The Formation of a Specific Inhibitor by Hydrolysis of Rabbit Antiovalbumin

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In a comparative study of rabbit antiovalbumin and the inert γ-globulin with which it is associated (Porter, 1950) no definite difference in structure was found to which the specific biological activity of the antibody could be attributed. The active and inactive material apparently exist as single long polypeptide chains of about 1500 amino-acid residues, the terminal tetrapeptide being, in each case, alanyl-leucyl-valyl-aspartyl-. There remain a great many possibilities of variation in structure, but the results already obtained suggest that considerable similarity may be found between normal γ-globulin and antibody and that antibody may differ from normal γ-globulin only in that segment of the chain which is responsible for combination with antigen. The lattice theory of antibody, antigen combination (Marrack, 1938) postulated that at least two combining areas should be present on each molecule to enable flocculation to occur. If the antibody molecule could be split to give fragments containing one such combining area these fragments should be capable of combining with the antigen, but not of precipitating with it. They would therefore be expected to inhibit the flocculation of antibody and antigen by competing for the combining area of the antigen. As the combining centre of the antibody may be small enough to permit detailed chemical examination it seemed worth while to try to produce an inhibitor by hydrolysis of antibody.

No such attempt to prepare an inhibitor from an antibody appears to have been reported, but Landsteiner (1942) studied the complementary problem of attempting to produce an inhibitor by hydrolysis of an antigen. He was successful, but it is difficult to interpret his results. The antigen, which was degummed silk, was brought into solution by concentrated hydrochloric acid and after several precipitations was finally adsorbed on charcoal before injection. Degradation may have occurred during preparation and the nature of the material injected is uncertain. However, specific inhibition of the antisera could be effected by the addition of dialysable products from partially hydrolysed silk, and Landsteiner (1942) estimated the average molecular weights of the inhibitors as being 600–1000. This work suggested that the combining area of an antibody, also, might be a small fragment of similar size.

After examining a wide variety of hydrolytic conditions it has been found that rabbit anti-ovalbumin will give, on treatment with papain-HCN, a specific inhibitor of antigen-antibody flocculation which had approximately one-quarter the size of the original molecule. Subsequent treatment with carboxypeptidase reduced the size further without destroying the inhibitory activity.

MATERIALS

No attempt was made to prepare pure antibody, but rabbit γ-globulin containing 25–35% specifically precipitating protein was used as starting material. This was prepared as described previously (Porter, 1950).

Enzyme preparations

Aminopeptidase. As a mixture of aminopeptidases with a broad specificity was required, a simple erepsin was prepared as suggested by Dr Emil L. Smith (private communication). Fresh hog gastric mucosa was ground with sand and water several times and the aqueous extracts were centrifuged. $(NH_4)_2SO_4$ was added to the opalescent solution to bring it to 40% saturation and the precipitate was discarded. The precipitate formed at 80% saturation was collected, dissolved in water and dialysed free of salt. Any precipitate formed was rejected and after adjusting to pH 8 the solution was stored at 4° under a film of toluene.

Carboxypeptidase. The enzyme was prepared from beef pancreas, according to Anson (1937*a*).

Chymotrypsin. The enzyme was a crystalline sample given by Mr D. M. Phillips.

Papain. A commercial preparation (Hopkins and Williams Ltd., London) was used without purification. It was activated by dissolving, to give a 10% solution, in 10% KCN and warming to 45° for 1 hr. The solution was centrifuged and stored for short periods at 4°.

Pepsin. A crystalline preparation was made from a commercial product according to the method of Northrop (1939).

Protease from Bacillus sporogenes. The protease was a crude preparation made according to the method of Maschmann (1938).

Trypsin. The enzyme was a crystalline sample from Armours Laboratories, Chicago.

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METHODS

In attempting to find the most advantageous conditions many types of hydrolysis were investigated.

Acid hydrolysis

12N-HCl at 37° from 1 to 72 hr. Boiling N-HCl from 1 to 4 hr. Boiling 0·1N-HCl from 1 to 6 hr. 0·05M-dodecylsulphonic acid at 37° from 4 to 72 hr. 0·025M-dodecylsulphonic acid at 20° from 1 to 4 hr.

Enzymic hydrolysis

Aminopeptidase. Hydrolysis was carried out in phosphate buffer, pH 8 at 37° and in the presence of 0·001M-Mn^{+++}. Assay of the enzyme was made using L-leucylglycine and L-leucyl-glycyl-glycine as substrates (Grassmann & Dyckerhoff, 1928). As a mixture of enzymes was deliberately used this method of assay with two substrates was probably of doubtful value. When attempts at prolonged hydrolysis were made fresh enzyme was added daily as the preparation was unstable at 37° in the presence of manganese.

Carboxypeptidase. Hydrolysis was effected at 37° in phosphate buffer, pH 8·5. Enzymic assay was made using a peptic hydrolysate of edestin as substrate, as described by Anson (1937*b*).

Chymotrypsin. Hydrolysis was at 37° in phosphate buffer pH 8.

Papain. Papain-HCN solution was added to the γ-globulin solution in phosphate buffer, pH 8 at 45° for 72 hr.

Pepsin. N-HCl was added to γ-globulin solution to reduce to pH 3 or 1·5. Crystalline pepsin was dissolved in 0·1N-HCl and added to the solution. Enzymic hydrolysis was carried out at 37°, for periods varying from 1 to 72 hr.

Protease from Bacillus sporogenes. γ-Globulin was incubated with the crude enzyme preparation for 16 hr. in phosphate buffer, pH 7·4, at 37°. The activity of the enzyme was tested under similar conditions using casein as substrate, and titrating in boiling 90% ethanol till alkaline to thymolphthalein as described by Willstätter, Waldschmidt-Leitz, Dünaiturria & Künster (1926).

Trypsin. Tryptic digestion was carried out in phosphate buffer, pH 7·4, at 37°.

Testing of the hydrolytic products for inhibitory activity

The hydrolytic products were in all cases neutralized, and if necessary dialysed, before testing for activity. Their ability to flocculate with ovalbumin was tested against serial dilutions of the antigen at 45°. If this was negative, their inhibitory power was tested by adding increasing amounts of the hydrolytic products to a known amount of egg albumin solution. Sufficient antiovalbumin γ-globulin solution, which as previously determined caused the most rapid flocculation, was added to all the tubes, and the effect of the hydrolysate on the rate of flocculation found. A blank tube without inhibitor was always included in the series. A delay in the time of flocculation caused by addition of hydrolytic solution was not considered significant, unless increasing amounts of the inhibitor caused increasing delay and finally completely prevented flocculation over, say, 24 hr. When such significant inhibition was found, the possible effect of non-specific factors was tested by adding similar increasing amounts of inhibitor to a different antibody-antigen flocculating system. Rabbit anti-horse sera were normally used for this purpose.

Quantitative assay of the antibody content of the γ-globulin preparations was carried out as follows: the optimal proportions were found by the α-procedure (Boyd, 1946) which involves serial dilutions of antigen. With these sera the optimum fell in the equivalence zone. Flocculation was allowed to continue for 1 hr. at 45° and overnight at 4°. The precipitate was centrifuged and washed three times with ice-cold saline, before estimation of total N in a micro-Kjeldahl apparatus.

End-group assay of the hydrolytic products was carried out as described by Sanger (1945) and Porter & Sanger (1948).

RESULTS

The results of this survey of the effects of different conditions of hydrolysis on immune γ-globulin, may be summarized by classifying into three groups, according to the biological activity of the end products.

Hydrolytic conditions after which flocculation occurred. After several types of treatment antibody was still capable of flocculation in presence of ovalbumin. These were tryptic digestion for varying periods of time; peptic digestion at pH 3 for 16 hr.; digestion with protease from *B. sporogenes* for 16 hr.; digestion with 12N-HCl for 1 hr.

Hydrolytic conditions after which no activity remained. When antibody was subjected to the following series of treatments it was incapable of flocculation with antigen or of prevention of flocculation of antibody with antigen: chymotrypsin digestion for 12 hr.; 12N-HCl for more than 2 hr.; boiling N-HCl for 2 hr. or longer; boiling 0·1N-HCl for 2 hr. or longer; 0·05M-dodecylsulphonic acid for 2 hr. or longer; 0·025M-dodecylsulphonic acid for 2 hr. or longer.

Hydrolytic conditions after which inhibitory power appeared. Only with papain-HCN and peptic hydrolysis was there any suggestion of the successful production of an inhibitor. Production of inhibition by peptic hydrolysis for 16 hr. at pH 1·5 was not always reproducible, and a very approximate estimation of particle size, by end-group assay after partial purification of inhibitor by salt fractionation, suggested that the active principle still had a molecular weight of about 100,000. The method was therefore abandoned in favour of papain-HCN hydrolysis which gave more satisfactory results.

Inhibitor produced by papain-hydrogen cyanide hydrolysis

(The abbreviation DNP is used in this paper for 2:4-dinitrophenyl.) The conditions of papain hydrolysis finally adopted were as follows.

An approximately 5% solution of immune γ-globulin in 0·1M-phosphate buffer, pH 8, was incubated at 45°, and to it was added one-tenth its volume of papain-hydrogen

cyanide solution prepared as described. Hydrolysis was allowed to go to completion, 72 hr. being required. The hydrogen cyanide appeared to be a satisfactory antiseptic. To this hydrolytic mixture, after dialysis, anhydrous sodium sulphate was added to 15% (w/v) concentration. The precipitate, which might be expected to contain any unchanged γ-globulin, had little inhibitory power and was discarded. The further precipitate formed by raising the sodium sulphate concentration to 18% (w/v), inhibited strongly and was collected. The precipitate, formed by raising the salt concentration still higher, had little activity. The active precipitate was dialysed and fractionated further with ethanol at $-5°$. The material insoluble in 50% (v/v) ethanol was inactive, while that thrown out on raising the ethanol concentration to 75% contained most of the activity.

End-group assay was carried out on the inhibitor produced by papain-hydrogen cyanide hydrolysis and on the inactive fractions. The active fraction had alanine as terminal amino-acid residue, and as judged by this criterion was about 90% pure as only traces of other coloured derivatives were present. No significant quantities of DNP-alanine could be found in the hydrolytic products of the DNP derivatives of the inactive fractions. In the active fraction about one terminal alanyl residue was present per unit of molecular weight 40,000, whereas in the original γ-globulin only one alanyl residue was found per unit of molecular weight 160,000 (Porter, 1950). Although end-group assay is inadequate as a test for homogeneity or of molecular size, it seems probable that the inhibitor represents the terminal quarter of the original polypeptide chain. The belief that the terminal alanyl residues in the inhibitor and in the original molecule are identical, is supported by the absence of alanine as an end group in the inactive fractions. It is probable that the purified inhibitor was mixed with similar but inactive terminal quarter molecules, which had arisen from the inert γ-globulin in the starting material. Petermann's finding that papain splits human γ-globulin into quarter molecules, as measured by ultracentrifuge studies (Petermann, 1946), agrees with the approximate molecular weight of 40,000 found for inhibitor.

Further hydrolysis of inhibitor. Attempts were now made to reduce the size of the inhibitor by treatment with other enzymes and acid without destroying its activity. No success was achieved with pepsin, trypsin and 12N-HCl, but carboxypeptidase hydrolysed the molecule slowly and after 72 hr. activity was still retained. Examination of the dialysate after hydrolysis, showed that all types of amino-acid were being liberated by this enzyme. Further treatment with carboxypeptidase, however, rapidly destroyed the activity, and it was believed that the combining centre of the inhibitor had then been reached. Repeated attempts were therefore made to remove amino-acids from the other end of the chain with aminopeptidase and reduce the molecule to a still smaller size without destroying the activity. The dialysate of the hydrolytic mixture continued, after days, to give a positive ninhydrin test suggesting that amino-acids were being liberated. On one occasion a dialysable inhibitor was apparently obtained, but this result could not be reproduced. The activity of the product was unaltered by treatment with aminopeptidases, but substantial reduction in size could not be effected.

Hydrolysis of specific precipitate. In an attempt to simplify purification by using purer starting material, the specific precipitate, containing about 90% antibody and 10% egg albumin, was hydrolysed with papain-HCN as before. The hydrolysate had no inhibitor activity and on testing the hydrolysate of egg albumin alone, it was found that this had inhibitory power. It is believed that papain-HCN hydrolysis does not destroy the combining centre of antibody or antigen and leaves the two potential inhibitors combined, and hence inactive when specific precipitate is used as starting material.

The properties of the inhibitor

For study of its properties, the inhibitor was prepared as described, but purified only by salt fractionation, the ethanol fractionation being omitted. Experiments were designed to study the interaction of antibody, inhibitor and antigen, to find whether the reactions are reversible and truly competitive, as would be expected, if the inhibitor was in fact a fragment of the original antibody molecule, in which the combining centre had remained intact.

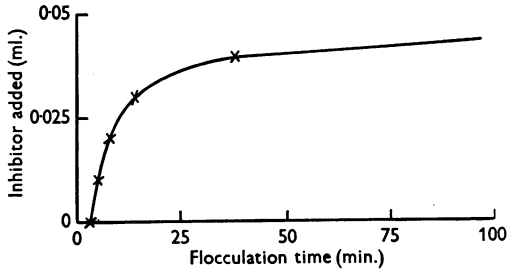

Fig. 1. Effect of increasing inhibitor concentration on flocculation rate. On addition of 0·05 ml. of inhibitor no flocculation was observed in 24 hr.

Effect of increasing inhibitor concentration. For a given solution of immune γ-globulin (1 ml. of 0·2% protein) the content of egg albumin to give the most rapid flocculation was found by serial dilution in the usual manner. Increasing quantities of inhibitor added to the antigen-antibody mixture increased the time of flocculation till none occurred even after standing in the ice chest for 72 hr. The shape of the graph (Fig. 1) and the failure to show even opalescence after a long time suggested that inhibition was in fact complete if sufficient material was added.

Table 1. *Effect of concentration of inhibitor on the weight of specific precipitate*

Exp. no.	Vol. of inhibitor solution added (ml.)	Wt. of egg albumin (mg. N)	Time of flocculation (min.)	Wt. of ppt. (mg. N)	Wt. of antibody (mg. N)
1	0	0·07	0·5	0·54	0·47
	0·4	0·07	20	0·49	0·42
2	0	0·017	1	0·148	0·131
	0·02	0·017	2	0·132	0·115
	0·08	0·017	4	0·126	0·109

A quantitative examination was made of the precipitate formed in the presence of inhibitor to determine whether the inhibitor was carried down when a precipitate was eventually formed, as happens with naturally occurring incomplete antibodies. Table 1 gives the results from two experiments; the precipitation was carried out at the optimal proportions which were in the equivalence zone.

As the inhibitor was not pure the relative content of inhibitor and antibody in the precipitate could not be found. From the data given in Table I it is apparent that less precipitate is formed in the presence of inhibitor than in its absence; it follows that inhibitor had not been carried down with the precipitate unless a considerable quantity of antibody had been left in solution. Owing to the presence of some inhibitor in the supernatant it was impossible to test satisfactorily for the presence of excess antibody or antigen.

Effect of varying antibody and inhibitor concentration relative to a fixed antigen concentration. An experiment was designed to test the competitive aspects of the inhibition. In enzymic experiments it can readily be established whether an inhibitor competes with the substrate or poisons essential groups of the enzyme. Owing to the complexity of the precipitin reaction, a direct quantitative test cannot be applied, but competition can be established qualitatively by studying the effects of varying proportions of the reactants competing for the antigen and the reversibility of these effects. A constant amount of antigen solution was run into a series of tubes and the times of flocculation measured after addition of varying amounts of antibody (in constant volume). This was repeated in the presence of two concentrations of inhibitor; in these experiments the antibody and inhibitor were added almost simultaneously. The results were plotted as indicated in Fig. 2.

Without inhibitor the system showed a flat optimum, as would be expected in this β-procedure with rabbit sera. With inhibitor the optimum occurred at a higher antibody concentration: thus in the presence of 0·1 ml. of inhibitor solution the antibody concentration necessary for precipitation in the minimum time was twice that required in its absence. The time of flocculation at the optimum was more than doubled. The effect of the inhibitor was not confined to binding a proportion of the antigen, otherwise the optimal proportion would have shifted to the right. The exact interpretation of such a complex system is not easy, particularly as the significance of the optimal proportions relationship in absence of inhibitor is not understood. It would be impossible to interpret quantitatively the more complex system of antigen, antibody and inhibitor. It would appear that a competitive mechanism is operative and that this would explain the shift in the zone of optimum proportions.

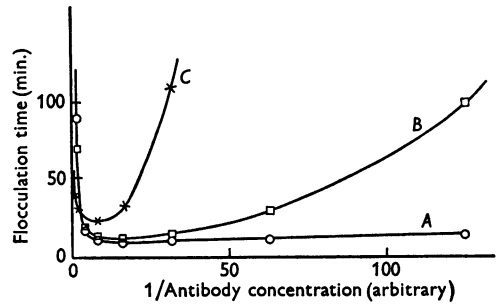

Fig. 2. Flocculation rates with varying antibody and inhibitor concentration. Constant amount of antigen throughout; ○, no inhibitor present; □, 0·02 ml. of inhibitor added; ×, 0·10 ml. of inhibitor added.

Reversibility of inhibition and precipitation. If we are dealing with competitive inhibition, the inhibition should be reversed by addition of excess antibody and conversely a specific precipitate should be capable of solution in excess inhibitor. It is apparent from Fig. 2 that, with relatively low antibody concentrations, an increase of antibody content lowers the flocculation time of a mixture containing fixed amounts of inhibitor and antigen. If the inhibitor is initially combined with antigen in the mixture, this effect would be produced by displacement of inhibitors by antibody. The forms of curves *B* and *C* are consistent with the assumption that there is a reversible equilibrium between inhibitor and antigen, such that inhibitor and antibody can compete for a limited quantity of antigen. A direct demonstration of this competition was achieved in the following manner: to a solution of antigen, inhibitor and antibody, which had failed to show any opalescence after 72 hr. in the ice chest, more antibody was added.

A precipitate formed readily; thus, a change in relative concentration had been sufficient to replace some of the antigen-inhibitor complex by antigen-antibody complex.

The reverse procedure, dissolution of the specific precipitate in excess inhibitor did not take place so readily. The solubility of the precipitate was slight and attainment of equilibrium between soluble inhibitor and precipitate was very slow; but by carrying out a quantitative experiment clear evidence of dissolution of the precipitate was obtained.

An equal volume of immune γ-globulin solution was added to each of six tubes, and to each equal amounts of egg albumin solution were added in optimal proportion. The floccules were allowed to stand at 45° for 1 hr. Two tubes were then stood at 4° overnight, the precipitate washed with cold saline three times and the N estimated in the normal manner. Two other tubes were incubated at 37° under a film of toluene for 4 days. Finally, excess inhibitor was added to the third pair of tubes which were also stored at 37° like the second pair. The total volumes of all tubes were equalized by addition of saline. After 4 days, solution of the precipitate by inhibitor was apparent in the third pair of tubes and the tubes were chilled to 4°. The precipitate was washed and estimated as before. There was good agreement between duplicates, and the results are summarized in Table 2.

Table 2. *Reversal of flocculation by addition of inhibitor to suspension of specific precipitate*

Wt. of egg albumin (mg. N)	Treatment of precipitate	Wt. of precipitate (mg. N)
0·07	16 hr. at 4°	0·53, 0·54
0·07	4 days at 37°	0·55, 0·53
0·07	Inhibitor added 4 days at 37°	0·36, 0·34

About 40 % of the specific precipitate had been dissolved and it seemed likely that, if sterility could be maintained at 37° for 1 or 2 weeks, the precipitate would be completely dissolved.

DISCUSSION

The existence of non-flocculating antibodies whose presence can be demonstrated by their ability to inhibit the flocculation (or agglutination) caused by 'normal' antibodies has been demonstrated in horse antiovalbumin sera (Pappenheimer, 1940), human anti-*Rh* sera (Wiener, 1941) and in other cases. The experimental transformation of precipitating antibodies to a non-precipitating form has been effected by heat (cf. Kleczkowski, 1941), reaction with diazo compounds (Eagle, Smith & Vickers, 1936), formaldehyde (Eagle, 1938) and light (Tyler, 1945). In those cases which have been studied it appears that the inhibition produced by natural or artificial non-flocculating antibodies consists of a delay of flocculation, precipitation occurring eventually. Quantitative examination of horse antiovalbumin sera (Heidelberger, Treffers & Meyer, 1940) showed that when precipitation occurred in the presence of the inhibitory material, this substance was also carried down, as the total nitrogen precipitated was higher than when precipitation took place in its absence. It is, in fact, accepted that the 'univalent' or 'incomplete' antibodies are carried down when precipitation with complete antibody occurs in their presence. The inhibitor produced from antibody by the methods described in this paper is distinct from inhibitors previously described, since there is no evidence that it is attached to the antibody-antigen complex. Probably, for the same reason, the inhibition it produces seems to be complete.

On the basis of the lattice theory of precipitation reaction, hydrolysis would be expected to produce two inhibitors arising from two combining centres in different parts of the molecule. From the limited criteria available it appears that only one inhibitor, from the terminal quarter of the molecule, is produced by papain-HCN hydrolysis. This result would seem to be more in keeping with the occlusion theory of precipitation (Boyd, 1942), but it is possible that a second combining centre was destroyed during hydrolysis.

The properties of inhibitor suggest that it competes with antibody for the combining centre of antigen, both combinations being reversible, though in one case the precipitation which follows combination removes the reactants from solution. It is presumed that the specific combining centre present in the original antibody molecule is contained in the inhibitor fragment and it is clear that specificity is the property of only a fragment of the molecule. The active centre is contained in the terminal quarter of the molecule and from its rapid destruction after 72 hr. treatment with carboxypeptidase it is probably quite localized. In view of the general importance of protein specificity in biological reactions it is desirable that the minimum chain length of the active polypeptide should be established. In the case of the antigen silk fibrin (Landsteiner, 1942) it appeared that the specificity was determined by a sequence of only six or eight amino-acid residues, and in the adrenocorticotropic hormone, the specific activity survives peptic hydrolysis and partial acid hydrolysis (Li, 1947). In neither case, however, was the polypeptide responsible for the specific action isolated.

In this case, reduction of the inhibitor to its minimum size seems to depend on finding an aminopeptidase preparation of sufficient stability and width of specificity. As there is no evidence to suggest that the combining centre will be in the same position in different antibody molecules, the use of other antibodies may be preferable. The minimum size of the specificity determining polypeptide will probably differ in different antibodies, and will pre-

sumably depend upon how far the specific configuration is determined by amino-acid sequence and how far by the influence of other spacially adjacent sections of the molecule.

SUMMARY

1. The effect of a wide variety of hydrolytic conditions on the immunological activity of rabbit anti-ovalbumin has been examined.

2. Papain-hydrocyanic acid hydrolysis produces a quarter molecule unable to flocculate with the antigen, but able to inhibit flocculation of the complete system.

3. This inhibitory fragment appeared to have arisen from the terminal quarter of the molecule.

4. Attempts to reduce the size of the inhibitor further without destroying its activity were only partially successful.

5. A study of the interactions of antibody, inhibitor and antigen showed that complete inhibition of flocculation could be produced. Inhibition or flocculation could be reversed and the conclusion was reached that inhibitor was probably competing with antibody for the same combining centres of the antigen.

6. There was no evidence to suggest that the inhibitor was carried down by the specific precipitate when flocculation occurred in its presence.

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The Components of the Dihydrocozymase Oxidase System

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It has been known for many years that cozymase is necessary for the oxidation of a number of intermediary metabolites by tissues. The cozymase (Co I) is reduced by the metabolite, activated by a specific dehydrogenase, to dihydrocozymase (Co I H_2). There are two well known ways whereby the Co I H_2 may be oxidized to reform Co I, viz. (1), by the anaerobic reaction with a substrate (S): $S + \text{Co I } H_2 \rightarrow \text{Co I} + SH_2$, which requires a single enzyme, SH_2 dehydrogenase; and (2), by the aerobic reaction

$$\text{Co I } H_2 + \tfrac{1}{2}O_2 \rightarrow \text{Co I} + H_2O,$$

which requires a complex of enzymes, which we may call the Co I H_2 oxidase system. The present investigation, which is primarily concerned with the elucidation of the components of this Co I H_2 oxidase system, led to a study of a less well known third method for the oxidation of Co I H_2, viz. its anaerobic oxidation by fumarate: Co I H_2 + fumarate → Co I + succinate. This reaction was demonstrated indirectly by Dewan & Green (1937*a*), who found an oxido-reduction linking β-hydroxybutyrate, which is a typical cozymase-requiring dehydrogenase, and succinic dehydrogenase, which does not require cozymase. Its mechanism has not hitherto been studied. It is shown below that this is a slow reaction, and it is somewhat doubtful whether it is of great quantitative importance *in vivo*.