The Reduction of Serum Albumin, Insulin and Some Simple Disulphides by Glutathione

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It was suggested by Hopkins & Morgan (1938) that glutathione (GSH) may function as a reducing agent maintaining certain enzymes in the reduced and active state, and it is usual to add glutathione, cysteine or some other thiol compound to systems depending on thiol groups for their activity. However, a considerable excess is added and the reduction of the disulphide bonds is not enzymic. In connexion with the above suggested biological function of GSH, there has been little work done on the enzymic reduction of disulphide bonds of oxidized enzymes by catalytic amounts of GSH in the presence of glutathione reductase and reduced nicotinamide-adenine dinucleotides (NADH₂ and NADPH₂). Such a system has, however, been used by Hülsmann & Lipmann (1960) to maintain an enzymic preparation in its active state.

Investigations on the enzymic reduction of intramolecular disulphide bonds of proteins have also been few. The reactions known so far are of two general types. In the first type GSH is the immediate reducing agent. Nahara & Williams (1959) give evidence for the enzymic reduction of insulin by two systems in liver by GSH, alone and with NADPH₂. In the second type, reduction proceeds enzymically at the expense of NADPH₂ without the necessity of GSH being present. Nickerson & Falcone (1956) report the reduction of disulphide bonds of yeast-cell-wall protein by particle preparations from yeast in the presence of a 'coenzyme concentrate'. Hatch & Turner (1960) found that extracts of pea seeds contain an enzyme capable of reducing several proteins, but the rate of reduction is very slow and the extent of the reaction is limited. Racker (1953) reported the reduction of insulin by NADPH₂ which is catalysed by liver extracts, and Black, Harte, Hudson & Wartofsky (1960) found a vigorous system in yeast which is capable of reducing all three disulphide bonds in insulin at the expense of NADPH₂, and in the absence of GSH.

The disulphide bonds of native proteins are resistant to reduction, unless the protein structure is first opened up by denaturing agents such as urea and guanidine (see review by Cecil & McPhee,

1959). After hydrolysis by pepsin disulphide bonds in ribonuclease (Ginsburg & Schachman, 1960b) and in antiovalbumin- γ -globulin (Nisonoff, Wissler, Lipman & Woernley, 1960) are more easily reduced by cysteine and other thiols. Pihl, Eldjarn & Bremer (1957) have shown that the 'reduction' of simple disulphides can be brought about by GSH and that the reaction can be followed by the reduction of the oxidized glutathione (GSSG) so formed, by NADPH₂ in the presence of glutathione reductase. Accordingly it was decided to investigate the ability of GSH to react with the disulphide bonds of serum albumin and insulin in their native state and after they had been partly digested by proteolytic enzymes.

MATERIALS AND METHODS

Materials. Crystalline bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Australia. Insulin-zinc was obtained from the Nordisk Insulin Co., Denmark. NADPH₂, glutathione reductase (6000 Racker units/mg.), GSH and GSSG were obtained from Sigma Co. Ltd., U.S.A. Cystamine, bisacetyl-L-cystine diethyl ester and $\beta\beta$ -dicarboxyethyl disulphide were gifts from Dr R. Cecil, Biochemistry Department, University of Oxford. L-Cystinylbis-L-valine, DL-cystinylbis-D-valine and bis- α -L-glutamyl-L-cystinylbis-L-valine were gifts from Dr D. Morris, National Institute for Medical Research, Mill Hill.

Enzymic hydrolysis of bovine serum albumin and insulin. Serum albumin (100 mg.) in 5 ml. of glass-distilled water was brought to pH 8.0 in a pH-stat (Radiometer, Copenhagen). After alkali consumption had ceased for 10 min., 0.1 ml. of water containing 1 mg. of chymotrypsin was added to the reaction vessel. The protons produced during the hydrolysis of the peptide bonds at this pH were titrated automatically with 0.2N-NaOH and recorded over a period of 7.5 hr. The solution was then freeze-dried and the powder was stored at -10° until required. In subsequent experiments, allowance was made in weighing for a small amount of water (less than 4%) and for the Na⁺ ion added during the titration procedure. A similar experiment was done with trypsin (2 mg./100 mg. of serum albumin) as the hydrolytic agent and also one where both enzymes were used. In this case chymotrypsin was allowed to act for 3.5 hr. and then trypsin for a further 5 hr. The control solution of serum albumin was kept in the pH-stat for 6 hr. at pH 8.0.

Insulin (48 mg.) in 10 ml. of glass-distilled water was adjusted to pH 8.0 in a pH-stat. In contrast with serum

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albumin there was a slow consumption of alkali for 2 hr. with this suspension. After this had ceased, 0.5 mg. of chymotrypsin in 0.1 ml. of water was added and the hydrolysis was followed for 5 hr. The control was kept in the pH-stat at pH 8.0 for 7 hr. The insulin existed in part as a coarse silky suspension and remained as such during the hydrolysis period and also in the control. In an attempt to overcome this problem of partial insolubility a digestion was also done at pH 8.7. Under these conditions the insulin existed as a very fine silky suspension. During the hydrolysis there was again no change in the appearance of the suspended matter. After the hydrolysis the solutions were handled in the same manner as serum albumin.

Estimation of disulphide. The following reactions were used to determine the reactivity of the disulphide bonds of some simple disulphides, serum albumin, insulin and enzymically hydrolysed products of these proteins.

Reaction (3) was followed by the disappearance of NADPH_2 , shown by the change in extinction at 340 μm in a manual spectrophotometer.

The system used in standard 3 ml. cuvettes (1 cm. light-path) was as follows: 1.2 ml. of potassium phosphate buffer (0.1M), pH 7.4, which was 10 mM with respect to EDTA; 0.1 ml. of buffer containing glutathione reductase (1.0 mg. unless otherwise stated); 0.1 ml. of buffer containing NADPH_2 giving an initial final concentration of 0.3 mM before the addition of disulphide; 0.1 ml. of buffer containing GSH (to give a final concentration of 0.625–6.25 mM); 0.1 ml. of buffer containing disulphide or protein preparation.

It was necessary to use a spacer to raise the cuvette in its holder in order to handle the small volume of 1.6 ml. On the addition of the first three solutions the extinction was about 1.7. The preparation of GSH used contained 1–2% of GSSG, and on the addition of 10 μmoles of GSH to the system there was a rapid drop in extinction due to the presence of the GSSG. After this reduction was complete there was a slow steady fall in extinction, partly due to decomposition of the NADPH_2 itself, but mostly due to reoxidation of GSH, which occurred even in the presence of EDTA. When a steady state had been reached (up to 30 min.) and the extinction was approx. 0.8–1.0, the disulphide to be tested was added and the fall in extinction plotted at suitable intervals of time (0.5–2 min.). The temperature in the cell housing was maintained at 28° by the circulation of water from a constant-temperature bath. A blank of suitable extinction (0.3) was used in the reference cell to permit the use of the sensitive range of the instrument down to an extinction of zero. For ease in presentation the extinctions in each experiment have been corrected to give a common point of departure as the point of addition of the disulphide.

RESULTS

Hydrolysis of serum albumin and insulin. Fig. 1 shows that the extent of hydrolysis of serum albumin increased in the following order: trypsin,

chymotrypsin, chymotrypsin plus trypsin. As the pK values of the amino groups liberated are unknown, no attempt has been made to relate protons titrated to amino groups liberated. However, on the assumption that these groups are substantially titrated at pH 8.0, slightly more than 20, 35 and 50 peptide bonds of a total of about 600 in serum albumin were hydrolysed by trypsin, chymotrypsin and chymotrypsin plus trypsin respectively. In the hydrolysis of insulin by chymotrypsin (on the assumption given above) approximately 1.4 bonds at pH 8.0 and 3 bonds at pH 8.7 were hydrolysed out of a total of 48. This latter value is in agreement with that obtained, at pH 8.7, by Ginsburg & Schachman (1960a).

Fig. 2 shows the results of the ultracentrifuge runs on the serum albumin used and on the three digested samples. Fig. 2A shows the presence of a small amount of a high-molecular-weight contaminant, possibly the dimer. Figs. 2B, 2C and 2D show that there was substantial digestion of serum albumin by the three treatments but that in each case some undegraded serum albumin or some other high-molecular-weight material remained.

Reactivity of the disulphide bonds of serum albumin. Fig. 3 shows that crystalline serum albumin contains 0.4–0.5 μmole of a disulphide that reacts quite rapidly with GSH. Fig. 3 shows the fall in extinction brought about by the addition of 0.125 μmole of GSSG to the system. After the initial rapid reduction of serum albumin there is a slow fall in extinction that is greater than the rate due to the background oxidation of GSH. This slow fall is possibly due to the slow reaction of GSH with one or more of the disulphide bonds of the protein. Serum albumin that has been kept in the pH-stat for 6 hr., as a control for the enzymically hydrolysed preparations, does not differ in its content of rapidly reacting disulphide, but shows a slight increase in the rate of reduction of the

Fig. 1. Hydrolysis of serum albumin by chymotrypsin (O), trypsin (□), and chymotrypsin followed by trypsin (Δ). The conditions are given in the text.

slower reacting disulphide bonds of the protein. This could be due to a minor change in structure.

Reduction of disulphides of partly hydrolysed serum albumin. Fig. 4 shows that after hydrolysis by chymotrypsin and trypsin there is a marked increase in the reactivity of many of the disulphide bonds of the preparations. Trypsin, although liberating fewer protons and presumably hydrolysing fewer peptide bonds than chymotrypsin, consistently gave products that reacted slightly faster. When the protein was hydrolysed by both enzymes there was a marked increase in the reactivity of the disulphide bonds. An extinction change of $0.35/0.1 \mu\text{mole}$ of GSSG (Fig. 4) is equivalent to approximately 5 disulphide bonds that have reacted with GSH. In other experiments not recorded the reaction has been followed for longer periods and with smaller amounts of hydrolysis products. Under these conditions nearly all of the 17 disulphide bonds were reduced, but as these

are probably present as a mixture of compounds no detailed kinetic analysis of the rates has been attempted. Fig. 5 shows that the rate of reduction of hydrolysis products is related to the GSH concentration and that it is still quite fast at 0.625 mM .

Reduction of disulphide bonds of insulin and partly hydrolysed products. Fig. 6 shows that the disulphide bond(s) of insulin have some reactivity toward GSH and that treatment in the pH-stat for the time the protein was subjected to hydrolysis at pH 8.7 did not alter the susceptibility of these bonds to reduction. Fig. 6 also shows that after the hydrolysis by chymotrypsin there was a marked increase in reactivity, the rate being greater with the products formed by hydrolysis at pH 8.7. After being freeze-dried, the hydrolysis products could not be completely dissolved, and the quantitative significance of the extent and rate of reduction therefore cannot be assessed accurately. However, as both solutions tested had the same

Fig. 2. Sedimentation patterns of: A, serum albumin; B, serum albumin digested by chymotrypsin; C, serum albumin digested by trypsin; D, serum albumin digested by chymotrypsin and then trypsin. Sedimentation is from right to left. Samples were spun as 1% solutions in 0.1 M-NaCl , pH 8.0, at a temperature of 20° . The duration of the runs was: A, 106 min.; B, 106 min.; C, 138 min.; D, 114 min.

extinction at $280\text{ m}\mu$, it can be concluded that the reactivity of the disulphide bonds after hydrolysis at pH 8.7 was considerably greater than that after hydrolysis at pH 8.0. This increased rate could

result from more extensive hydrolysis at the higher pH and perhaps from hydrolysis taking a different course. This aspect has not been investigated further. The change in extinction recorded in Fig. 6 is equivalent to the reduction of approximately 1.2 disulphide bonds/molecule of insulin added. As the products of hydrolysis did not all dissolve this value would if anything be low.

Reduction of simple disulphides. Fig. 7 shows the reduction of three simple disulphides: GSSG is reduced directly, and the remaining two are reduced indirectly by GSH; L-cystinylbis-L-valine

Fig. 3. Reduction of reactive disulphide bonds of serum albumin ($0.1\text{ }\mu\text{mole}$) by GSH (6.25 mM). ●, Crystalline serum albumin; ○, crystalline serum albumin incubated in a pH-stat for 6 hr.; □, $0.125\text{ }\mu\text{mole}$ of GSSG. Serum albumin and GSSG were added at the time indicated by the arrow. The conditions are given in the text.

Fig. 4. Reduction of reactive disulphide bonds of serum albumin ($0.05\text{ }\mu\text{mole}$) preparations by GSH (6.25 mM). ●, Control serum albumin; ○, serum albumin hydrolysed by chymotrypsin; □, serum albumin hydrolysed by trypsin; Δ, serum albumin hydrolysed by chymotrypsin and then trypsin. The protein preparations were added at the time indicated by the arrow. Other conditions are given in the text.

Fig. 5. Reduction of reactive disulphide bonds of serum albumin hydrolysed by chymotrypsin followed by trypsin. GSH concentration: ○, 0.625 mM ; □, 3.13 mM ; Δ, 6.25 mM . The serum albumin hydrolysate was added at the time indicated by the arrow. The conditions are given in the text.

Fig. 6. Reduction of reactive disulphides of: ●, crystalline insulin; ○, insulin which had been held in a pH-stat for 7 hr.; □, insulin hydrolysed by chymotrypsin at pH 8.0; Δ, insulin hydrolysed by chymotrypsin at pH 8.7. The preparations were added at the time indicated by the arrow. The conditions are given in the text.

and bis- α -L-glutamyl-L-cystinylbis-L-valine were found not to be substrates for glutathione reductase. Apart from a lag in time the final rate of reduction of these disulphides is the same or slightly faster than that of GSSG itself. To slow the rate of reaction the experiment was repeated with the concentration of GSH lowered from 6.25 to 0.625 mM. Fig. 8 shows the rate of reduction of a number of simple disulphides mediated by GSH at this concentration. Under these conditions GSSG is reduced at the greatest rate, as would be expected, under conditions where its concentration is not rate-limiting. The next fastest rate is given

Fig. 7. Reduction by GSH (6.25 mM) of: Δ , GSSG; $\square$, L-cystinylbis-L-valine; $\circ$, bis- α -L-glutamyl-L-cystinylbis-L-valine. The compounds were added at the time indicated by the arrow. Glutathione reductase (20 μ g.) was present.

Fig. 8. Reduction by GSH (0.625 mM) of: +, $\beta\beta$ -dicarboxyethyl disulphide; $\bullet$, trypsin hydrolysate of serum albumin; $\circ$, bis- α -L-glutamyl-L-cystinylbis-L-valine; $\blacksquare$, bisacetyl-L-cystine diethyl ester; $\square$, cystamine; $\blacktriangle$, L-cystinylbis-L-valine; Δ , GSSG. Glutathione reductase (20 μ g.) was present. The compounds were added at the time indicated by the arrow. Other conditions are given in the text.

by cystine derivatives with a free amino group. There was no difference in rate between L-cystinylbis-L-valine and DL-cystinylbis-D-valine. Blocking of the amino groups of cystine with glutamyl groups greatly decreased the rate of reduction. A trypsin hydrolysate compared on the same basis of disulphide content was reduced quite slowly. Dicarboxyethyl disulphide was reduced at scarcely a measurable rate and it is of interest to compare the rates of this compound possessing a neighbouring negative charge with those of the positively charged cystamine.

With all the compounds tested except for GSSG there was a definite lag before the final linear rate of reduction was attained, and there was also a general positive relationship between the lag time and the final linear rate obtained. This lag is probably due to a slow build-up in concentration of the mixed disulphide GSSX which then reacts with glutathione to form GSSG, the compound responsible for the fall in extinction. When the concentration of the mixed disulphide reaches a certain level, steady-state conditions are established and the overall rate is linear.

DISCUSSION

Serum albumin has been shown by many workers to have a thiol content of approximately 0.7 group/molecule and this departure from unity has been puzzling. Dimer formation accounts in part for this discrepancy, and recently two other pieces of evidence provide a further explanation. Eagle, Oyama & Piez (1960), from nutritional studies on certain cells in culture, found that serum albumin contains bound cystine which can be released by various treatments. An investigation by King (1961) has shown that serum albumin contains both cysteine and glutathione as mixed disulphides with the protein. In the present work the sample of serum albumin used was shown to contain 0.4–0.5 reactive disulphide bond/molecule of protein. It is possible therefore that this is due to a mixed disulphide of the type discovered by these workers. In this connexion cystine is measured by the technique used in the present work.

The marked increase in the reactivity of the disulphide bonds of insulin and serum albumin after hydrolysis of less than 10% of the peptide bonds of the proteins shows that the peptide structure protects the disulphide bonds from reduction. In the present work GSH was used at concentrations from 0.625 to 6.25 mM and found to be effective even at the lowest concentration. The GSH content of various tissues has been determined (Tallan, Moore & Stein, 1954; Thomson & Martin, 1959; Martin & McIlwain, 1959; Bhattacharya, Robson & Stewart, 1956): the values range from 0.9 μ mole/g.

for muscle to 4.0–8.8 $\mu\text{mole/g.}$ for liver, with intermediate values for brain, pancreas and kidney. The experiments described in the present paper, therefore, bear a relationship to the reducing power of tissues when considered with the widespread distribution of glutathione reductase in tissues and a dynamic supply of NADH_2 and NADPH_2 . With the appropriate hydrolytic enzymes (cathepsins?) present, reduction of all of the disulphide bonds of proteins could thus be achieved.

The results of experiments on the reduction of the simple disulphides indicate that if the peptides are small enough they can be reduced. The reasons for the resistance of the disulphide bonds of the intact proteins investigated are not known with any certainty. In the present work there is evidence supporting the view of Cecil & McPhee (1959) that a negative charge close to the disulphide bond could repel an approaching negatively charged thiol group (GS^-) and so slow the rate of reaction. In this connexion amino acid analysis of bovine serum albumin (Hughes, 1954) shows the presence of 90 carboxyl groups that could conceivably be in a position to protect electrostatically the 17 disulphide bonds from reduction by GSH. The results of Fava & Illiceto (1958), which have been discussed by Boyer (1960), suggest a further explanation for the resistance of protein disulphide to reduction. This is, that the most successful approach to the disulphide bond by a reducing agent is made from an angle of 180° to the $-\text{S}-\text{S}-$ bond. Apart from the expected inaccessibility to the reducing compound due to the general protein structure, the additional directional problem would confer by enhancement an even greater protection of the disulphide bonds against reduction. The resistance of the protein thiols to reduction may therefore be due to both the charge and steric environment of the disulphide bonds.

Racker (1955) and Thompson, Black & Hudson (1960) have shown that preparations of glutathione reductase contain an enzyme catalysing disulphide-exchange reactions. The possibility exists, therefore, that the differences in the rates of reaction of the simple disulphides used are due not only to charge and steric effects but also to characteristics of this enzyme. However, it is known with certainty (see Cecil & McPhee, 1959) that chemical reactions of this type are common. Further, the interaction of glutathione and serum albumin (native and digested with chymotrypsin) has been followed polarographically by the disappearance of glutathione in the absence of glutathione reductase (R. Frater & F. J. R. Hird, unpublished work). These results show that the reaction of GSH with the digested protein can proceed non-enzymically and rapidly. However, it is not possible to assess the contribution of enzyme specificity to the present results.

SUMMARY

1. The reduction of disulphide bonds in bovine serum albumin, insulin and a number of simple disulphides by physiological concentrations of glutathione has been investigated. Glutathione reductase and reduced nicotinic-adenine dinucleotide phosphate were used to follow the reactions spectrophotometrically.

2. Serum albumin has been shown to have approximately 0.5 of a rapidly reducible disulphide bond/molecule of protein. The remaining disulphide bonds of the native protein and also those of insulin are only slowly reduced by glutathione.

3. On the hydrolysis of less than 10% of the peptide bonds of these proteins by chymotrypsin or trypsin or both there is a marked increase in the rate of reduction of the disulphide bonds.

4. Reduction of simple disulphides with a nearby negative charge proceeds more slowly than that of other types.

5. The factors concerned in the reduction of disulphide bonds of proteins by glutathione are discussed.

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REFERENCES

- Bhattacharya, S. K., Robson, J. S. & Stewart, C. P. (1956). *Biochem. J.* **62**, 12.
- Black, S., Harte, E. M., Hudson, B. & Wartofsky, L. (1960). *J. biol. Chem.* **235**, 2910.
- Boyer, P. D. (1960). *Brookhaven Symp. Biol.: Protein Structure and Function*, **13**, 1.
- Cecil, R. & McPhee, J. R. (1959). *Advanc. Protein Chem.* **14**, 255.
- Eagle, H., Oyama, V. I. & Piez, K. A. (1960). *J. biol. Chem.* **235**, 1719.
- Fava, A. & Illiceto, A. (1958). *J. Amer. chem. Soc.* **80**, 3478.
- Ginsburg, A. & Schachman, H. K. (1960a). *J. biol. Chem.* **235**, 108.
- Ginsburg, A. & Schachman, H. K. (1960b). *J. biol. Chem.* **235**, 115.
- Hatch, M. D. & Turner, J. F. (1960). *Biochem. J.* **76**, 556.
- Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
- Hughes, W. L. (1954). In *The Proteins*, vol. 2, part B, p. 663. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
- Hülsmann, W. C. & Lipmann, F. (1960). *Biochim. biophys. Acta*, **43**, 123.
- King, T. P. (1961). *J. biol. Chem.* **236**, rc 5.
- Martin, H. & McIlwain, H. (1959). *Biochem. J.* **71**, 275.
- Nahara, H. T. & Williams, R. H. (1959). *J. biol. Chem.* **234**, 71.

- Nickerson, W. J. & Falcone, G. (1956). *Science*, **124**, 318.
- Nisonoff, A., Wissler, F. C., Lipman, L. N. & Woernley, D. L. (1960). *Arch. Biochem. Biophys.* **89**, 230.
- Pihl, A., Eldjarn, L. & Bremer, J. (1957). *J. biol. Chem.* **227**, 339.
- Racker, E. (1953). *Fed. Proc.* **12**, 711.
- Racker, E. (1955). *J. biol. Chem.* **217**, 867.
- Tallan, H. H., Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 927.
- Thompson, J. F., Black, S. & Hudson, B. (1960). *Fed. Proc.* **19**, 33.
- Thomson, G. G. & Martin, H. (1959). *Symp. biochem. Soc.* **17**, 17.

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Enzymic Oxidation of Vitamin A Aldehyde to Vitamin A Acid by Rat Liver

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Vitamin A acid, first synthesized by Van Dorp & Arens (1946*a*), was shown to have vitamin A activity in the rat (Van Dorp & Arens, 1946*b*); Sharman (1949) confirmed that the acid can support growth of rats. However, no vitamin A acid or alcohol could be detected in the liver of rats dosed with the acid (Van Dorp & Arens, 1946*b*; Sharman, 1949), which led Moore (1957) to suggest that the acid might be able to fulfil some of the functions of vitamin A, though not being able to replace it in the visual process. This suggestion was fully substantiated by Dowling & Wald (1960), who demonstrated conclusively that vitamin A acid can indeed replace the classical vitamin A in the general metabolism of the rat except perhaps in the visual cycle. More recent observations, however, indicate that vitamin A acid fails to maintain the reproductive performances of vitamin A-deficient male and female rats (Thompson, Howell & Pitt, 1961*a, b*).

It is being increasingly recognized that the classical vitamin A alcohol is not the actual active form of the vitamin for the general metabolic needs of the animals, and the consensus of opinion is that the active compound might be derived from vitamin A acid, if the acid by itself is not the active metabolite. Dmitrovsky (1961) has shown that the aldehyde oxidase prepared from pig liver can oxidize retinene to vitamin A acid, and a preliminary report by Elder & Topper (1961) claims that a NAD-linked aldehyde dehydrogenase in rabbit liver can catalyse the conversion of retinene into vitamin A acid. Further, several workers have shown that vitamin A aldehyde is distributed in the tissues of many species (Plack & Kon, 1961; Winterstein & Hegedus, 1960). It is now demonstrated that rat liver does contain retinene as well as two enzymes that independently catalyse the

reduction of retinene to vitamin A alcohol and the oxidation of retinene to vitamin A acid, and that the two enzymes can be separated by ammonium sulphate fractionation of rat-liver preparations. Since the properties of the alcohol dehydrogenase which catalyses the oxidation of vitamin A alcohol to retinene are well established (Bliss, 1951), in the present paper some of the properties of the retinene-oxidizing enzyme are described and the implications of these findings are discussed with respect to the possibility of the formation of vitamin A acid *in situ* for metabolic needs.

MATERIALS AND METHODS

Materials. Retinene was prepared from vitamin A alcohol and was purified by chromatography on 10% deactivated alumina according to Ball, Goodwin & Morton (1948). The purified product had an absorption maximum at 381 m μ in ethanol (Fig. 1*b*). GSH, FAD, *p*-chloromercuribenzoate, NAD, NADH₂ and NADP were from Sigma Chemical Co., St Louis, Mo., U.S.A., thiobarbituric acid from L. Light and Co. Ltd., Colnbrook, Bucks, and thiourea from Hopkin and Williams Ltd., London. Crystalline vitamin A alcohol and acid were from Hofmann-La Roche. The sources of other materials and reagents were as described by Mahadevan, Murthy, Krishnamurthy & Ganguly (1961).

Separation, identification and estimation of vitamin A alcohol, aldehyde and acid. Two methods, as described below, were used for the chromatographic separation of these components.

(*a*) Adsorption chromatography. When a mixture of these components (0.5 mg. each), dissolved in a small volume of light petroleum, was passed through a 10% deactivated alumina column (1 cm. $\times$ 5 cm.), the retinene could be first eluted with 20 ml. of 2% (v/v) acetone in light petroleum (b.p. 40–60°) and the alcohol with 20 ml. of 8% (v/v) ethanol in light petroleum (b.p. 40–60°). The column was