REACTION OF ENZYMES AND PROTEINS WITH MUSTARD GAS (BIS(β-CHLOROETHYL)SULFIDE)*

By ROGER M. HERRIOTT, M. L. ANSON, AND JOHN H. NORTHROP

(From the Laboratories of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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I. Rate of Reaction of Mustard Gas (H) with Different Enzymes and Proteins

Thirteen different proteins[1] have been treated with mustard (H)[2] in aqueous solution. In every instance the H reacted with the protein as shown by either an increase in bound sulfur or a change in enzymatic activity, or both. We have failed to find any protein which did not react. Some enzymes were more rapidly inactivated than others, as shown by the value of their inactivation constants (Tables I and II). Dixon, Van Heyningen, and Needham (4, 45) have concluded that enzymes fall into two classes; those which react and those which fail to, but our results would indicate a scale of reaction rates with chicken pepsin near the faster end and chymotrypsin at the slower end. Crystalline yeast hexokinase is intermediate.

Effect of the Method of Addition of H.—Two methods have been used for the addition of H to the reaction mixture. In one case—Needham and Dixon (5)—the H was dissolved in alcohol or a similar organic solvent and added all at once

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[1] Crystalline swine pepsin and pepsinogen, chicken pepsin, crystalline chymotrypsinogen and crystalline chymotrypsin, yeast sucrase and crystalline yeast hexokinase, crystalline egg albumin, human serum albumin, human serum globulin, human fibrinogen, gelatin, and zein.

[2] The H used in the experiments was a highly purified sample prepared from thiodiglycol (TDG). Technical preparations give quite different results, reacting much faster with SH groups.

TABLE I

Inactivation Rates of Swine Pepsin and Hexokinase at 25°C. in M/25 pH 5.8 Acetate Buffer at Various Concentrations of Protein, H, and by the Stirring and Dixon Methods

Enzyme	Method	Curve of Fig. 1	Protein concentration	H Concentration	*KC**
			mg./ml.	*millimolar*	
Swine pepsin	Constant H by stirring	A	1	1.9	36
		B	16	2.5 ± 1	29
		C	1	0.1	38
	Dixon's (H in cellosolve)	D	1.8	2.0 (initial)	30
		E	1.8	4.0 (initial)	26
Hexokinase highly purified but not crystalline	Constant H by stirring	F	1	0.27	13
		G	1	1.7	14
		H	10	1.7	16
Crystalline hexokinase	Dixon's (H in cellosolve)	I	0.1	2 (initial)	12
		J	0.1	4 (initial)	12
	Constant H by stirring	K	3.0	2.4	18

* KC = fraction inactivated per minute per mol H per liter. When Professor Dixon's method (5) was used the inactivation constant was obtained by substituting in the formula $KC = \frac{1}{H}\frac{\Delta \ln P}{\Delta t}$ where the change in activity was determined over short intervals of time; *e.g.*, in curve E between 2 minutes and 4 minutes the pepsin activity dropped from 81 per cent to 69 per cent. During this time interval the gas concentration was calculated to be 3.2 and 2.4 millimolar, therefore $KC = \frac{1}{0.0028}\ln\frac{81}{69}\frac{}{2}$ or 29. The values for KC in Table I, using Professor Dixon's method represent averages of values for several intervals.

TABLE II

Reaction Constants of Various Enzymes with H and Their Values Relative to That of Water

Substance	*KC* (bimolecular)		$k = \frac{E_0 - E}{E[H_0]}$ by Dixon's method		$\frac{KC\text{ (enzyme)}}{KC\text{ (water)}}$	
	pH 6.0	pH 7.8	pH 6.0	pH 7.8	pH 6.0	pH 7.8
Swine pepsin	37		380		1.5×10^4	
Chicken pepsin	150	300	2800	6000	6.2×10^4	1.25×10^5
Yeast hexokinase	15		75		6.2×10^3	
Swine pepsinogen	3.5	12	70	160	1.5×10^3	5×10^3
Yeast sucrase	2–3	60	30	900	1×10^3	2.5×10^4
Beef chymotrypsin	2	3 (?)	<10	38	8×10^2	1.3×10^3
Water (M/25 acetate)	0.0024					

to the reaction mixture. In the stirring method, liquid H was stirred with the protein solution. It has been suggested (6) that variation in the results has been due to the fact that the reaction proceeds differently in the presence of liquid H, even though the concentration of dissolved H is the same.

The rate of inactivation of pepsin and of hexokinase by H using both methods was therefore determined. No significant differences were found, as may be seen in Fig. 1 and Table I. The different results obtained in this laboratory and in Professor Dixon's laboratory are therefore not due to the different meth-

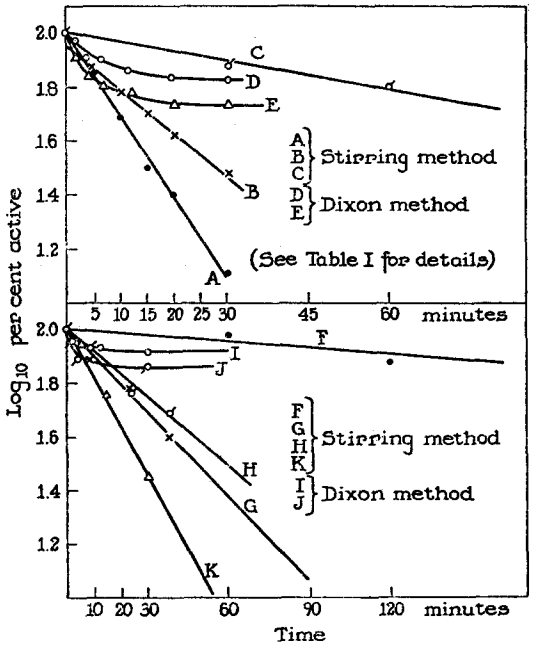

FIG. 1. Inactivation of swine pepsin and yeast hexokinase by various concentrations of mustard at 25° C. in M/25 pH 5.8 acetate buffer using the Dixon or the stirring method.

ods. The differences are probably due to differences in the enzyme preparation or in the hydrogen ion concentration.

Experimental Results.—In the Dixon method small amounts of alcohol or ethyl cellosolve solutions of H were added to the aqueous enzyme solution, accompanied by rapid stirring to minimize the separation of the free H oil. The quantity of H in the organic solvent was such that when mixed with the protein the H concentration was less than saturated, 6×10^{-3} molar. In general a final concentration of $1\text{–}2 \times 10^{-3}$ molar H has been used. The H concentration decreased with time due to hydrolysis as well as through action with the protein and was nearly zero in 30 minutes at 25°C. if no inhibiting sub-

stances, such as chlorides were present. In this method there was only one phase; *i.e.*, the system was homogeneous.

The Dixon method is much simpler to carry out but the results are not so readily interpreted kinetically.

In the stirring method an excess of H is stirred at a constant rate in the protein solution.[3] The concentration of the dissolved H can be varied from a saturated solution (6×10^{-3} M) to practically zero by varying the rate of stirring, but during any given experiment the dissolved H concentration can be kept relatively constant. In holding the H concentration constant the mathematical treatment of the kinetics is simplified, as will be seen from the discussion of kinetics in the next section. Using the stirring method one may treat a protein with large quantities of H by merely extending the time of stirring. Ball (personal communication) has found similarly that the results on different enzymes by the two methods are about the same.

Thus, as long as other conditions are comparable the same relative sensitivity of enzymes to H is obtained by either method.

II. Kinetics of Inactivation of Enzymes by H

(*a*) *Kinetics Using the Stirring Method.*—In comparing the effect of H on the activity of different enzymes it was necessary to choose conditions in which all the enzymes were stable, and also to keep at a minimum the possible complicating reactions. Because of its physiological importance it would have been desirable to work at pH 7.0–8.0. However, both swine pepsin and crystalline yeast hexokinase are rapidly inactivated above pH 7.0 so that pH 6.0, where these and other enzymes are stable, was used. The buffer was M/25 acetate throughout. In a few experiments at pH 7.8, 0.014 M veronal buffer was used. The stirring method holds the H concentration fairly constant but analyses were usually made.[3]

Under the above conditions the drop in activity of all the enzymes tested was logarithmic when plotted against time. This is illustrated in Fig. 1 by experiments on crystalline swine pepsin and crude or crystalline yeast hexokinase. It was also true for crystalline chymotrypsin, chicken pepsin, yeast sucrase, and swine pepsinogen. As may also be seen in Fig. 1 and Table I the slope of this logarithmic curve is practically independent of the protein concentration but is directly proportinal to the dissolved H concentration.

(*b*) *Derivation of a Bimolecular Equation.*—The bimolecular equation derived below fits all the facts just described when the H concentration is held constant throughout the experiment; *i.e.*, when the stirring method is used.

In order to prevent confusion between the symbol for hydrogen ion and for activated H the symbol DH is used for mustard gas and DH* for the activated form of it.

[3] *Cf.* experimental methods, page 208.

It seems probable that the active mustard unit is not pure DH but an "activated" or "cyclized" compound DH* formed by a reaction with water Thus: $DH + H_2O \rightarrow DH \cdot H_2O \rightarrow DH^* + Cl^-$. When [DH] is held constant the reaction to the right will reach a steady state and [DH*] will be constant, or

$$[DH^*] = C[DH]$$

With protein P, n molecules of DH^* may react, then

$$n[DH^*] + P \rightarrow P[DH^*]_n \qquad \text{and}$$

$$\frac{-dP}{dt} = K[P][DH^*]^n \qquad \text{or}$$

$$\frac{-dP}{P} = K[DH^*]^n\, dt$$

If, as in our experiments the [DH] is held constant then $[DH^*]^n$ is also constant, and on integrating between P at 0 time and P at time t the following equation is obtained

$$K = \frac{1}{t[DH^*]^n} \cdot \ln \frac{P_0}{P_t}$$

but $$[DH^*] = C[DH]$$

therefore $$KC = \frac{1}{t[DH]^n} \cdot \ln \frac{P_0}{P_t}$$

KC has the dimensions of mols P reacting per mol P per liter per mol of $[DH]^n$ per liter per minute.

This equation predicts that:

1. The inactivation of an enzyme or the decrease in concentration of any reactant other than DH will be logarithmic. This relationship would not hold if [DH] decreased during the reaction as it does in the Dixon method.

2. The fraction of the protein inactivated at a given time will be independent of the concentration of the protein.

3. The fraction inactivated per unit time will be proportional to $[DH]^n$.

4. The relative rates of reaction of two different compounds cannot be predicted unless n (the number of mols of H combined per mol of other reactant) is known.

The results of some experiments with crystalline swine pepsin, chicken pepsin crude and crystalline yeast hexokinase, yeast sucrase (invertase), crystalline chymotrypsin, and swine pepsinogen are shown in Fig. 1 and Tables I and II, along with the reaction constants calculated from the above equation. It may be seen that the predictions are adequately fulfilled.

Since, as is shown in Table III, many mustard molecules react with a given protein molecule, *i.e.* $n > 1$, it was expected that the fraction of enzyme in-

TABLE III

*Analyses of Dialyzed Proteins Treated with 1–3 × 10^{-3} Molar H at 25°C. in M/25 pH 6.0 Acetate Buffer**

Protein	Molecular Weight	Time	Volume	N/ml.	Activity	Change in No. of groups/mol protein					
						H		COOH	NH_2	Tyrosine + tryptophane	
						Total S‡	Alkali-labile S			Before alkali	After alkali
		hrs.	*ml.*	*mg.*	*per cent*						
Crystalline swine pepsin	36,000	0.67	50	5.5	28	§	7	7	0	1	0
		1.75			5		15	13	0	7	1
Alkali-denatured swine pepsin at pH 8.0		0.5	100				7	7		5–8	0
		1.0					11–12	12		8–11	1
		2.0		5.7			19–21	22		14–16	3
Crystalline hen's egg albumin	43,000	0.75					4	4.5	0	4	0
		2.0	50	7		11	8	8	0	7	0
		10–15					15–18	22		>10	
Crystalline chymotrypsinogen	37,000	0.75	50	8	90		3	2.4	0	Technical	0
		2.0			63	3	4.4	4.8	0	difficulties	0
Crystalline yeast hexokinase	97,000	0.3	10	3.5	20	35	7	11	31‖	2	
		1.0¶			<5	30	14	28	20‖	0	
Gelatin	62,000	7.5	200	6.5		5	4		0	No color	
		18				14	10–12	9	0		
Crystalline swine pepsin + benzyl H	36,000	12	30	3.5	4		17	15		10	

* These analyses were performed on dialyzed, but unfractioned, reaction mixtures. The purpose for which this work was carried out and the pressure of other problems did not warrant the effort necessary to fractionate and isolate relatively pure mustard-protein compounds.

‡ The total sulfur by micro Carius is not very reproducible with proteins. Thus, the sulfur content of crystalline hexokinase varied from 0.7 to 0.9.

§ No total sulfur analysis was performed on this H-pepsin but several others have been analyzed and the increase in total sulfur has always agreed with the alkali-labile S, within the error of the total sulfur measurement. See Experimental methods.

‖ These analyses were performed on solutions of hexokinase after they had been used for carboxyl titration. The protein was denatured.

¶ This sample was taken from a different but similarly performed experiment.

activated would vary as some higher power of the DH concentration. However, as may be seen from Fig. 1 and Table I, the fraction inactivated varies directly

with the H concentration as though $n = 1$. Any of the following assumptions will predict this result.

1. The first DH molecule to react with the enzyme causes complete inactivation.

2. The reaction of the enzyme with the first mol of DH is the slowest or pacesetting reaction.

3. Each group of the enzyme reacts independently with DH, due probably to the fact that the groups are far apart in the protein molecule. In this case n is the number of DH mols which react with one *equivalent* of protein; *i.e.*, $n = 1$.

It is not possible from the present data to decide among these possibilities, although 3 is probably correct.

(*c*) *Rates of Action of H with Enzymes Compared to the Rate with Water.*—It is sometimes desirable to compare the rates of action of H on various materials to that on some one common substance such as water. This was the purpose of the competition theory (7). If the reaction of H with water is really monomolecular, as is usually assumed, then comparing the bimolecular protein reactions to the water reaction has no particular physical significance. Holiday, Ogston, Philpot, and Stockton (7) have concluded that the reaction of H with water is purely monomolecular and there is considerable evidence for this assumption. It fails, however, to predict the decrease in hydrolysis rate when the water concentration is decreased by the addition of alcohol or other non-aqueous solvents. Thus, in terms similar to those used above for enzymes, the bimolecular constant for water at 25°C. is

$$KC = \frac{0.13}{55} = 0.0024$$

The rate of action of H on an enzyme such as swine pepsin compared to its action on water may, therefore, be expressed as a ratio of the bimolecular constants or

$$\frac{KC(\text{pepsin})}{KC(\text{water})} = \frac{37}{0.0024} \text{ or } 15{,}000.$$

Table II shows this ratio for the various enzymes we have studied.

(*d*) *Kinetics Using Dixon's Method.*—In this method the reaction goes to completion and the per cent of the enzyme inactivated varies with the product of the active enzyme left and the initial H concentration. This holds over a considerable range of H concentration for many enzymes, as may be seen in Fig. 2. The formula

$$K = \frac{\frac{E_0}{E} - 1}{[H_0]} \text{ or } \frac{(E_0 - E)}{[H_0]E}$$

describes the reaction. E is the active enzyme left after reaction with $[H_0]$ the initial gas concentration. K in this formula is the slope of the plot of $\frac{E_0}{E}$ against $[H_0]$. No theoretical derivation of this equation has been found. The expected relation is

$$K = \frac{a}{[H_0]} \ln \frac{E_0}{E}$$

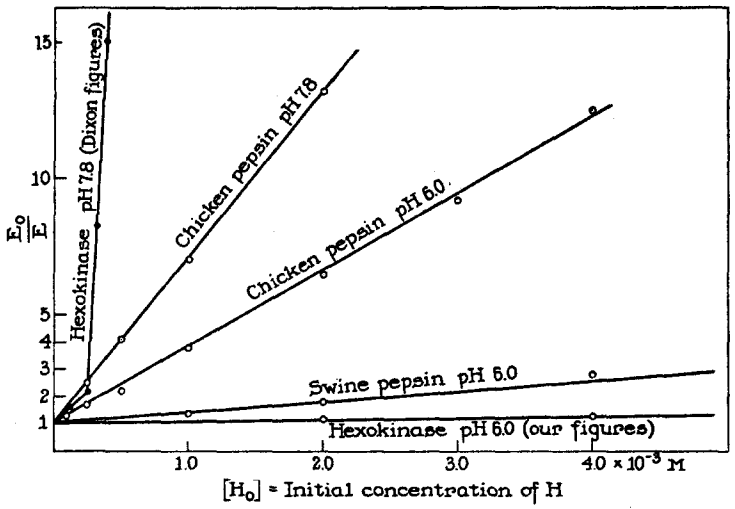

FIG. 2. Inactivation of various enzymes by mustard using the Dixon method.

Experimental Procedure for Fig. 2

Various amounts of 2 per cent (0.157 M) H dissolved in ethyl alcohol or ethyl cellosolve were added to 5 ml. of enzyme solution in M/25 pH 6.0 acetate buffer or 0.014 M pH 7.8 veronal buffer. The enzyme solutions were allowed to stand for 30 minutes at 25°C. after which the activity was determined. In Fig. 2, E_0 = activity when only cellosolve and no H have been added while E = activity after standing 30 minutes with H.

where a = the hydrolysis constant of H under the conditions of the experiment. This relation does not hold as well as the simple reciprocal equation above.

The values reported by Dixon (6) for hexokinase have also been plotted in Fig. 2. It may be seen that his values do not give a straight line. His experiment was carried out at pH 7.8 and 39°C., whereas ours was at pH 6.0 and 25°C. Our crystalline preparation was rapidly inactivated at pH 7.8 in the absence of H so it was decided to avoid these conditions. We have carried out the inactivation of chicken pepsin and yeast sucrase by the Dixon method at

pH 7.8 as well as at pH 6.0 and this is shown in Fig. 2 and Table II. With chicken pepsin the value of *KC* at pH 7.8 was about twice that at pH 6.0, while with sucrase it was thirty times. Thus, no quantitative prediction can be made about the effect of pH on the rate of inactivation of enzymes by H.

(*e*) *H Inactivation Constants of Yeast Enzymes in Vivo and in Vitro.*—It was shown by Nelson, Palmer, and Wilkes (8) that Fleischmann's yeast sucrase (invertase) would hydrolyze sucrose solutions while the enzyme was still a part of the living yeast cell. They found the kinetics of this enzyme reaction to be the same *in vivo* and *in vitro.*

Fig. 3 shows the effect of H on the *in vivo* and *in vitro* enzymatic activity of yeast sucrase at pH 6.0. Freshly washed Fleischmann's bakers' yeast was used. The bimolecular equation has been applied and the constants for the two cases

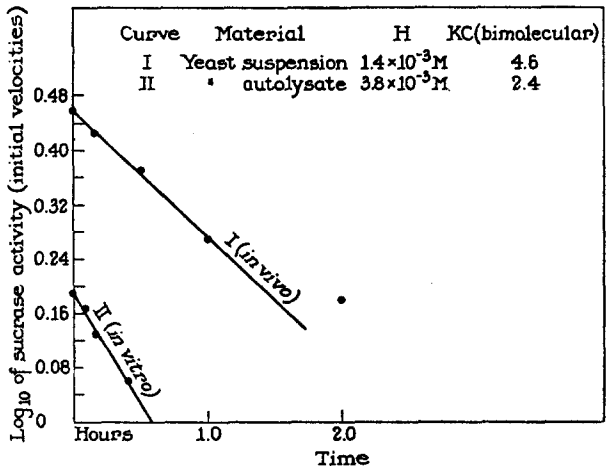

FIG. 3. Inactivation of yeast sucrase (invertase) by mustard *in vivo* and *in vitro.*

were of the same order of magnitude, being 2–3 for the *in vitro* reaction and 4–5 for the *in vivo* reaction.

Anaerobic glycolysis of yeast was similarly examined and a constant of 9 was obtained. This is not far from the value of 12–15 obtained *in vitro* with yeast hexokinase which is supposed to be the enzyme involved in anaerobic glycolysis.

Inhibition of yeast glycolysis by mustard and other war gases has been observed by Massart and Peeters and reported for them by Bacq (33).

III. Protein Groups Reacting with H

The groups in proteins that may be available for chemical reaction are carboxyl, amino, tyrosine phenol, tryptophane indol, histidine imidazole, arginine guanido, aliphatic hydroxyl of serine, threonine, hydroxy-proline, etc., SH of

cysteine, the disulfide of cystine, and the thiomethyl ether linkage of methionine.

The work in this paper is primarily concerned with the carboxyl, amino, tyrosine phenol, and tryptophane indol groups of enzymes and proteins. Preliminary experiments with amino acids and peptides indicated that all four of the groups react with mustard. Ball, Doering, and Linstead (9) have found that carboxyl groups of many acids react with H.

Several other workers (1, 3 10) have suggested that carboxyl groups of proteins react with H. Since our preliminary report containing titrimetric evidence that H had reacted with carboxyl groups of pepsin and gelatin, Ball, Davis, and Ross (1) have shown that hemoglobin when treated with H at pH 5.5 loses many of its carboxyl groups as evidenced by the change in titration curve. When the reaction was carried out at pH 7.5 then, in addition to carboxyl groups, the imidazole group of histidine reacted.

Hellerman (11), Bergmann (12), and Bacq (34) have shown that H reacts with the SH group of cysteine. Moritz, Henriques, *et al.* (13), Ball, Davis, and Ross (1), and du Vigneaud, Stevens, and McKennis (14) have found the imidazole of histidine to react if the reaction media was held between pH 6.0–8.0. Bergmann (15) has reported that a sulfonium compound is formed by the action of H on the sulfur of methionine.

Cogan, Grant, and Kinsey (16) have fed rats a hydrolysate of H-treated casein. The results of these feeding experiments indicated that the histidine, lysine, methionine, and threonine were not "available." In another paper (17) they suggest that in the case of threonine the lack of "availability" is not due to a direct reaction of the threonine residue with H.

Voegtlin and collaborators (44) found that H combined with SH and NH_2 groups. These experiments were carried out in "slightly alkaline" solution. The H-carboxyl group compound might not be formed under these conditions since in some instances this linkage has been hydrolyzed in solutions more alkaline than pH 9.0.

Hellerman's experiments (38) revealed that the SH groups of denatured egg albumin and certain SH groups of native urease react with chemically pure mustard at pH 7.0. Bacq and his associates, Fischer and Desreux, have also found that H reacts with SH groups of denatured egg albumin at pH 8.0 (35), with crystalline lens protein (36), and urease (37). Bacq attributes the inactivation of enzymes by H to this reaction. Banks, Wormall, *et al.* (43) have also reported that H reacts with the SH groups of egg albumin. They found no extensive reaction with NH_2 groups (46).

Two other laboratories have analyzed the action of H on proteins. Bergmann, Fruton, Irving, Moore, and Stein (2) studied the action of H on some amino acids and peptides as well as on some intracellular and serum enzymes. Ball and Ormsbee (3) using pig skin found that about half the fixed radioactive mustard was alkali-labile. This they suggested, was attached to protein carboxyl groups.

In the present experiments various aqueous protein solutions were stirred with H at pH 6.0. Samples were removed at various time intervals. These samples were dialyzed to remove thiodiglycol (TDG), salts, etc., and analyzed for activity (in case of enzymes), alkali-labile sulfur, carboxyl, amino, tyrosine

plus tryptophane groups, and, in some instances, total sulfur. The results of some of these experiments are shown in Table III.

As can be seen from this table, in four of the five different proteins the change in the number of free carboxyl groups resulting from treatment with H equalled within the experimental error the increase in number of alkali-labile H residues bound. In most instances the labile sulfur was nearly the same as the total sulfur. In these same four proteins there was no decrease in amino nitrogen content. There was a decrease in the Folin phenol color value which, although indicating that some of the tyrosine moieties have reacted with H, is probably a secondary effect; perhaps due to steric hinderance. This will be discussed more fully in a later section.

The results on crystalline yeast hexokinase stand out as being different since they indicate a reaction at the NH_2 groups. This result depends, however, on a single analysis. Unfortunately this experiment could not be repeated owing to lack of material. With gelatin, pepsin, egg albumin, and chymotrypsinogen, large quantities of protein permitted several runs and duplicate analyses involving large differences but the measurements and differences obtained with hexokinase were small. On the other hand, it may well be that hexokinase is different from other proteins and the H reacts with its amino groups even at pH 6.0.

The carboxyl groups and tyrosine plus tryptophane groups were determined as follows:

(*a*) *Carboxyl Group.*—Carboxyl groups of proteins have been determined by titrating untreated and H-treated proteins over the range of acidity from pH 6.0 to 2.0. The number of groups combined or lost was calculated from the difference in titration equivalents. Except for small amounts of bound phosphoric acid in certain proteins and the overlapping between pH 5.0 and pH 6.0 of the histidine titration—Cohn, Green, and Blanchard (18)—only carboxyl groups of proteins are titrated over this range of acidity. Ball, Davis, and Ross (1) have found that hemoglobin, a protein rich in histidine, treated with H at pH 5.5 showed no change in its titration curve in the region where the imidazole group of histidine in proteins is usually titrated. It, therefore, seems reasonable to interpret our titration differences in terms of carboxyl groups.

Although it has been assumed that the H is linked directly to the carboxyl group, it is conceivable that the H could attach itself in a secondary position to a carboxyl and so affect its ionization that to include it the titration curve would have to extend beyond the arbitrary pH 6.0–2.0 range.

Titrations from pH 2.0–1.0 on H treated proteins have been made along with those on the untreated proteins and there is not the slightest divergence to indicate a change in any group in this region. In fact, it is doubtful if there are any groups in these proteins that react on the acid side of pH 2.0. Titration curves were not carried into more acid solutions owing to the very large error involved in correcting for the free hydrogen ion.

Figs. 4 and 5 are titration curves of the normal and H-treated pepsin and gelatin respectively. These figures show the effect of H on the titration curves. The curves were all started arbitrarily at pH 6.0 where it is assumed all carboxyl groups are ionized. In practice when determining the number of carboxyl groups the complete curve was not run. The solutions were first adjusted to pH 6.0, then an amount of acid was added all at once to bring the solution close to pH 2.0, after which final adjustment was made to exactly pH 2.0. This tended to reduce the secondary changes in the protein by the acid. The H-treated proteins often precipitated during titration with less acid or more completely than did the untreated protein. This, we believe, is additional evidence that the titratable groups have been altered by the H.

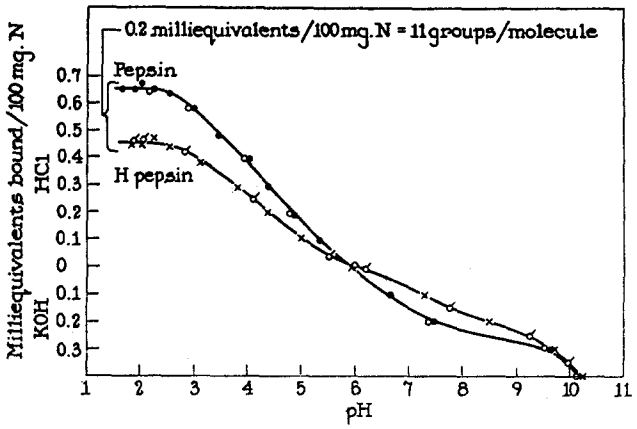

FIG. 4. Titration curves of pepsin and H-pepsin.

Materials and Experimental Procedure Used in Figs. 4 and 5

Pepsin.—Dialyzed at pH 6.0; 4.3 mg. nitrogen per ml.

H-Pepsin.—100 ml. of a pepsin solution containing M/25 pH 6.0 acetate were stirred with H for 7 hours during which time the pH was held close to pH 6.0 with M/1 bicarbonate. The preparation was dialyzed free of TDG and salts and analyzed. The nitrogen concentration was 4.3 mg. nitrogen per ml. These analyses showed it to be only 16 per cent active, to have 12 alkali-labile H residues, to have 6 fewer chromogenic groups calculated as tyrosine.

Gelatin.—Dialyzed; 6.5 mg. nitrogen per ml.

H-Gelatin.—200 ml. dialyzed gelatin were stirred with H at constant pH 6.0 for 2 hours after which time it was dialyzed. Analyses showed it to contain 14 total sulfurs, 12 alkali-labile sulfurs, and no loss of amino nitrogen.

Technique and Apparatus.—This is described in the section on Experimental methods.

The titrimetric differences are not artefacts reflecting differences in the solubility of the parent and derived proteins. This was demonstrated by com-

paring untreated and H-treated alkali-denatured pepsin. In this instance both proteins were completely insoluble in solutions more acid than pH 4.0. It was found that the decrease in titration equivalents in the mustardized protein agreed quantitatively with the number of H residues bound (see Table III).

(*b*) *Tyrosine and Tryptophane Groups.*—The experiments and their results will be presented first, followed by a discussion of the possible interpretations.

Folin's Phenol Reagent.—This reagent, a phosphotungstic-phosphomolybdic complex (19) is reduced by tryosine and tryptophane in alkaline solution to give a blue color. The intensity of the blue color is quantitatively related to the concentration of tyrosine and tryptophane. The reaction is not stoichiometric. The phenol reagent is most rapidly reduced in strong alkali but can be reduced at pH 7.5–8.0. At this low pH esters of phenols are not hydrolyzed and do not

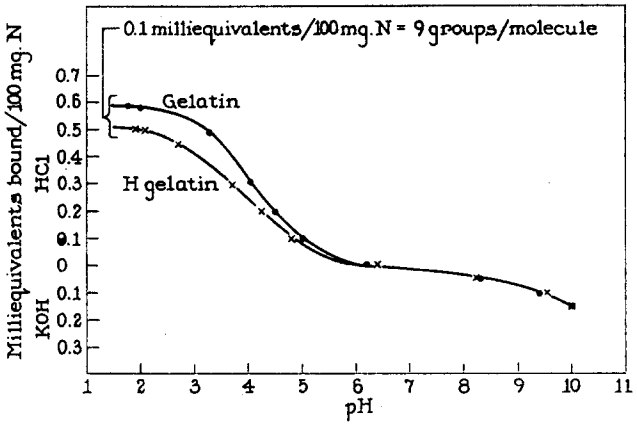

FIG. 5. Titration curves of gelatin and H-gelatin.

reduce the reagent, whereas in stronger alkali the esters are hydrolyzed and the free phenols reduce the reagent (20). As will be shown later, certain linkages of H with proteins are readily broken by alkali so that if tyrosine or tryptophane groups of proteins are covered by H it would be important to estimate the extent of reaction with the phenol reagent at pH 8.0.

(*c*) *Effect of H on Phenol Color of Proteins.*—Proteins which contain tyrosine or tryptophane give a blue color with Folin's phenol reagent (Table IV). These proteins gave less color after they were treated with H. The color returned when the H-protein complex was allowed to stand in dilute alkali (Tables III and IV). As stated earlier, the loss of carboxyl groups when these proteins were treated with mustard gas was approximately equivalent to the number of H residues attached. Thus, not enough H groups were attached to the protein to combine with both tyrosine and carboxyl groups.

In the case of pepsin (Table III, first line) the carboxyl groups decreased at first in proportion to the number of H residues attached. During this stage of reaction little change occurred in the phenol color. As more carboxyl groups reacted, the change in phenol color became greater.

Gelatin contains no tyrosine or tryptophane and hence gave no color with the phenol reagent (Table III). Hexokinase, on the other hand, yields a color with the reagent but no change occurred on treatment with H.

(*d*) *Reaction of H with Peptides.*—Ball, Davis, and Ross (1) found that H does not react with phenol groups of tyrosyltyrosine. Du Vigneaud *et al.* (21) reported that the Folin's reagent color value of tryptophane was lowered by forming the methyl ester of the carboxyl group and was increased again when the ester was hydrolyzed.

Tyrosylglycine and glycyltyrosine, on the other hand, gave about the same phenol color so that the effects were not constant but depended on the nature of the linkage formed.

Herriott and Northrop found in preliminary experiments that glycyltyrosine on treatment with H lost over 75 per cent of its pH 8.0 phenol color and that little recovery was observed even after 20 hours in N/10 alkali (pH 13.0) (see Table IV). Du Vigneaud and Stevens (22) have reported similar findings, and consider that this might be expected because of the ether type linkage between the phenolic group and H. Recently we have prepared an H-glycyltyrosine compound which also has only a fraction of the original phenol color value but in this case the color was almost completely recovered by treatment with dilute alkali for 10 minutes. Thus, this second H-glycyltyrosine compound behaved like H pepsin and many other H proteins on treatment with dilute alkali. The method of preparing these two H peptides differed only in the concentration of peptide. In the earlier work the concentration was 5 mg. per ml. while for the new derivative the concentration was 100 mg. per ml. The pH was 7.5–8.0 in both cases.

The new H-glycyltyrosine product was not crystallized and was not pure as shown by fractionation experiments. The following properties of the reaction mixture indicate something of the nature of the compound:

1. The compound was soluble in dilute acid between pH 2.0 and 3.0, but insoluble above or below this region. 2. It was relatively insoluble in chloroform and in alcohol but soluble in glacial acetic acid.

3. Most of the amino groups were no longer determinable by the Van Slyke method.

4. Hypochlorite and bromine titrations showed that there were one or two H residues per mol peptide.

These properties indicate that the amino group of the glycyltyrosine reacted with H. The insolubility in neutral as well as acid region suggests that the carboxyl group also reacted. As yet the structure of this product is not

known.[4] However, it is probable that glycyltyrosine can react with H to give a compound having properties resembling those of H proteins.

(*e*) *Rate of Recovery of Phenol Color of Various Proteins in Dilute Alkali.*—Mann and Pirie (10), Peters and Wakelin (23), Moritz, Henriques, *et al.* (13), and Ball and Ormsbee (3) have shown that H proteins are unstable in dilute alkali and that some of the H is hydrolyzed even at pH 9.0–10.0.

TABLE IV

Protein	Amino acid content		Reduction in color by H	pH of hydrolysis media	Time for complete recovery of color at 35°C.
	Tyrosine	Trypto-phane			
			per cent		
Pepsin*.....	8.7	2.4	30	11	< 0.5 min.
Egg albumin‡.....	4.2	1.3	41	11	1 hr.
Serum albumin‡.....	4.7	0.5	35	11	4 hrs.
Chymotrypsinogen§.....	2.9	5.4	50	11	> 70 hrs.
Chymotrypsinogen§.....				13	2 hrs.
Glycyltryptophane			40	13	6 hrs.
Glycyltyrosine			75	13	Not in 20 hrs.
Zein‡‖.....	5.9	0.2	Nearly 100	13	Not in 20 hrs.

* Brand, E., unpublished results.

‡ Schmidt, C. L. A., Chemistry of Amino Acids and Proteins, Springfield, Illinois, Charles C. Thomas, 1938.

§ Brand, E., and Kassel, B., *J. Gen. Physiol.*, 1941, **25**, 167.

‖ This preparation was almost completely insoluble.

Experimental Procedure for Table IV

Protein solution or peptides were treated with H by stirring method for 2 hours or until the pH 8.0 phenol color had shown a definite drop. The protein solutions were then dialyzed against distilled water.

Aliquots of the various solutions were mixed with an equal volume of 0.2 molar borate buffer so that the final pH = 11.0. These were kept at 35°C. and at various intervals of time samples were removed and analyzed along with samples of the corresponding normal or untreated protein solution similarly diluted with buffer, and the samples examined for pH 8.0 color value.

Similarly, as indicated in Table IV, some of the solutions were treated with 0.2 N sodium hydroxide so the final pH = 13.0. All color values are expressed as per cent of the control.

Figs. 6 and 7 show the hydrolysis curve of H derivatives of pepsin and gelatin at pH 9.0–10.0, and 11.0. Gelatin, it must be remembered, has neither tyrosine nor tryptophane. However, the H residues hydrolyze from H-gelatin at pH 9.0 at approximately the same rate as from H-pepsin. The return of the phenol color was somewhat faster from H-pepsin than was the hydrolysis of the H residues.

[4] These experiments were discontinued owing to pressure of other problems.

Table IV contains the rate of recovery of the phenol color of several proteins during alkaline hydrolysis. There is a great difference in these rates. Pepsin recovers all its color in a few seconds at pH 11.0 while zein shows very little recovery at pH 13.0 in many hours and there are many rates in between these extremes. Unfortunately, we do not have the corresponding rates of hydrolysis of the H residues or the liberation of carboxyl groups from these H-proteins. It appears, however, that the rates of reaction of similar groups in different proteins may vary enormously.

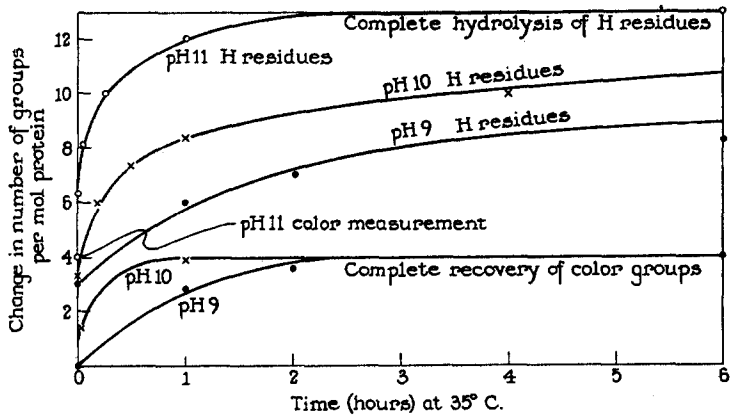

FIG. 6. Alkaline hydrolysis of H-pepsin at various pH.

Materials and Experimental Procedure Used in Figs. 6 to 8

The pepsin H-pepsin, gelatin, and H-gelatin were the same as that described for the experiments shown in Figs. 4 and 5. The alkali-denatured pepsin and its H derivative were prepared as follows.—

Alkali-Denatured Pepsin.—1.8 ml. M/1 sodium hydroxide were added to 200 ml. of dialyzed pepsin, 5.7 mg. nitrogen per ml. at pH 6.0. The pH was 8.5. The solution stood 2 hours at 25°C.

H-Alkali-Denatured Pepsin.—1 ml H was added and stirred with 100 ml. of the above alkali-denatured pepsin. The pH was kept between 7.5 and 8.0 by the addition of M/1 sodium hydroxide. After 2 hours the solution was separated from excess H, dialyzed, and analyzed. Analyses showed 19 to 21 alkali-labile H residues bound, 22 fewer carboxyl groups, than the original protein, and 15 fewer chromogenic groups calculated as tyrosine.

Procedure.—5 ml. of protein were mixed with 5 ml. of a 0.2 molar buffer + 0.05 to 0.15 ml. N/1 sodium hydroxide to the desired pH. The temperature was 35°C. At various intervals of time samples were removed and except in the case of gelatin, the pH 8.0 color value was determined. The free TDG in solution was determined after precipitation of the protein with 0.3 N trichloracetic acid by titrating the trichloracetic acid supernatant with hypochlorite solution as described in the Experimental methods.

(*f*) *Discussion of the Change in Phenol Color.*—It is evident from the preceding experiments that interpretation of the observed results is complicated since both phenol groups and carboxyl groups have been affected, whereas less than an equivalent number of H residues were combined. Several suggestions have been made to account for this anomaly.

1. One mol of H reacts with both OH and COOH groups. This reaction is unexpected from kinetic theory; also, benzyl-H (C_6H_5-CH_2-S-C_2H_5-Cl), which has only one reacting group has the same effect on the phenol color as does H itself. In this case also the loss in carboxyl groups was equivalent to the combined benzyl-H (TableIII).

2. It was suggested by du Vigneaud and Stevens (24) that the change in phenol color is due to denaturation. It is true that the rate of denaturation of

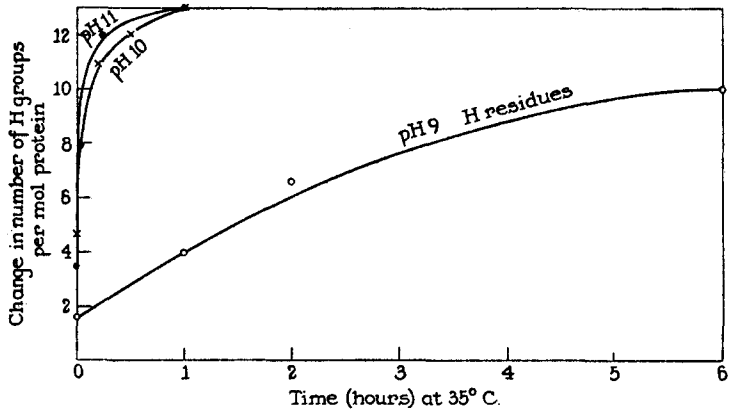

FIG. 7. Alkaline hydrolysis of H-gelatin at various pH.

protein-H-compound is different from that of the original protein. This cannot account for the present results, however, since pepsin denatured before reaction with H behaves just as does native pepsin (Fig. 8, Table III, 2nd line). Also, egg albumin, serum albumin, and chymotrypsinogen are not denatured during the reaction and yet show the same results as pepsin. Evidently these results cannot be explained by denaturation.

3. Esterifying the carboxyl groups affects the phenol color indirectly. Du Vigneaud *et al.* (21) have shown that this is experimentally true in the case of tryptophane but our experiments showing that tyrosylglycine had the same molar color as glycyltyrosine indicate that esterifying the carboxyl group *per se* will not account for the results. The nature of the ester must be considered. There is not enough tryptophane present in some proteins to account for the observed color change and hence tyrosine residues must be involved. With other derivatives of tyrosine, such as the diiodo (41) and N-acylated (39, 40),

the phenol color value is appreciably lower than tyrosine yet there is probably no direct chemical union with the phenol group. A decrease in color, therefore, is not necessarily evidence that some of the tyrosine phenol groups have been linked with mustard molecules.

4. H reacts with the OH groups and the reaction changes the pK of carboxyl groups so that they are not titrated. Gelatin, which contains neither tyrosine nor tryptophane, would have to be considered a special case if this proposal were valid. We were unable to find carboxyl groups of H-proteins ionizing below pH 2.0.

5. A sulfonium salt of H and protein phenolic OH or COOH groups is formed, (Rydon (25, 26)). An H molecule is assumed to combine directly with protein COOH or OH groups. A second H molecule then combines with the first to

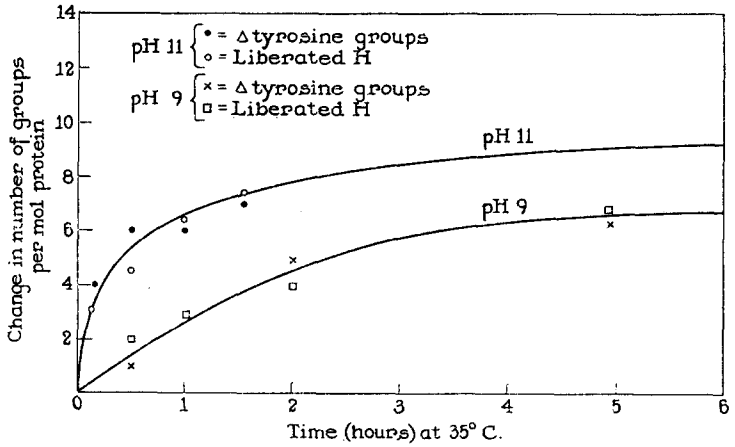

FIG. 8. Alkaline hydrolysis of H-alkali-denatured pepsin.

form a sulfonium salt. Such a compound would be expected to be split from the protein by alkali more rapidly than an H-phenol compound and in this respect the results with proteins agree with the facts.

The following predictions may be made from this assumption:

1. More than one H will combine for each protein group lost.

2. There will be one non-ionized chlorine bound for each sulfonium complex formed.

3. The amount of chloride ion produced during the reaction of H and protein will be less than if the same amount of H reacts with water.

4. Hydrolysis of the H-protein by alkali will take place in at least two steps: (*a*) Decomposition of the sulfonium salt. (*b*) Decomposition of the other type linkages.

These predictions could not be confirmed, although only a small number of experiments were carried out.

1. The first prediction is not true (see Table III). The number of H residues bound is never more than the total number of groups covered but either equal or less.

2. One pepsin preparation which had been reduced to 30 per cent ofitsoriginal activity by treatment with H contained less than one molof cholorine per mol protein.

3. Experiments using a silver-silver chloride electrode (Fig. 9) show that the amount and rate of formation of chloride ion is the same in the presence of 6 mg. of pepsin per ml. as in its absence. This amount of protein was such as to have bound a determinable amount of chlorine in the unionized sulfonium complex if this type reaction took place.

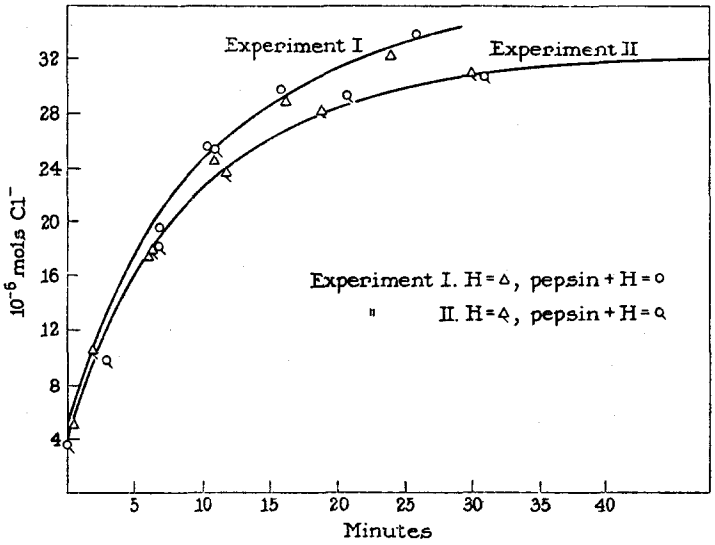

FIG. 9. Rate of formation of Cl ions from H in 0.1 M sodium nitrate with and without pepsin. Pepsin concentration 1 mg. P.N. per ml.

4. Results on the alkaline hydrolysis of H-treated proteins (see Figs. 6 to 8) while not being ideal curves of single type linkage, do not show a change of slope sufficient to indicate the presence of two reaction rates. It is true that the color value returns faster than H groups are hydrolyzed. This might be considered as supporting the theory of Rydon but there may be two types of carboxyl groups to which H is attached, namely those which are associated in some way with the chromogenic properties of the protein and those that are not.

Rydon (27) has recently commented on our experimental evidence given above. He suggests that the amount of H bound to a protein as a sulfonium complex is only a small fraction of the total H bound and that it was too small to be detected. It is true that we would not have detected small units of sul-

fonium compound. On the other hand, the number of phenol OH groups which apparently disappear is quite large and if this reaction is due to a sulfonium salt, as Rydon assumes, we would have detected it.

Rydon also states that our prediction 4 is incorrect since sulfonium compounds do not hydrolyze in two steps. This is due to a misunderstanding. We did not intend the prediction to apply to a two step hydrolysis of the sulfonium compound itself but to a difference in rate between the hydrolysis of the sulfonium bond and other bonds.

The discussion pertaining to the reversible change in phenol color of proteins on treatment with H may be summarized as follows: (1) There are not enough H residues bound to H-proteins to account for the loss of both carboxyl and tyrosine groups. (2) It is necessary to suppose either that the reaction of H with carboxyl groups affects the phenol color or the reaction of H with phenol groups affects the ionization of certain carboxyl groups. (3) The bulk of the evidence favors the hypothesis that the H residues are bound to the carboxyl groups in proteins and that the lower phenol color is a secondary or referred effect.

IV. Materials and Experimental Methods

MATERIALS.—Chemically pure mustard prepared from thiodiglycol (TDG) has been used in most of this work. In those instances where the distilled Levinstein product was used it has been identified in the protocols as HS.

Enzymes.—The crystalline enzymes and precursors, swine pepsin and pepsinogen and beef chymotrypsinogen and chymotrypsin, have been prepared as previously described (28).

The chicken pepsin was freshly activated from chicken pepsinogen prepared as described by Herriott, Bartz, and Northrop (29). The pepsinogen was then dialyzed, frozen and dried at 0°C.

The sucrase (invertase) was prepared from fresh Fleischmann's bakers' yeast treated as follows: 5 ml. of toluene were stirred into 10 gm. of yeast and the mixture incubated at 37°C. for an hour or two. 100 ml. of water were then added and the mixture allowed to stand in the ice box overnight after which it was centrifuged and filtered through Whatman's number 42 paper.

The crystalline hexokinase was prepared by Kunitz and McDonald (30).

Measurement of Enzyme Activity.—The determination of enzyme activity of swine pepsin, chicken pepsin, chymotrypsin, and swine pepsinogen after activation, was by the milk-clotting method (31). Sucrase activity was determined by adding aliquots of sucrase to 25 ml. of 12.5 per cent sucrose in M/100 pH 4.5 acetate buffer and stopping the reaction after 10 minutes at 25°C. with 1 drop of 5 N sodium hydroxide. The concentration of enzyme was such that on reading the sugar solution in a 2 dm. polariscope tube there was about a 2° change in rotation. Under these conditions the concentration of enzyme is roughly proportional to the change in rotation.

The hexokinase activity was according to Kunitz and McDonald (30).

Other Proteins.—The human serum fractions: globulin, albumin, and fibrinogen were supplied to us by Dr. E. J. Cohn's laboratory of Harvard Medical School.

The crystalline egg albumin was prepared by fractional crystallization in a mixture containing M/10 phosphate pH 6.8, and approximately half-saturated ammonium sulfate. After 4 or 5 recrystallizations the crystals were then dissolved and the solution dialyzed.

The gelatin was an isoelectric, dialyzed product prepared by Dr. M. Kunitz.

The zein was prepared by Harris Laboratory of Tuckahoe, New York.

METHODS.—

Hypochlorite Titration of H or TDG.—To 1 to 2 ml. of a 10^{-4}–10^{-3} M solution of either H or TDG are added 1 drop of 5–10 M sulfuric acid and 1 drop of dilute methyl red (we have used 0.02 per cent). Hypochlorite of 10^{-4} or 10^{-3} M is run in from a burette until the indicator is nearly bleached then a second drop of indicator is added and the addition of hypochlorite continued until the tube matches a tube containing water but no indicator. A titration blank is deducted for the water or buffer solution used. Care must be taken that distilled water used in diluting solutions is not contaminated with even small amounts of H_2S or similar substances, particularly if 10^{-4} M hypochlorite is employed.

A Fisher fluorescent titrating lamp helps in observing the end point.

2 mols of hypochlorite are required for 1 mol of H or TDG.

Hypochlorite may be obtained by diluting either zonite or clorox. Standardization of the hypochlorite may be carried out with either known H or TDG solutions. "Kromfax solvent," a product of Union Carbon and Carbide Company, is a nearly pure preparation of thiodiglycol. It may be taken as 10 molar.

Bromine water may be substituted for hypochlorite in the determination. In this case 1 mol of H or TDG uses 1 mol of bromine. Bromine reacts with fewer substances than does hypochlorite.

Estimation of Free H in Aqueous Solution.—The following methods are based on a combination of properties which make the estimation highly specific for H. Thus, H is readily extracted from water by chloroform but in 30 minutes at room temperature a water solution of H changes into TDG which is not extractable by chloroform. The estimation of the concentration of free H in solution is made by either of two methods depending on the nature of the other substances in the solution.

1. If the blank titration for the solution (containing no H) is small, the determination is as follows: An aliquot of the solution to be analyzed is put into each of 2 test tubes. One aliquot is extracted with a small amount (1/10 volume) of chloroform immediately and the other after standing 0.5 to 1 hour at 25°C. Aliquots of the supernatants are then titrated with hypochlorites as described above. The difference in the titration is the amount of H originally present

2. If the blank titration is high then an aliquot (3 to 10 ml.) is extracted with 0.5 to 1.0 ml. of chloroform. The chloroform layer is transferred to another tube, 10 ml. water added, and the tube immersed in water at 65°C. until the chloroform has evaporated. This last step is accomplished as follows: An alundum chip is added to prevent super heating and the tube immersed in the water bath only as far as the top of the chloroform layer. As the chloroform is driven off the H is hydrolyzed by the water. When the last trace of chloroform is gone the water is cooled, reextracted with chloroform, and the aqueous supernatant titrated with hypochlorite. This last extraction with chloroform is necessary to remove all chloroform-soluble material other than H which might titrate with hypochlorite.

Another method, proposed by Anson, is based on the reaction of thiosulfate with an aqueous solution of H. Anson measured iodimetrically the amount of thiosulfate used up in 30 minutes by an aliquot of the unknown H solution. This method requires larger amounts of H.

Determination of Total H Bound to Proteins.—A protein solution which has been treated with H is first dialyzed to remove excess H or TDG. A sample containing about 1 mg. of protein is then titrated with hypochlorite in acid solution in the presence of 30 mg. of Duponol P. C. The titration of an equal amount of untreated protein is similarly performed and this value subtracted to get the titration of the bound H. The Duponol P. C. should first be treated in acid solution with hypochlorite to eliminate the large blank.

Determination of Alkali-Labile H Bound to Proteins.—An aliquot of a protein solution plus half its volume of N/1 sodium hydroxide are left 10 minutes at 25°C. after which approximately 5 volumes of 0.3 N trichloracetic acid are added and the suspension centrifuged. An appropriate aliquot of the trichloracetic acid supernatant is then titrated with 10^{-4} to 10^{-3} M hypochlorite. A control of untreated protein should be run parallel. Table V shows the results of two such titrations by these two methods.

TABLE V

Protein	$\frac{\Delta S}{N}$		
	By *S* analysis	By direct titration on protein	By titration of TDG liberated by sodium hydroxide
Pepsinogen	0.105	0.105	0.075
Pepsin	0.08	0.085	0.08

Stirring Method of Treating Proteins with H.—This consists merely of stirring an excess of pure H with the desired solution. The concentration of dissolved free H is dependent on the speed of stirring and the surface area of the saturating H. Therefore, depending on the volume of solution, rate of stirring, and number and size of H droplets any concentration of dissolved H can be obtained from saturation (6×10^{-3} M) downward. The exact concentration of dissolved H is dependent on so many factors that it is difficult to reproduce conditions and apparatus accurately. The shape and position of the stirrer with respect to the H-water boundary markedly affects the speed required of the stirrer to break up the H into droplets. Calibration of apparatus and method must precede final experiments if the concentration of dissolved H must be within narrow limits. Having found by experiment the steady concentration of H maintained by a certain stirring system, the time necessary to attain that steady state may be considerably shortened by adding to the starting solution enough water or buffer saturated with H to bring the final volume to the desired H concentration.

A V-shaped flask with a vertical opening for the stirrer and a side arm was found convenient for treating proteins by the stirring method. The side arm permits a constant examination of the pH as well as removal of samples and introduction of more H. Foaming or mixing much air into the protein solution must be avoided at all times.

The following table gives an idea of the reproducibility of the H concentration in a system stirred 500 R.P.M. at 25°C.

Volume of H	Volume solution	Dissolved H
ml.	*ml.*	10^{-8} M
0.1	10	1
0.1	10	2
0.1	10	1.7
0.1	10	2.4
0.3	80	0.4
0.8	100	0.3
0.8	120	0.1

Determination of Tyrosine and Tryptophane Groups by the pH 8.0 Phenol Color Method (20)

Reagents.—Folin's phenol reagent (19) diluted 1/3 in water.

Alkaline phosphate: 34 ml. N/1 sodium hydroxide + 60 ml. M/2 disodium phosphate + 6 ml. water.

Procedure.—3 ml. of the diluted phenol reagent were mixed rapidly with 5 ml. of the alkaline phosphate solution and this mixture poured immediately into a flask or tube containing the protein sample to be studied. The tube or flask was then set in a water bath at 37°C. for 15 minutes and compared colorimetrically with either a standard tyrosine solution or a protein solution of known tyrosine content. In general enough protein was used to give the same color as 0.3 mg. of tyrosine under the same conditions.

When a precipitate formed the suspension was kept 13 minutes at 37°C. and then chilled in ice water followed by filtration through number 42 Whatman paper in the ice box. The filtrate was then warmed to room temperature and read immediately.

When it was necessary to treat the protein with alkali before estimating the pH 8.0 color value, the protein sample was mixed with the 5 ml. of alkaline phosphate for a known time after which the phenol reagent was added and the color developed as usual. This introduced no new substances into the solution. However, a control protein solution must be similarly treated.

Calculation of Number of Tyrosine Plus Tryptophane Groups

Tyrosine and tryptophane and peptides containing them reduce the phenol reagent. It is not known with certainty, however, that all tyrosine and tryptophane residues in protein react equally in this respect. Using a known amount of pure tyrosine as a standard, the color produced by a known amount of protein was found to be roughly related to the tyrosine plus tryptophane content of the protein obtained from direct amino acid analyses after acid hydrolysis. In alkaline solution (pH 12.0–13.0) the color produced approaches that expected from the amino acid content while in the pH 8.0 method only 59 per cent of that value was obtained (20, 39, 42). Since this was true for several different proteins this decrease in color at pH 8.0 is not a peculiarity of any one protein but is intrinsic in the method.

The color value of a protein can be expressed as the number of groups (tyrosine plus tryptophane) known to be present in the protein. Then the number of groups lost or covered can be calculated from the per cent drop in color value. Another method is to determine the amount of tyrosine that will give the same color. Then the color can be calculated as tyrosine equivalents. The factor 1.6 should be used in this latter calculation for, as stated above, at pH 8.0 only 59 per cent of the color of a protein is produced as compared to pure tyrosine.

When the tyrosine and tryptophane content of a protein is not known, as was the case with crystalline hexokinase, the latter method must be used. However, when analyses are known the first method is somewhat simpler.

The absolute number of tyrosine or tryptophane groups given in the tables may not be correct but the relative changes in the numbers are significant.

Method of Determining Carboxyl Groups and Titration Curves

Apparatus.—A glass electrode of the type described by Mouquin and Garman (32) was used.

Material.—Protein solutions containing, when possible, at least 2 mg. protein nitrogen per ml. were titrated to pH 6.0 and dialyzed against flowing distilled water for 18 hours.

Procedure.—The glass electrode was first standardized against standard M/10 pH 4.0 acetate and M/15 pH 7.6 phosphate buffers. 0.5 ml. of saturated potassium chloride was then added to an aliquot of protein solution containing 50 to 100 mg. of nitrogen and the solution diluted to 23 ml. This made the initial ionic strength about 0.1 M and the change during titration was neligible. The solution was then adjusted to pH 6.0 with 1.0 M potassium hydroxide or 1.0 M hydrochloric acid. 1.0 M hydrochloric acid was run in with constant stirring from a burette followed by duplicate determinations of the pH. When the entire titration curves were desired small aliquots of acid were added. When only the total titration equivalent between pH 6.0 and pH 2.0 was desired, an amount of acid thought to be sufficient to titrate the solution from pH 6.0 to pH 2.0 was added all at once. Exact adjustment to pH 2.0 was then made with the necessary small amounts of acid or alkali. After each run the electrode was again checked against standard buffer.

Correction for the Water Blank.—Instead of making the usual calculations for the amount of acid free at various pH, an empirical blank run was made. Using the same reagents and amounts and the same apparatus, a curve was run for both acid and alkali. The acid or alkali used was plotted against the pH of the solution. From this curve it was a simple matter to correct the protein solution for acid necessary to adjust the solvent to any pH.

V. SUMMARY

1. The rate of reaction of mustard gas (H) with thirteen proteins has been determined. The extreme variation in reaction rates is about 100:1.

2. No qualitative difference in the results was observed when the treatment with H was carried out by the Dixon or stirring methods.

3. The kinetics have been analyzed and a bimolecular equation derived which fits the facts.

4. The carboxyl groups of all proteins reacted when the reaction with H was carried out at pH 6.0 in M/25 acetate buffer. In most cases the number of carboxyl groups covered was approximately equal to the number of H residues bound.

5. The amino groups of proteins failed to react with the possible exception of yeast hexokinase.

6. The color obtained when proteins were mixed with Folin's phenol reagent at pH 8.0 decreased as the protein was treated with H. The color returned on treatment of the H-protein with alkali and many of the combined H groups were hydrolyzed. Similar results were observed when a concentrated glycyltyrosine solution was treated with H.

REFERENCES

1. Ball, E. G., Davis, S., and Ross, W. F., OSRD Formal Report No. 1630, June 7, 1943.
2. Bergmann, M., Fruton, J. S., Irving, G. W., Moore, S., and Stein, W. H., OSRD Formal Report No. 1439, 1943.
3. Ball, E. G., Ormsbee, R. A., and Henriques, F. C., Jr., OSRD Formal Report No. 1825, 1943.
4. Dixon, M., von Heyningen, R. and Needham, D. M., No. 11, British Report V 17890.
5. Needham, D. M., and Dixon, M., No. 1 British Report U 24815, 1941.
6. Dixon, M., British Report Y 7483.
7. Holiday, E. R., Ogston, A. G., Philpot, J. StJ., and Stocken, L., Peters Report No. 1.
8. Nelson, J. M., Palmer, E. T., and Wilkes, B. G., *J. Gen. Physiol.*, 1932, **15**, 491. Wilkes, B. G., and Palmer, E. T., *J. Gen. Physiol.*, 1932, **16**, 237.
9. Ball, E. G., Doering, W. E., and Linstead, R. P., OSRD Formal Report No. 1094, December 9, 1942.
10. Mann, I., and Pirie, A., British Report V 12478.
11. Hellerman, L., OSRD Informal Progress Report, May, 1942.
12. Bergmann, M., OSRD Informal Progress Report, December, 1942.
13. Moritz, A. R., Henriques, F. C., Jr., *et al.*, OSRD Informal Progress Report, August, 1942.
14. du Vigneaud, V., Stevens, C. M., and McKennis, H., Jr., OSRD Informal Progress Report, November, 1942.
15. Bergmann, M., OSRD Informal Progress Report, July, 1942.
16. Cogan, D. G., Grant, W. M., and Kinsey, V. E., Cogan Report No. 18, NDRC, November 20, 1943.
17. Cogan, D. G., Grant, W. M., and Kinsey, V. E., Cogan Report No. 19, NDRC, November 20, 1943.
18. Cohn, E. J., Green, A. A., and Blanchard, M. H., *J. Am. Chem. Soc.*, 1937, **59**, 509.
19. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, **73**, 627.
20. Herriott, R. M., *J. Gen. Physiol.*, 1935, **19**, 283.
21. du Vigneaud, V., McKennis, H., Jr. *et al.* Informal Report, NDRC 9:3:2, February 10, 1943.

22. du Vigneaud, V., and Stevens, C. M., OSRD Informal Progress Report, October, 1942.
23. Peters, R. A., and Wakelin, R. W., British Report V 14978, 1943.
24. du Vigneaud, V., and Stevens, C. M., OSRD Informal Progress Report, December, 1942.
25. Rydon, H. N., Porton Report No. 2483, February 19, 1943.
26. Crane, C. W., and Rydon, H. N., Porton Report No. 2554, October 26, 1943.
27. Rydon, H. N., WA 999, 4, 4a, September 25, 1943.
28. Northrop, J. H., Crystalline Enzymes. The Chemistry of Pepsin, Trypsin, and Bacteriophage. Columbia Biological Series, No. 12, New York, Columbia University Press, 1939.
29. Herriott, R. M., Bartz, Q., and Northrop, J. H., *J. Gen. Physiol.*, 1938, **21,** 575.
30. Kunitz, M., and McDonald, M. R., *J. Gen. Physiol.*, 1946, **29,** 143.
31. Herriott, R. M., *J. Gen. Physiol.*, 1938, **21,** 501.
32. Mouquin, H., and Garman, R. L., *J. Ind. and Eng. Chem., Analytical Edition*, 1937, **9,** 287.
33. Bacq, Z. M., *Enzymologia*, 1941, **10,** 48.
34. Bacq, Z. M., *Acta biol. belgica*, 1942, **4,** 430.
35. Bacq, Z. M., and Fischer, P., *Bull. Soc. roy. sc., Liége*, 1943, **7.**
36. Bacq, Z. M., and Desreux, V., *Acta biol. belgica*, 1942, **3,** 369.
37. Fischer, P., *Bull. soc. sc., Liége*, 1943, **4.**
38. Hellerman, L., 1944, personal communication.
39. Tracy, A. H., and Ross, W. F., *J. Biol. Chem.*, 1942, **142,** 871.
40. Miller, G. L., *J. Biol. Chem.*, 1942, **146,** 345.
41. Herriott, R. M., *J. Gen. Physiol.*, 1941, **25,** 185.
42. Miller, G. L., and Stanley, W. M., *J. Biol. Chem.*, 1941, **141,** 905.
43. Banks, T. E., Boursnell, J. C., Francis, G. E., Hopwood, F. L., Wormall, A., W. 16505, 1942.
44. Voegtlin, C., *et al.*, (A-1114) Toxic action of B B'dichloroethyl sulfide (mustard gas), National Cancer Institute, August 30, 1943.
45. Dixon, M., and Needham, D. M., *Nature*, 1946, **158,** 432.
46. Banks, T. E., Boursnell, J. C., Francis, G. E., Hopwood, F. L., and Wormall, A., *Biochem. J.*, 1946, **40,** xlvi.