at pH 7.5–8 and at 37° has been studied by the use of radioactive S (S^{35}) as a tracer element.

2. Determination of the S^{35} content of the H -treated proteins has given a measure of the amounts of H which have reacted with the various proteins. The results have fully confirmed the view that H readily reacts with many widely differing proteins.

3. The maximum amount of H which combines with the human-serum proteins is about 22 or 23 mmol./16 g. of protein-N, equivalent to 22 or 23 mol. of H or H -‘residue’/mol. of protein, assuming a mean mol.wt. of 100,000 for the serum proteins. This amount is sufficient to account for the new immunological specificity of H -treated proteins.

4. No attempt has been made to determine the maximum amount of H which can combine with all the proteins used in these experiments, but values higher than those for the mixed human-serum proteins have been obtained for H -nucleoprotein (31 mmol./16 g. of protein-N) and for H -treated human-serum albumin (34 mmol./16 g. of protein-N).

5. When a gelatin solution and diluted human serum containing the same percentage of protein are separately treated with the same small amount of H , the H -serum proteins produced contain about 3 times as much S^{35} as does the H -gelatin.

6. When human serum is treated with small amounts of H , over 50 % of the added H may combine with the protein and the reaction may be complete in 15 min. With larger amounts of H , more S enters the protein molecule (up to the limits given

above), but the reaction requires 9 hr. or longer to reach completion, and a much higher percentage of the H (90 % in some cases) undergoes hydrolysis or some similar change.

7. There is a marked reduction in the free SH and the titratable SH (free plus ‘masked’) of various proteins as a result of the action of H . The amount of S entering the serum proteins may, however, be at least 10 times the equivalent of the reduction in titratable SH. Reaction with SH groups can therefore account for a small part only of the combination of H with the serum proteins.

8. The blocking of the free NH_2 -groups of a protein by phenylisocyanate does not reduce the capacity of the protein to react with H . From this and other evidence it is concluded that H does not act mainly, if at all, on the free amino-groups of proteins.

9. H which has been hydrolyzed in a slightly alkaline medium until all detectable vesicant power has disappeared, combines with proteins, but to a much smaller extent than does unhydrolyzed H . Thiodiglycol, one of the chief hydrolysis products of H , does not combine with proteins to any significant extent.

10. $\beta\beta'$ -Dichlorodiethyl sulphone (HO_2) readily reacts with proteins, chiefly with the amino-groups, but $\beta\beta'$ -dichlorodiethyl sulphoxide (HO) does not.

11. A comparison has been made of the action of a small amount of HO_2 with that of an equivalent amount of H on plasma proteins. Under the conditions of this experiment the reaction with H was complete in about 30 min. and that with HO_2 in about 1 hr., but the HO_2 -protein contained considerably more S^{35} than did the final product of the H -plasma reaction. These results are briefly discussed in relation to the results of the injection experiments.

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Studies on Mustard Gas ($\beta\beta'$ -Dichlorodiethyl Sulphide) and some Related Compounds

5. THE FATE OF INJECTED MUSTARD GAS (CONTAINING RADIOACTIVE SULPHUR) IN THE ANIMAL BODY†

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Young (1941) showed that when H^* (mustard gas containing radioactive sulphur) is applied to the tail of a rat, some of the S^* (radioactive S) derived from the H^* passes from the site of application, and is later found in the liver, kidneys and urine of the animal, and Salter (1942) found considerable amounts of S^* in the bone marrow of rats 3 hr. after H^* had been applied to the skin. Cameron (1941) has described the systemic damage occurring in H -treated animals, and Needham, Cohen & Barrett (1943), in investigations on the systemic effects of H , have drawn attention to the fact that there is inhibition of glycolysis in bone marrow, and that there is an appreciable delay in the onset of this inhibition, parallel with the delay in the appearance of histological damage. They also showed that, in contrast, the action of H on the enzymic systems of bone marrow *in vitro* is almost immediate. One possible explanation for the delay, discussed very tentatively by Needham *et al.*, is that H or some derivative of it might be stored in the fat of the bone marrow or elsewhere in the body and released slowly over a long period. In the hope of throwing some light on these problems, we have carried out investigations involving the injection of H^* into rabbits and the determination of the distribution of S^* in the blood and other tissues of the animals at various intervals (up to 12 hr.) after the injections. In other experiments the excretion of S^* in the urine

and the bile of rabbits injected with H^* has been investigated.

In these experiments, two types of determination of the S^* content of a tissue have been made, one involving a determination of the total S^* and the other the 'fixed' or firmly combined S^* . For the total S^* determinations tissues were dried *in vacuo* but were not extracted with any solvent; these results give a measure of the combined and uncombined H^* plus any other S^* -containing substance present in the tissue at the time that the rabbit was killed. The 'fixed' S^* determinations were made on the minced tissues after they had been exhaustively extracted with various solvents, to remove loosely attached H^* and related compounds, and then dried; it is believed that these values give a measure of the amount of H^* which has combined with the proteins and perhaps other constituents of the tissues. The use of the term 'fixed' is not intended to imply that the attached S^* will necessarily remain fixed in the tissues *in vivo*, and, indeed, evidence is presented to the contrary, for in some of the experiments the results suggest that there is, in the intact animal, fairly rapid removal of this 'fixed' S^* from the tissues. In the majority of cases where determinations were made of both total S^* and 'fixed' S^* , the two values were of the same order when both results were given per g. of material dried over CaCl_2 *in vacuo*; occasionally, however, the 'fixed' S^* value was appreciably higher than the total S^* , presumably due to the extraction by the solvents of fatty substances containing little or no S^* .

† The investigations described in this paper were the subject of reports to the Ministry of Supply in 1943-5.