The Reactivity of the Disulphide Bonds of Purified Proteins in Relationship to Primary Structure

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1. With the aid of a coupled system involving glutathione reductase, the reaction of glutathione with the disulphide bonds of purified proteins has been studied. 2. Bovine serum albumin, conalbumin, lysozyme, trypsin inhibitors from egg white, lima bean and soya bean either did not react with glutathione or reacted only slightly. With these proteins reactivity was markedly increased by limited proteolysis. 3. Bovine and human γ -globulins, fibrinogen and β -lactoglobulin exhibited some reactivity (less than 15%) with glutathione and again this was increased by limited proteolysis. Pepsin, trypsin and chymotrypsin exhibited greater reactivity than the proteins previously mentioned. Di-isopropylphosphoryl-chymotrypsin exhibited less reactivity than chymotrypsin, suggesting that autolysis under the experimental conditions used contributed towards the reactivity of this protein. Proteolysis also increased the reactivity of these proteins. The three disulphide bonds of insulin were reduced by glutathione. 4. Above 35° the disulphide bonds of serum albumin show a progressive increase in reactivity and at 55° half of the bonds become accessible to glutathione. 5. From the results obtained with the proteins investigated, the conclusion reached is that the disulphide bonds of native proteins are structurally protected and do not react with glutathione under physiological conditions.

The disulphide bonds of proteins are relatively unreactive with thiols and sulphite (Cecil & McPhee, 1959; Cecil, 1963). The thiol compounds most commonly used have been thioglycollate and β -mercaptoethanol. As GSH is the principal naturally occurring thiol, its rate and extent of reactivity with the disulphide bonds of proteins is of special interest. In this connexion, also, it is difficult to extrapolate from the results obtained with other simple thiols as the rate of reactivity of various thiols with disulphides (Fava, Iliceto & Camera, 1957) and vice versa (Pihl, Eldjarn & Bremer, 1957) has been found to vary.

Certain enzymes containing disulphide bonds are inactivated by reduction, and in addition thiols, such as GSH, can initiate thiol-disulphide exchange reactions that may result in polymerization and gelling of protein systems (Huggins, Tapley & Jensen, 1951). These processes would obviously cause difficulties to the cell. On the other hand, GSH may function in a series of disulphide exchange reactions to reduce enzymes in the inactive disulphide form. In such reactions, glutathione reductase

maintains GSH and thiol-requiring enzymes in the reduced state.

Previous investigations have shown that at neutral pH values there is little reaction of GSH with native serum albumin to form mixed disulphides (Frater & Hird, 1963; Libenson & Jena, 1963) or to reduce completely any of the disulphide bonds of serum albumin and bovine pancreatic ribonuclease A (Hird, 1962; Davidson & Hird, 1965). Insulin, on the other hand, has been shown to be slowly reduced (Hird, 1962). If, however, the protein structure is disrupted by limited proteolysis or thermal treatment, most or all of the disulphide bonds become reactive (Hird, 1962; Frater & Hird, 1963; Davidson & Hird, 1965).

As the information available on the interaction of protein disulphide bonds with GSH is not yet extensive enough to enable a generalization to be made it was decided to extend these investigations to a wider range of purified proteins.

MATERIALS AND METHODS

Materials. Soya-bean trypsin inhibitor (cryst.), egg-white trypsin inhibitor, lima-bean trypsin inhibitor, lysozyme (cryst.) and conalbumin (iron-free) were obtained from the

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Table 1. *Reduction of proteins and protein digests by GSH*

Protein	Mol.wt.	Amino acid residues/mole	Total SH found (moles/mole)	Total S-S (mole/mole)		Details of proteolysis			Details of reduction		
				Found	Reported	Enzyme (% of substrate)	μ moles of titrant/ μ mole of protein*	% of peptide bonds split†	μ moles of reactive S-S/ μ mole of protein	% of total S-S reduced	
Bovine serum albumin	65000 ^b	620†	0.7	16.6	17 ^a	None	—	—	0.40	2	
						Chymotrypsin (1%)	32	5.2	7.8	47	
						Trypsin (2%)	19	3.1	7.3	44	
						Chymotrypsin (1%) + trypsin (2%)	50	8	10.7	64	
Conalbumin	87000 ^b	753 ^c	0.8	13.4	13.8 ^b	pH-stat control	—	—	0.24	1	
						None	—	—	0	0	
						Trypsin (1%)	48	6.4	7.5	56	
						pH-stat control	—	—	0.47	4	
Soya-bean trypsin inhibitor	21500 ^d	192 ^d	0	2.0	2 ^d	None	—	—	0.26	13	
						Pronase (0.25%)	15	7.8	1.3	65	
						pH-stat control	—	—	0.33	16	
Egg-white trypsin inhibitor	28800 ^e	165 ^e	0	6.8	8.0 ^e	None	—	—	0	0	
						Chymotrypsin (1%)	8.1	4.9	3.5	51	
						pH-stat control	—	—	0.08	1	
Thyroglobulin	650000 ^f	6200†	2.8	100	110 ^f	None	—	—	5.5	5.5	
						Trypsin (1%)	303	4.9	94	94	
						pH-stat control	—	—	6.3	6	
Lysozyme	14307 ^g	129 ^g	0	3.8	4.0 ^g	None	—	—	0	0	
						Pronase (1%)	5.6	4.3	0.76	20	
						pH-stat control	—	—	0	0	
Lima-bean trypsin inhibitor	10000 ^h	95†	0	4.7	—	None	—	—	0	0	
						Chymotrypsin (1%)	2.4	2.5	0.12	3	
						Pronase (0.5%)	3.1	3.3	0.10	2	
						pH-stat control	—	—	0.07	2	
Human γ -globulin	160000 ⁱ	1520†	0.8	14.3	14.9 ^j	None	—	—	0.97	7	
						Trypsin (1%)	16.2	1.1	3.2	22	
						Pepsin (1%)	11.8	2.2	10.9	76	
						pH-stat control (pH 3)	—	—	2.2	15	
Bovine γ -globulin	169000 ⁱ	1610†	1.1	18.9	17 ^k	None	—	—	1.4	7	
						Pepsin (1%)	16.3	2.0	19.7	104	
						pH-stat control	—	—	2.1	11	
α -Chymotrypsin	25200 ⁱ	242 ⁱ	0	4.5	5.0 ⁱ	None	—	—	0.66	15	
						Di-isopropylphosphoryl-chymotrypsin	—	—	0.31	7	

References: ^a Kolthoff, Anastasi & Tan (1958); ^b Tristram & Smith (1963); ^c Lewis, Snell, Hirschmann & Fraenkel-Conrat (1950); ^d Wu & Scheraga (1962); ^e Lineveaver & Murray (1947); ^f Derrien, Michel, Pedersen & Roche (1949); ^g Canfield (1963a,b); ^h Fraenkel-Conrat *et al.* (1952); ⁱ Phelps & Putnam (1960); ^j Crumpton & Wilkinson (1963); ^k Markus & Karush (1957); ^l Hartley (1964); ^m Desnuelle (1960); ⁿ Walsh, Kauffman, Sampath Kumar & Neurath (1964); ^o Blumenfeld & Perlmann (1959); ^p Piez, Davie, Folk & Gladner (1961); ^q Ryle, Sanger, Smith & Kitai (1955).

Fibrinogen (human)	341 000 [†]	3240 [‡]	0.8	29	—	Pepsin (0.5%)	5.1	4.2	1.1	24
						Pepsin (1%)	9.7	8.0	2.4	53
						pH-stat control	—	—	0.63	14
						None	—	—	4.3	15
Trypsin	23 000 ^m	223 ^a	0	5.9	6 ^a	Trypsin (1%)	69	2.1	9.2	32
						pH-stat control	—	—	Insoluble	—
						None	—	—	2.0	34
Pepsin	36 200 ^o	341 ^o	0	2.9	2.9 ^o	Pepsin (1%)	11	10	3.6	61
						pH-stat control	—	—	3.3	56
						None	—	—	0.8	28
β-Lactoglobulin	36 600 ^p	350 [‡]	1.4	4.9	4 ^p	Trypsin (1%)	6.0	1.8	2.2	76
						pH-stat control	—	—	1.0	34
						None	—	—	0.75	15
Insulin (amorphous, Zn ²⁺ -free)	5 734 ^q	51	0	3.0	3.0 ^q	Trypsin (1%)	7.9	2.3	3.0	61
						pH-stat control	—	—	1.1	22
						None	—	—	2.6	87
Insulin (cryst.)	5 734	51	0	3.0	3.0	None	—	—	2.6	87

* Values in this column have been corrected for any uptake of titrant by the control preparation.

† It has been assumed that the hydrolysis of a peptide bond at pH 8 produces one proton, and that at pH 3, 0.5 of a proton is used to titrate the carboxylate ion.

‡ Assuming an average residue weight of 105.

Sigma Chemical Co., St Louis, Mo., U.S.A. Bovine serum albumin (crystallized 15 times), bovine γ-globulin, human γ-globulin, human fibrinogen (65% protein, 22% glycine, 5% sodium citrate and 8% moisture; the N was 85–90% clottable) and bovine insulin (crystalline and amorphous grades, the latter Zn²⁺-free) were obtained from the Commonwealth Serum Laboratories, Parkville, Vic., Australia. β-Lactoglobulin was obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.; N-acetyl-L-tyrosine ethyl ester from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Thyroglobulin was fractionated with (NH₄)₂SO₄ by the method of Derrien, Michel & Roche (1948) and purified chromatographically by the method of Ui & Tarutani (1961). The hydrolysates of bovine serum albumin were the samples prepared by Hird (1962). Other materials have been specified previously (Davidson & Hird, 1965).

Preparation of di-isopropylphosphoryl-chymotrypsin. The method used was based on that described by Schaffer, Simet, Harshman, Engle & Drisko (1957). The extent of coupling was determined by assaying for chymotryptic activity at 5 min. intervals up to 20 min. when it was completed. Excess of di-isopropyl phosphorofluoridate and buffer salts were removed by dialysis against water.

Assay of chymotrypsin. This was done titrimetrically in a pH-stat (Radiometer, Copenhagen, Denmark) with N-acetyl-L-tyrosine ethyl ester as substrate (Parks & Plaut, 1953). The substrate concentration was 18 mM in 10 mM-potassium phosphate buffer, which was made 10% (v/v) in ethanol. At 25° and pH 7.9 the rate of uptake of 30 mM-NaOH from 0.5 min. to 3.5 min. after the addition of the enzyme (0.7 μg.) was almost constant and was proportional to the concentration of enzyme.

Preparation of protein hydrolysates. The protein solution (25 mg./5 ml.) was equilibrated at 30° in the pH-stat (pH 3.0 for peptic and pH 8.0 for other digestions) for 20 min. The proteolytic enzyme was then added in 0.05 ml. of water and the hydrolysis followed by the uptake of HCl (50 mM) at pH 3 or NaOH (20 mM to 0.1 N) at pH 8. When the reaction was complete, or after 15 hr., the mixture was freeze-dried. Controls were treated in exactly the same way except that no proteolytic enzyme was added. Weights of freeze-dried powders for analysis were corrected for water content and any additions of alkali or acid.

Other analytical methods. Disulphide bonds reacting with GSH were estimated by the method previously described (Davidson & Hird, 1965).

Total thiol and disulphide contents were estimated by amperometric titration with methylmercuric iodide, by using the indirect method of Frater & Hird (1965).

RESULTS

Bovine serum albumin. The reduction of both the native protein and the hydrolysates have been reported previously (Hird, 1962). It was observed then that enzymic hydrolysis of serum albumin led to a marked increase in the rate of reduction of the disulphide bonds by GSH. In the present work the use of an anaerobic technique (Davidson & Hird, 1965) permitted an estimation of the number of bonds reduced in the system, and for serum albumin this is about 50% of the total (Table 1).

Fig. 1. Effect of temperature on the reduction of disulphide bonds of bovine serum albumin. The experimental conditions for determining reactive disulphide bonds were described previously (Davidson & Hird, 1965).

The effect of temperature on reactivity was investigated, and Fig. 1 shows that reduction starts to increase at 35° and half the bonds react at 55°. Inactivation of glutathione reductase at this temperature made it impossible to extend the investigation to the remaining disulphides. At all temperatures the reaction was not complete after 2 hr. and was still proceeding at approx. 30% of its maximal rate. Decomposition of both NADPH_2 and glutathione reductase precluded the use of longer reaction periods.

The results do not show any stepwise increase in reactivity, which would indicate preferential reduction of susceptible bonds. In contrast with ribonuclease (Davidson & Hird, 1965) the increase in reactivity is spread over a wide temperature range. This may be an expression of the greater molecular size and complexity.

Conalbumin, soya-bean and egg-white trypsin inhibitors, thyroglobulin and lysozyme. These proteins all behaved similarly, having no to little reactivity with GSH in the native state, but hydrolysis of 4–8% of the peptide bonds led to a proportionately greater increase in the reactivity of the disulphide bonds (Table 1). A time-course of reduction of lysozyme and conalbumin and digestion products is given in Fig. 2. This reaction curve,

which goes to a finite state, is typical of these proteins.

Lima-bean trypsin inhibitor. Lima-bean trypsin inhibitor did not react at all with GSH in the native state and was resistant to enzymic hydrolysis, a property reported also by Fraenkel-Conrat, Bean, Ducau & Olecott (1952). There was very little reaction with GSH after the limited proteolysis that was obtained (Table 1). No published value could be found for the disulphide content and on the basis of a molecular weight of 10000 there are probably 5 cystine residues/mol. No thiol groups were detected.

Human and bovine γ -globulins. One disulphide bond in human γ -globulin reacted with GSH, but the reaction with the bovine protein probably involved 2 bonds, as reaction was still incomplete at 80 min. (Table 1). With both globulins, proteolysis resulted in an increased rate and extent of reaction with GSH. With thiol-containing proteins such as these and thyroglobulin (Table 1) it would be possible for oxidation to occur during incubation in the pH-stat and with subsequent freeze-drying, thereby forming additional and perhaps reactive disulphide bonds. Such oxidation could account for the increase in the reactivity of the disulphide bonds in the control preparation of these proteins.

α -Chymotrypsin. The result obtained with chymotrypsin indicates the presence of less than one

Fig. 2. Reaction of GSH with untreated and partly hydrolysed conalbumin and lysozyme. One of the following protein samples was added at the time indicated by the arrow: A, 0.016 μmole of conalbumin, or B, 0.050 μmole of lysozyme; C, 0.050 μmole of a Pronase digest of lysozyme; D, 0.016 μmole of a tryptic digest of conalbumin. The results have been adjusted to start from the same extinction and corrected for the decomposition of NADPH_2 . The experimental details have been described previously (Davidson & Hird, 1965).

Fig. 3. Reaction of GSH with α -chymotrypsin, trypsin and pepsin. One of the following protein samples was added at the time indicated by the arrow: A, 0.20 μ mole of trypsin; B, 0.090 μ mole of pepsin; C, 0.15 μ mole of chymotrypsin. The results have been adjusted to start from the same extinction and corrected for the decomposition of NADPH₂. The experimental conditions have been described previously (Davidson & Hird, 1965).

reactive bond (Table 1 and Fig. 3). Autolysis of chymotrypsin would be expected to yield protein fragments that contain reactive disulphide bonds. To minimize autolysis chymotrypsin was dissolved at pH 3 before the reaction with GSH at pH 7.8 was studied. However, it is possible that over the period of estimation some autolysis could have occurred to expose reactive disulphide bonds. Accordingly di-isopropylphosphoryl-chymotrypsin was prepared to inactivate the protein and its reactivity with GSH determined. The value (Table 1) was less than that for uncoupled chymotrypsin and suggested that some autolysis had occurred with the untreated protein. The small amount of reactivity in di-isopropylphosphoryl-chymotrypsin could still be due to autolysis and denaturation occurring during preparative procedures. In this context commercial samples of ribonuclease A contain various amounts of reactive disulphide bonds (Davidson & Hird, 1965). It is possible therefore that native α -chymotrypsin has no disulphide bonds that react with GSH.

Digestion of chymotrypsin by pepsin increased the reactivity with GSH. The effect of temperature on the reactivity of the disulphide bonds of di-isopropylphosphoryl-chymotrypsin was also examined, but precipitation of both di-isopropylphosphoryl-chymotrypsin and reduction products occurred above 42°. At this temperature 1 disulphide bond/mol. reacted with GSH.

Fibrinogen, trypsin, pepsin and β -lactoglobulin.

In contrast with the proteins discussed above these, in their untreated form, had more than 15% of disulphide bonds that reacted with GSH. The reaction of β -lactoglobulin was unusual in that it continued at a steady rate over 80 min. (Fig. 4). Suitable controls indicated that this was not due to decomposition of NADPH₂ by either β -lactoglobulin or an impurity; therefore at least one disulphide bond in β -lactoglobulin is reactive.

The reactivity found with trypsin (Fig. 3) may be partly due to autolysis as occurred with chymotrypsin, even though the precaution of dissolving it at pH 3 was taken to prevent such autolysis taking place before and during the estimation.

The reactivity of pepsin shown in Fig. 3 and Table 1 indicates that at least one disulphide bond is reactive and possibly more. This may have resulted from denaturation, as pepsin is rapidly denatured at pH values greater than 6 (Bovey & Yanari, 1960).

Insulin. Table 1 shows that, in the system used, nearly all of the disulphide bonds of insulin were slowly reduced over a 3 hr. period. Because the reduction products were precipitated from solution at higher concentrations only small quantities of insulin could be analysed. The reactivity of less than 100% of the bonds was possibly due to precipitation that was visually undetected.

DISCUSSION

The reaction of GSH with the disulphide bonds of a protein and the subsequent reduction of GSSG by NADPH₂ are shown by the following equations:

Fig. 4. Reaction of GSH with β -lactoglobulin (A) and its tryptic digest (B). The protein sample (0.036 μ mole) was added at the time indicated by the arrow. The experimental conditions have been previously described (Davidson & Hird, 1965).

$$K = 10^3 \text{ (Rost \& Rapoport, 1964)}$$

Considerations of the appropriate equilibrium constants for these reactions ($K_1 = \text{approx. } 3$ for cystine; Kolthoff, Stricks & Kapoor, 1955) indicate that, at equilibrium in the experimental system used, only 0.0003% of a small-molecular-weight disulphide compound such as cystine remains in the oxidized form. This complete reduction is also found experimentally.

Similar calculations indicate that, in the present context, a disulphide bond would be regarded as unreactive if the equilibrium constant for reaction (1) were less than 10^{-7} to 10^{-8} , or unless the reaction were very slow despite a favourable equilibrium constant.

The presence of NADPH_2 and glutathione reductase makes this analytical system analogous to the situation that probably exists *in vivo* where (in cerebral tissue) GSH has been shown to be predominantly in the reduced form (Martin & McIlwain, 1959). In addition, the concentration of GSH used (6mM) is in the physiological range and the reactivity of protein disulphide bonds measured in this study can therefore be regarded as approximating to their behaviour *in vivo*.

With most of the proteins investigated less than 10% of the disulphide bonds were reactive, and in many cases there was no reactivity. Two of the most reactive proteins, trypsin and pepsin, were special cases in that trypsin was probably autolysed to some extent and pepsin is unstable at neutral and alkaline pH values. In addition, these proteins are formed by partial proteolysis of a precursor and the results obtained indicate that limited proteolysis of a protein usually leads to increased reactivity of disulphide bonds. It might be expected, then, that trypsinogen and pepsinogen are unreactive. For technical reasons of stability and purity it was not possible to examine these proteins.

Previous observations showed that only the two interchain disulphide bonds of insulin are reduced electrolytically or react with sulphite (Cecil & Weitzman, 1964; Cecil & Wake, 1962) and that an average of 1 bond/mol. reacts with thioglycollate (Lindley, 1955). In the present study either the NADPH_2 -glutathione reductase system provides a greater 'pull' to bring the reaction to completion, or GS^- can approach the disulphide bond more successfully than the reagents previously used.

Reduction of the disulphide bonds of insulin can also be effected by the GSH-insulin transhydrogenase found in liver by Narahara & Williams

(1959). This system also catalyses the reduction of all three bonds of insulin by GSH (Katzen, Tietze & Stetten, 1963). Also, at least the two interchain disulphide bonds of insulin can be slowly reduced by serum (Ensink, Coombs, Williams & Vallance-Owen, 1964).

The reactivity of the disulphide bonds of insulin is therefore well established and may be consequent to its mode of action as a hormone, since this is thought to involve attachment to cell membranes by thiol-disulphide exchange reactions (Cadenas, Kaji, Park & Rasmussen, 1961; Fong, Silver, Popenoe & Debons, 1962). Such a reaction would require a readily accessible disulphide bond.

The reactivity of the disulphide bonds of insulin, in conjunction with its two-chain structure and the observation that proteolysis generally increases reactivity, is consistent with the possibility that insulin, like trypsin and chymotrypsin, is derived by hydrolysis from a precursor molecule. If such a precursor exists the work of Wang & Carpenter (1965) suggests that it is present at less than 10% of the total insulin concentration in pancreas.

Some possible explanations of the lack of reactivity of protein disulphide bonds have been discussed previously (Cecil & McPhee, 1959; Boyer, 1960; Hird, 1962). It was suggested that the important factors were steric hindrances and negative charges adjacent to the bonds. Also, the hydrophilic nature of GSH and its charged reactive group ($-\text{S}^-$) suggests that the presence of hydrophobic regions around a disulphide bond, or the approaches to it, would prevent reaction by exclusion of the solvent (water).

Since the reactive species in reaction (1) are the thiol anions, a reaction would also be made unfavourable at physiological pH values if the $\text{p}K$ of the protein thiol group were markedly lower than that of GSH.

The general lack of reactivity of the disulphide bonds of native proteins with GSH (Hird, 1962; Frater & Hird, 1963; Libenson & Jena, 1963; Davidson & Hird, 1965; this paper) can be regarded as a protective device whereby enzymes containing disulphide bonds can remain active in the presence of GSH, an essential cellular component. It is also likely that the disulphide bonds do not react with the thiol groups of other proteins to form intermolecular bonds. Polymerization and consequent gelling of cellular proteins is therefore prevented.

Since proteolysis has been found to increase the reactivity of protein disulphide bonds it follows that any catheptic hydrolysis of proteins containing disulphide bonds would result in their reduction by GSH. The resultant opening up of the protein structure would lead to further proteolysis, so that the two processes (proteolysis and reduction) might be expected to facilitate one another. Such a

facilitation has been found in the peptic hydrolysis of γ -globulin in the presence of GSH (Putnam, Easley & Lynn, 1962). The presence of the GSH-GSSG reductase system *in vivo* also ensures that disulphide bonds exposed by proteolysis do not react with protein thiols and subsequently polymerize.

However, there may be certain cases, e.g. the formation of blood clots and keratins, such as in wool fibres and skin, where limited proteolysis in the absence of glutathione reductase and NAD(P)H₂ and subsequent polymerization through thiol-disulphide exchange reactions are part of a normal physiological mechanism. The large increase in reactivity of the disulphide bonds of some proteins found after the hydrolysis of a small proportion of the peptide bonds indicates that such exchange reactions could require only a small structural change for their initiation.

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REFERENCES

- Blumenfeld, O. O. & Perlmann, G. E. (1959). *J. gen. Physiol.* **42**, 553.
- Bovey, F. A. & Yanari, S. S. (1960). In *The Enzymes*, 2nd ed., vol. 4, p. 63. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Boyer, P. O. (1960). *Brookhaven Symp. Biol.* **13**, 1.
- Cadenas, E., Kaji, H., Park, C. R. & Rasmussen, H. (1961). *J. biol. Chem.* **236**, 663.
- Canfield, R. E. (1963a). *J. biol. Chem.* **238**, 2691.
- Canfield, R. E. (1963b). *J. biol. Chem.* **238**, 2698.
- Cecil, R. (1963). In *The Proteins*, 2nd ed., vol. 1, p. 379. Ed. by Neurath, H. New York: Academic Press Inc.
- Cecil, R. & McPhee, J. R. (1959). *Advanc. Protein Chem.* **14**, 255.
- Cecil, R. & Wake, R. G. (1962). *Biochem. J.* **82**, 401.
- Cecil, R. & Weitzman, P. D. J. (1964). *Biochem. J.* **93**, 1.
- Crumpton, M. J. & Wilkinson, J. M. (1963). *Biochem. J.* **88**, 228.
- Davidson, B. E. & Hird, F. J. R. (1965). *Biochem. J.* **96**, 890.
- Derrien, Y., Michel, R., Pedersen, K. O. & Roche, J. (1949). *Biochim. biophys. Acta*, **3**, 436.
- Derrien, Y., Michel, R. & Roche, J. (1948). *Biochim. biophys. Acta*, **2**, 454.
- Desnuelle, P. (1960). In *The Enzymes*, 2nd ed., vol. 4, p. 119. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
- Ensinnck, J. W., Coombs, G. J., Williams, R. H. & Vallance-Owen, J. (1964). *J. biol. Chem.* **239**, 3377.
- Fava, A., Iliceto, A. & Camera, E. (1957). *J. Amer. chem. Soc.* **79**, 833.
- Fong, C. T. O., Silver, L., Popenoe, E. A. & Debons, A. F. (1962). *Biochim. biophys. Acta*, **56**, 190.
- Fraenkel-Conrat, H., Bean, R. C., Ducay, E. D. & Olcott, H. S. (1952). *Arch. Biochem. Biophys.* **37**, 393.
- Frater, R. & Hird, F. J. R. (1963). *Biochem. J.* **88**, 100.
- Frater, R. & Hird, F. J. R. (1965). *Biochem. J.* **96**, 895.
- Hartley, B. S. (1964). *Nature, Lond.*, **201**, 1284.
- Hird, F. J. R. (1962). *Biochem. J.* **85**, 320.
- Huggins, C., Tapley, D. F. & Jensen, E. V. (1951). *Nature, Lond.*, **167**, 592.
- Katzen, H. M., Tietze, F. & Stetten, D. (1963). *J. biol. Chem.* **238**, 1006.
- Kolthoff, I. M., Anastasi, A. & Tan, B. H. (1958). *J. Amer. chem. Soc.* **80**, 3235.
- Kolthoff, I. M., Stricks, W. & Kapoor, R. C. (1955). *J. Amer. chem. Soc.* **77**, 4733.
- Lewis, J. C., Snell, N. S., Hirschmann, D. J. & Fraenkel-Conrat, H. (1950). *J. biol. Chem.* **186**, 23.
- Libenson, L. & Jena, M. (1963). *Arch. Biochem. Biophys.* **100**, 441.
- Lindley, H. (1955). *J. Amer. chem. Soc.* **77**, 4927.
- Lineweaver, H. & Murray, C. W. (1947). *J. biol. Chem.* **171**, 565.
- Markus, G. & Karush, F. (1957). *J. Amer. chem. Soc.* **79**, 134.
- Martin, H. & McIlwain, H. (1959). *Biochem. J.* **71**, 275.
- Narahara, H. T. & Williams, R. H. (1959). *J. biol. Chem.* **234**, 71.
- Parks, R. E. & Plaut, G. W. E. (1953). *J. biol. Chem.* **203**, 755.
- Phelps, R. A. & Putnam, F. W. (1960). In *The Plasma Proteins*, vol. 1, p. 143. Ed. by Putnam, F. W. New York: Academic Press Inc.
- Piez, K. A., Davie, E. W., Folk, J. E. & Gladner, J. A. (1961). *J. biol. Chem.* **236**, 2912.
- Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.
- Putnam, F. W., Easley, C. W. & Lynn, L. T. (1962). *Biochim. biophys. Acta*, **58**, 279.
- Rost, J. & Rapoport, S. (1964). *Nature, Lond.*, **201**, 185.
- Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
- Schaffer, N. K., Simet, L., Harshman, S., Engle, R. R. & Drisko, R. W. (1957). *J. biol. Chem.* **225**, 197.
- Tristram, G. R. & Smith, R. H. (1963). *Advanc. Protein Chem.* **18**, 227.
- Ui, N. & Tarutani, O. (1961). *J. Biochem., Tokyo*, **50**, 508.
- Walsh, K. A., Kauffman, D. L., Sampath Kumar, K. S. V. & Neurath, H. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 301.
- Wang, S. & Carpenter, F. H. (1965). *J. biol. Chem.* **240**, 1619.
- Wu, Y. V. & Scheraga, H. A. (1962). *Biochemistry*, **1**, 698.